Abstract

Nickel as a mutagen and as an enabler of EMS genotoxicity in *Caenorhabditis elegans*

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Nickel is found naturally and vital for survival of some organisms. Nickel occurs as a metal as insoluble compounds and soluble compounds. Nickel exposure has increased due to urbanization from various sources including use of metal alloys and car exhaust. Soluble nickel (II) is a divalent cation that travels through waterways and binds to soils and sediments. Toxic nickel exposure levels results in rashes, respiratory conditions and cancer in exposed individuals. The projected increased nickel exposure from expanding urbanization would suggest that there is a need to study its toxicity and its interactions with other environmental toxins and carcinogens. In this research, both heritable genotoxic effects of nickel exposure and how nickel may interact with other environmental mutagens, using Ethyl methanesulfonate (EMS) (a standard laboratory mutagen) is investigated. A genetic screen was conducted to compare the mutagenicity of EMS, nickel, and EMS plus nickel as compared to a control group exposed to PBS, a standard laboratory salt solution. Furthermore, the same treatments were applied to worms that are unable to undergo premature cell death or apoptosis to take into account the non-genotoxic effects of nickel on the screen. The screens were performed using the animal developmental genetic model for *C. elegans* and scoring the production of morphological mutants (including Dpy, Bli, Unc, Muv, and Pvul). This research suggests that nickel is a mutagen causing heritable genetic

changes in animals. It may work synergistically with EMS and its mechanism of action involves more than just genotoxicity and likely triggers programmed cell death in compromised germ cells.

Nickel as a mutagen and as an enabler of EMS genotoxicity in *Caenorhabditis elegans*

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Introduction

Nickel

At low levels, nickel is necessary for life. Natural nickel exposure occurs through the consumption of many foods including nuts, cocoa and spinach (Lu, 2005). In the earth's crust, nickel is one of the most common transition metals but is typically found in low levels in the natural environment. Natural occurrences are from volcanic eruptions and weathering (about 150,000 metric tons per year) (Lu, 2005, Kasprzak, 2003). In comparison, however, most nickel exposure occurs from anthropogenic sources (about 180,000 metric tons per year) (Lu, 2005). Six enzymes needed for organisms have nickel in the active site of the enzyme and include methyl-coenzyme M reductase, acetyl-coenzyme A synthase, superoxide dismutase, carbon monoxide dehydrogenase, hydrogenase, and urease (Ragsdale, 1998). A coenzyme, coenzyme F_{430} has also been identified as requiring nickel (Walsh, 1987).

At high levels, nickel is toxic. Industry workers in alloy production, welding and electroplating are most likely to be exposed to nickel. However, individuals who live in urban areas are also exposed to high quantities of airborne nickel that is released by fossil fuel combustion (Lu, 2005). As nickel is also found in cigarette smoke, individuals who smoke are also exposed to nickel by smoking (Lu, 2005, Sunderman, 1968). Therefore as the world becomes more urbanized it is important to consider how people and other organisms are affected by nickel. Nickel has been classified as a Group 1 carcinogen (Lu, 2005, Kasprzak, 2003). Inhaled nickel is concentrated in the spinal cord, brain, diaphragm, heart and lungs (Lu, 2005). Ingested nickel is primarily stored in the kidneys and eliminated from the body in the urine and feces (Lu, 2005, Sunderman, 1968). Once ingested, typically one to two percent of nickel is absorbed into the body. Long-term exposure to nickel leads to kidney disease, cardiovascular

disease, nasal cancer, lung fibrosis and other cancers (Lu, 2005). Other health issues include contact dermatitis, nickel allergy and organ system toxicity (Lu, 2005).

In order to have its effect on the body, nickel must first enter cells. Once nickel in both insoluble and soluble forms enters the cell, it can affect many cellular processes. Nanoparticles of insoluble nickel are phagocytized and enter the lysosomes where exposure to an acidic environment results in slow release of nickel over time, thus serving as a long-lasting source of nickel ion exposure (Kuehn, 1982). More general transporters that also move nickel include the HypB transporter can also store nickel and release it when nickel becomes limiting (Ragsdale, 1998, Olson, 1997). Soluble nickel can enter cells in two ways. One way that soluble nickel can enter cells is through calcium ion channels. The Divalent Metal Ion Transporter (DMT1) also transports soluble nickel ions into the cell (Lu, 2005). Additionally, there are two types of high-affinity nickel transporters for soluble nickel that have been identified for incorporation into the cell. One type of nickel transporter is the one-component nickel transporters including HoxN, HupN, NixA and UreH. The other type of nickel transporter is a multi-component transporter system that binds to ATP (Ragsdale, 1998). Once nickel has enters the cell it has been shown to promote lipid peroxidation, increase proto-oncogene expression and silence genes (such as tumor-suppressor genes such as p16, p21 and Rb) (Lu, 2005).

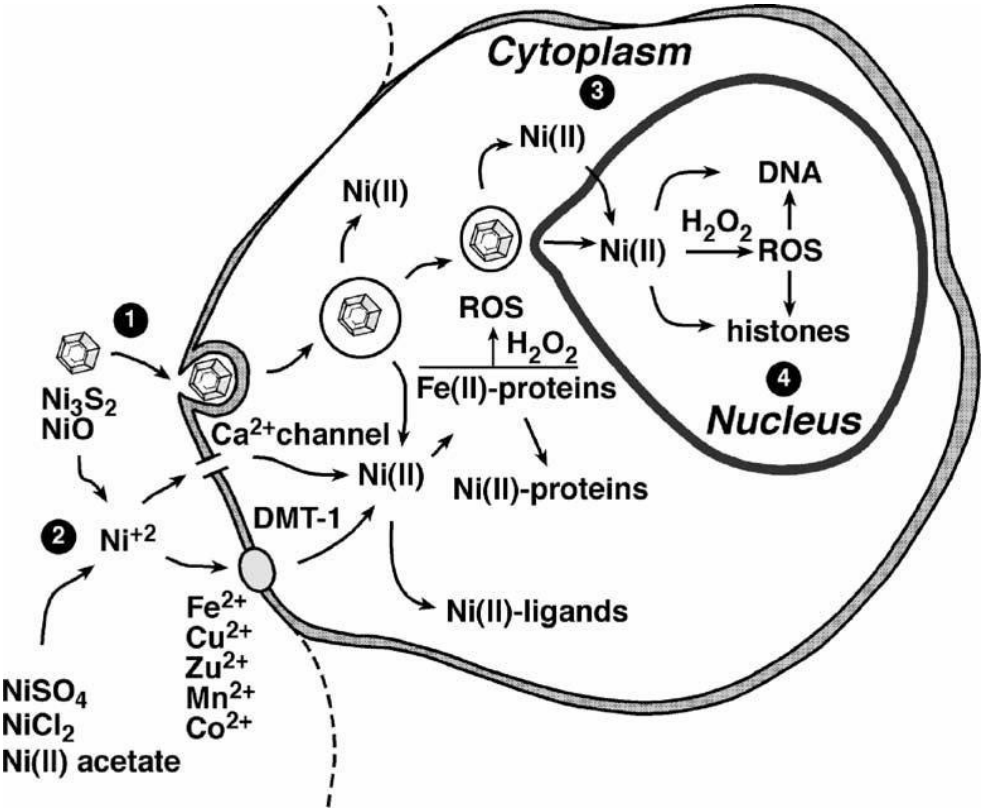


Figure 1: The path of soluble and insoluble nickel into and through the cell. Image from Kasprzak, et al. 2003.

Nickel has a complex, multi-pathway molecular mode of toxicity. Nickel interferes with cell signaling, intracellular communication and apoptosis due to cellular damage. Nickel appears to activate the NF-κB cellular pathway and stimulates inflammation through the intracellular adhesion molecules EMAM-1, VACM-1, and ICAM-1 (Lu, 2005, Pulido, 2003). It has been shown that nickel (in the form of NiS_2) can change G to T at guanosine residues in the K-ras gene (Lu, 2005, Higinbotham, 1992). Little research has been done on nickel's effect on the mitogen-activated protein kinases (MAP kinases) that facilitate cell signaling pathways but the studies that have been conducted indicate that they are affected too (Lu, 2005). As shown in Figure 1, nickel produces reactive oxygen species (ROS) that affect histones and therefore affects the packaging of DNA in the nucleus. Finally, in cells that were transformed by nickel

exposure, the phosphorylation of retinoblastoma protein, which helps controls the transcription regulator c-myc, decreases (Lu, 2005). Cells exposed to nickel can become immortalized and have inhibited intracellular communication (Lu, 2005, Miki, 1987).

Exposure to nickel induces apoptosis, perhaps through activation of several triggering pathways as shown in Figure 2. These pathways include the DNA damage pathway, pathogen response pathways, physiological pathways, and physiological cytoplasmic stress pathways (Gartner, et al., 2008). Any one or a combination of these pathways leads to apoptosis.

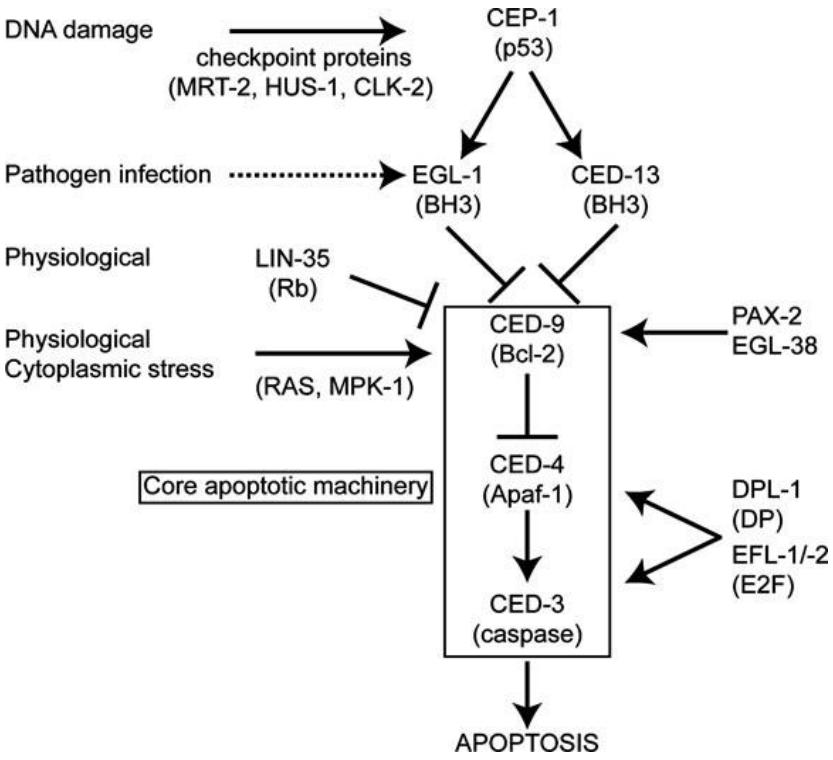


Figure 2: Different pathways in *C. elegans* leading to apoptosis. Image from Gartner, et al., 2008.

Previous studies have indicated that nickel increases the harmful effects of other toxins and damaging agents (Dubins, et al., 1986, Hartwig, et al., 1987, Hartwig, et al., 1989, Hu, et al., 2004) which could have long-term effects. By inducing histone modification in DNA (Ke, et al. 2006) nickel may work as an enabler of other mutagens by indirectly causing harm by perhaps

unwinding the DNA packaging which makes the DNA vulnerable to additional environmental toxins (Dubins, 1986). Nickel could also work indirectly by affecting the DNA repair machinery that otherwise would have repaired the damage to the DNA if nickel was not present. The harmful effects of nickel have been shown to be transferred to the offspring in *C. elegans* indicating that the effects of nickel could last multiple generations (Wang, et al., 2008) in a heritable fashion. Therefore any damage that occurs from the interaction of nickel and other toxins could increase in each generation.

It is still not completely understood how nickel, a known carcinogen, interacts with other mutagens and how it impacts the DNA of organisms. This project investigates those topics. People and other organisms are increasingly exposed to nickel and other environmental chemicals from various sources due to anthropogenic activity and this research seeks to understand how nickel may impact human health in a heritable manner. Furthermore, although previous studies have considered the effects of nickel using individual cells and bacteria (Dubins, et al., 1986, Hartwig, et al., 1987, Hartwig, et al., 1989, Hu, et al., 2004), overall mutagenicity of nickel has not been well studied in a multicellular animal either alone or in combination with other mutagenic agents.

The literature suggests that not only is nickel toxic by itself but that it may expose DNA so that other environmental toxins can have greater access to the DNA sequence and therefore can increase the harmful effects of environmental toxins. Therefore it is expected that mutant phenotypes of *C. elegans* will occur after worms are exposed to nickel alone. Furthermore, it is expected that if nickel is an enabler of direct acting genotoxic agents, the frequency of mutant phenotypes will greatly increase when worms are exposed to nickel and a mutagen of this type at the same time as compared to those that are exposed to nickel and the second mutagen

separately. This is illustrated below in Figure 3, with EMS only, nickel only and both an additive effect and a synergistic effect of nickel with EMS.

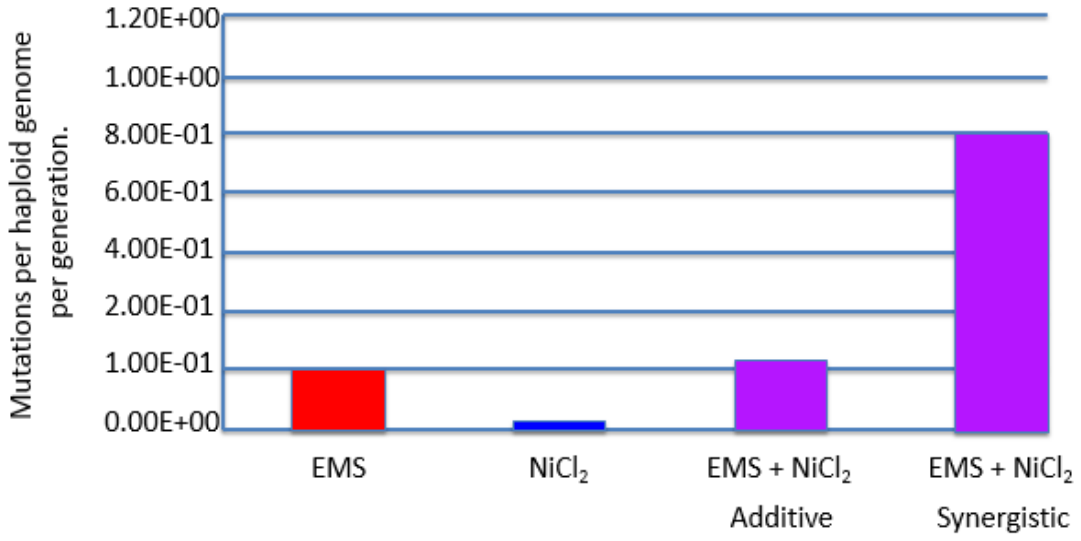
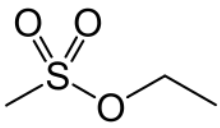


Figure 3: Hypothesized conceptual model of the number of mutations per haploid genome per generation for those groups exposed to each treatment based on previous research studies. Both potential results of an additive and a synergistic effect are shown for the EMS plus nickel group.

Ethyl methanesulfonate (EMS)

Ethyl methanesulfonate (EMS), as explained further in the Methods section, was chosen as the mutagen in this study because it is a commonly used mutagen and carcinogen in the literature. It has been used across many species and types of organisms including *C. elegans* nematodes to study phenotypic mutations when worms are exposed to EMS (Brenner, 1974). Once entering the cells of organisms, the EMS molecule primarily causes harm to the organism by reaching the DNA molecule where the ethyl group from the EMS molecule is transferred and attaches to the guanine of the DNA molecule, forming 6-Ethylquanine (Sega, 1984). Instead of now pairing with cytosine, the new base incorrectly pairs with thymine as shown in Figure 4.

This results in mutations in the DNA molecule that can further lead to transition, insertion and deletion mutations in the DNA through chromosomal breakage and eventually lead to phenotypic changes (Sega, 1984).



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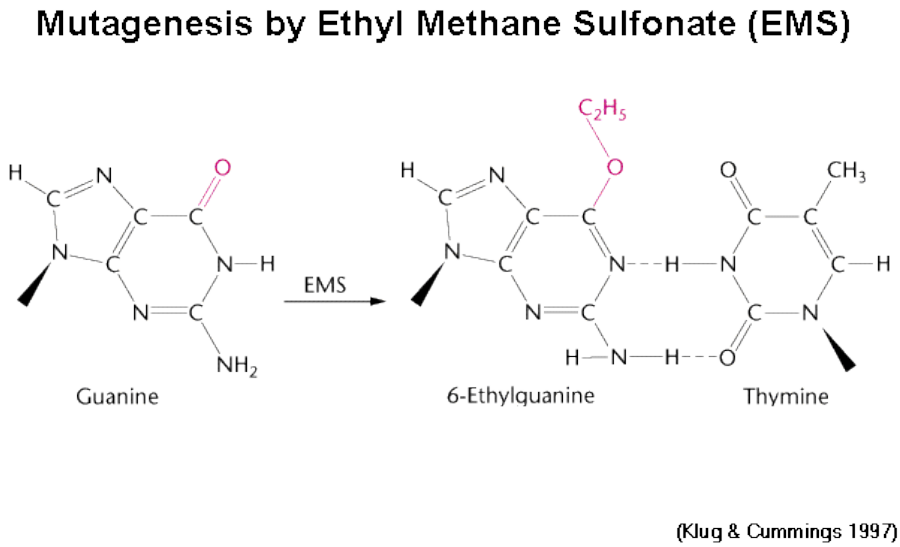

http://www.mun.ca/biology/scarr/EMS_Mutagenesis.html

Figure 4: The structure of EMS (top) and the mechanism of the ethyl group pairing with guanine on a DNA molecule resulting in mutations (bottom).

Objectives

The objectives of this research were to:

1. Explore the genotoxic interaction of nickel alone.
2. Explore the interactions between nickel and another mutagen (EMS).
3. Determine if nickel has an additive or synergistic effect when combined with another mutagen.
4. Determine if non-genotoxic effects from nickel are having an impact on the production of the next generation.

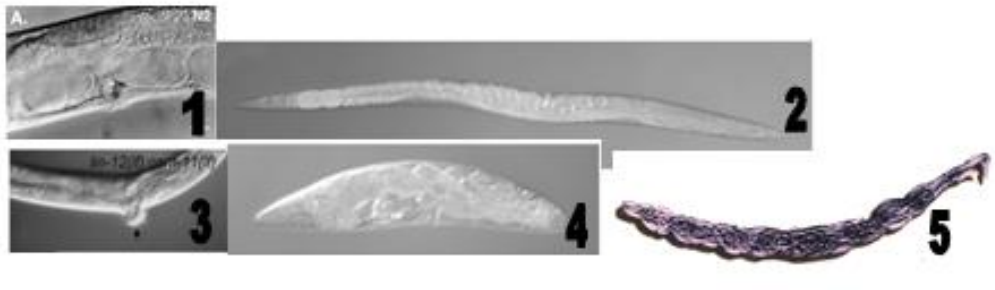

Figure 5: Examples of mutant phenotypes of *C. elegans*: (1) wild-type vulva, notice the relatively smooth appearance around the vulva; (2) wild-type phenotype, notice the smooth and long appearance; (3) protruding vulva (Pvul), notice the vulva protrudes as compared to wild-type in 1; (4) dumpy (Dpy) phenotype, notice the shorter and fatter appearance as compared to 2; and (5) Egl (egg-laying defective) phenotype, notice due to a vulva mutation where the eggs cannot be laid outside the body, the young worms hatch and develop inside the adult hermaphrodite. Images from Eisenmann, et al. 2000, Komatsu, et al. 2008, Page, et al. 2007 and Blaxter, 2010.

Materials and Methods

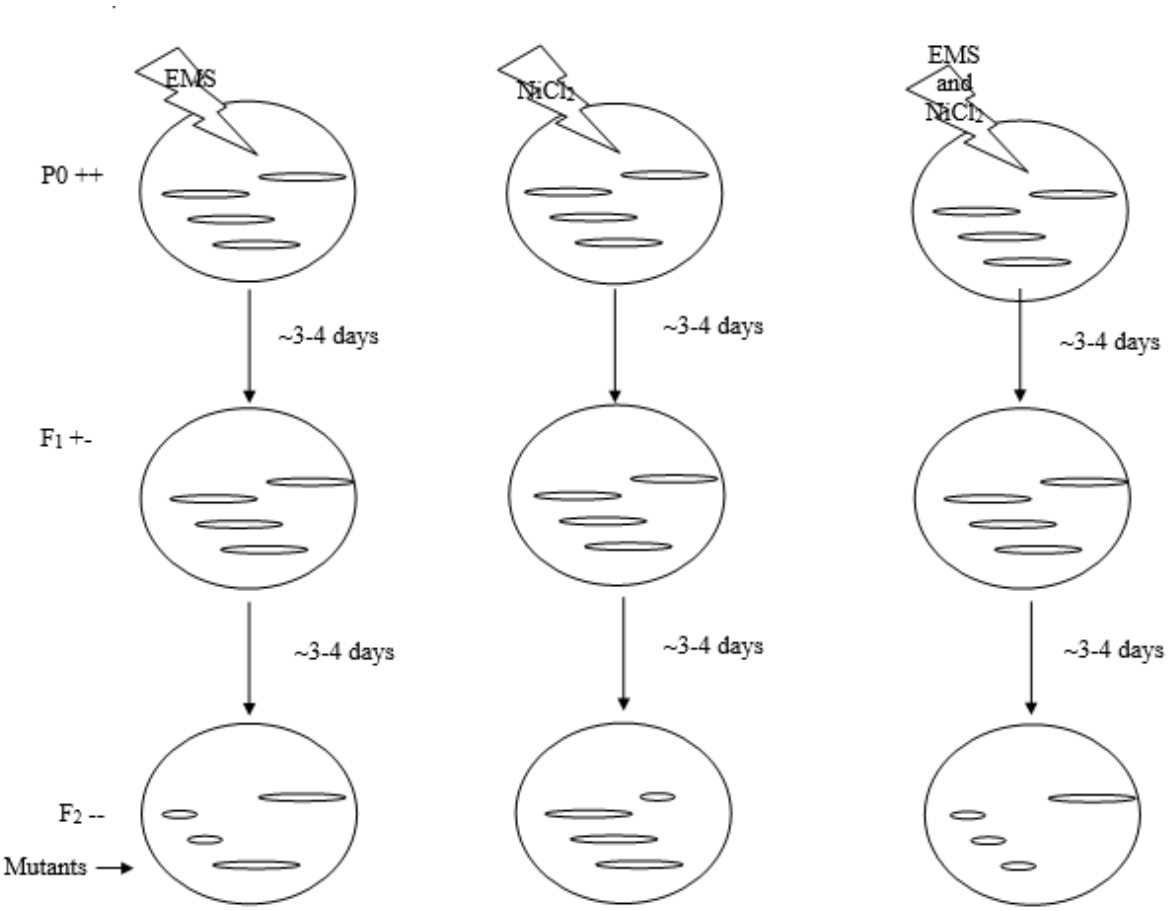

Figure 6: Schematic of the mutagenesis protocol. As an example the dpy phenotype is given. Dpy phenotypes are the shortened worms and the normal phenotypes are the longer worms.

C. elegans was chosen as an appropriate model organism to observe the effect of nickel in this study due to its ease of care, short generation time, and its widespread use in the scientific community. Two strains were used for this study including N2, the wild-type strain, and KX84, a strain containing a loss-of-function mutation in *ced-3* (n2452) and therefore cannot prematurely

undergo apoptosis. During development *C. elegans* has four larval stages (named L1, L2, L3 and L4) before reaching the adult stage. It is at the adult stage that individual worms become reproductively mature and can mate. Individual worms were chosen from the L4 stage because it is at this stage that they are virgins and would not have been able to mate.

In order to test the mutagenic effects of nickel, a genetic screen was performed using the nematode *C. elegans* animal developmental genetic model. For this study, three treatments were conducted: one group was exposed to nickel only, another group was exposed to a mutagen only, and the final group was exposed to nickel and the mutagen together (Fig 6). Two generations later, when the mutagenic effects were expressed, the number and kind of morphological mutants in the nickel-exposed group were compared to both those in the EMS-exposed group and those in the nickel plus EMS group. The parental group of worms that were directly exposed to nickel and/or the standard mutagen were designated as the P0 generation, the progeny of the P0 generation were designated as the F1 generation, and the progeny of the F1 generation were designated as the F2 generation. In order to compare treatments, the offspring (the F2 generation) of 1,200 F1s worms will be screened for each of the three treatments. As *C. elegans* is diploid, this translates into 2,400 haploid genomes being screened per treatment.

$NiCl_2$, a soluble form of nickel, was used, as the nematodes are grown in the isolated environment of the petri dish so the worms were exposed to constant Nickel (II) concentration levels. Preliminary studies were conducted in order to determine which concentration of $NiCl_2$ to use. In those preliminary studies, groups of *C. elegans* were grown on different concentrations of $NiCl_2$ and their response was analyzed. The worms appeared to grow normally when grown on 25 µg/L $NiCl_2$ plates. However, when grown on plates with 50 µg/L of $NiCl_2$, the worms grew

slowly, and at 100 µg/L, the worms did not survive. Therefore, 25 µg/L of $NiCl_2$ was selected as the dose before development and survival became compromised.

Ethyl methanesulfanate (EMS) was chosen as a commonly used mutagen found in the literature that primary causes direct changes to exposed DNA molecules. It has been shown that EMS as a carcinogenic compound can produce transition, insertion and deletion mutations in the DNA through chromosomal breakage (Sega, 1984). EMS has shown to primarily cause ethylation changes of the DNA molecule (Sega, 1984) resulting in nucleotides incorrectly pairing as explained in the Introduction.

Individual Trial Protocols

These protocols were used for the N2 strain and then repeated in the KX84 strain. As mentioned above, the N2 strain is the wild-type strain and the KX84 ((*ced-3*(n2452) strain has a non-functional ced-3 gene.

For EMS only mutants

A *C. elegans* hermaphrodite culture was grown to maturity on a 60 mm petri dishes according to Brenner, et al., 1974. A plate populated with *C. elegans* worms (+/+) was used. The worms were washed off the plate with PBS into a 10 ml conical tube, which was centrifuged at 6°C at 1200 rpm for five minutes. The nematodes were subsequently washed three times with PBS and centrifuged at 1200 rpm in the same conical tube. Bacteria tends to remain in the supernatant as the worms pellet, thus separating the worms from the majority of the bacterial food contaminant from the petri dish. The worms were incubated with 4 mL of 0.2 M EMS in PBS. The top of the conical tube was wrapped with parafilm and mixed by inversion. The worms were mixed on a rocker for four hours to mix in the EMS mixture at lab room temperature. Afterwards, they were washed four times with PBS. The worms were then put onto a large plate with a glass pipette. The worms were left to recover for an hour. Twenty young P0 adults were plated individually onto twenty small plates. Worms were grown for three more days at 20°C. Three days later, ten L4 F1s (potentially m/+) each were taken from the twenty different plates and plated onto new small individual plates for a total of 200 worms on 200 plates. In some cases, not all the P0 worms grew and the remaining F1s were taken from the other plates. The worms were left to grow for four days at 20°C temperature. After about four days, the F2s (potentially m/m) were screened for mutant phenotypes. It is expected based upon our EMS concentration and treatment

to find about 20 mutants per 100 F1s for the EMS only group based on previous research (Brenner, 1974).

For $NiCl_2$ only mutants

A *C. elegans* hermaphrodite culture was grown to maturity on a 60 mm petri dishes according to Brenner, et al., 1974. A plate populated with *C. elegans* worms (+/+) was used. The worms were washed off the plate with PBS into a 10 ml conical tube. The worms in the conical tubes were centrifuged at 6°C at 1200 rpm for five minutes. The nematodes were subsequently washed three times with PBS and centrifuged at 1200 rpm in the same conical tube. Bacteria tends to remain in the supernatant as the worms pellet, thus separating the worms from the majority of the bacterial food contaminant from the petri dish. Subsequent to that, worms were placed onto a seeded 25 µg/L $NiCl_2$ plate for 48 hours at lab room temperature. The worms were washed off the nickel plate with PBS into a 10 ml conical tube, which was centrifuged at 6°C at 1200 rpm for five minutes four times. The worms were incubated with 4 mL of 25 µg/L $NiCl_2$. The top of the conical tube was wrapped with parafilm and mixed by inversion. The worms were mixed on a rocker for four hours to mix in the nickel mixture at lab room temperature. Afterwards, they were washed four times with PBS. The worms were then put onto a large plate with a glass pipette. The worms were left to recover for an hour. Twenty young P0 adults were plated individually onto twenty small plates. Worms were grown for three more days at 20°C. Three days later, ten L4 F1s (potentially m/+) each were taken from the twenty different plates and plated onto new individual small plates for a total of 200 worms on 200 plates. In some cases, not all the P0 worms grew and the remaining F1s were taken from the other plates. The worms were left to grow for three days at 20°C temperature. After about four days, the F2s (potentially m/m) were screened for mutant phenotypes. Although it isn't known how many mutants to expect for the

nickel only group, few mutants are expected if nickel works indirectly as an enhancer of other mutagens.

For EMS and $NiCl_2$ mutants

A *C. elegans* hermaphrodite culture was grown to maturity on a 60 mm petri dishes according to Brenner, et al., 1974. A plate populated with *C. elegans* worms (+/+) was used. The worms were washed off the plate with PBS into a 10 ml conical tube. The worms in the conical tubes were centrifuged at 6°C at 1200 rpm for five minutes. The nematodes were subsequently washed three times with PBS and centrifuged at 1200 rpm in the same conical tube. Bacteria tends to remain in the supernatant as the worms pellet, thus separating the worms from the majority of the bacterial food contaminant from the petri dish. Subsequent to that, worms were placed onto a seeded 25 µg/L $NiCl_2$ plate for 48 hours at lab room temperature. The worms were washed off the plate with PBS into a 10 ml conical tube, which was centrifuged at 6°C at 1200 rpm for five minutes. The nematodes were subsequently washed three times with PBS and centrifuged at 1200 rpm in the same conical tube. As mentioned before, bacteria tends to remain in the supernatant as the worms pellet, thus separating the worms from the majority of the bacterial food contaminant from the petri dish. The worms were incubated with 4 mL of 0.2 M EMS in 25 µg/L $NiCl_2$ PBS. The top of the conical tube was wrapped with parafilm and mixed by inversion. The worms were mixed on a rocker for four hours to mix in the EMs and nickel mixture at lab room temperature. Afterwards, they were washed four times with PBS. The worms were then put onto a large plate with a glass pipette. The worms were left to recover for an hour. Twenty young P0 adults were plated individually onto twenty small plates. Worms were grown for three more days at 20°C. Three days later, ten L4 F1s (potentially m/+) each were taken from the twenty different plates and plated onto new individual small plates for a total of 200 worms on 200 plates. In some

cases, not all the P0 worms grew and the remaining F1s were taken from the other plates. The worms were left to grow for three days at 20°C temperature. After about four days, the F2s (potentially m/m) were screened for mutant phenotypes. Although it isn't known how many mutants to expect for the nickel plus EMS group, exponential numbers of mutants are expected if nickel works indirectly as an enhancer of EMS and other mutagens.

For control group

A *C. elegans* hermaphrodite culture was grown to maturity on a 60 mm petri dishes according to Brenner, et al., 1974. A plate populated with *C. elegans* worms (+/+) was used. The worms were washed off the plate with PBS into a 10 ml conical tube, which was centrifuged at 6°C at 1200 rpm for five minutes. The nematodes were subsequently washed three times with PBS and centrifuged at 1200 rpm in the same conical tube. Bacteria tends to remain in the supernatant as the worms pellet, thus separating the worms from the majority of the bacterial food contaminant from the petri dish. The worms were incubated with 4 mL of PBS. The top of the conical tube was wrapped with parafilm and mixed by inversion. The worms were mixed on a rocker for four hours to mix in the PBS at lab room temperature. Afterwards, they were washed four times with PBS. The worms were then put onto a large plate with a glass pipette. The worms were left to recover for an hour. Twenty young P0 adults were plated individually onto twenty small plates. Worms were grown for three more days at 20°C. Three days later, ten L4 F1s (potentially +/+) each were taken from the twenty different plates and plated onto new small individual plates for a total of 200 worms on 200 plates. In some cases, not all the P0 worms grew and the remaining F1s were taken from the other plates. The worms were left to grow for four days at 20°C temperature. After about four days, the F2s (potentially +/+) were screened for mutant

phenotypes. It is expected as a control the number of mutants found reflects the number of spontaneous mutations for each strain.

Results

N2 (wild-type) strain

The screens of the EMS exposed group found a similar number of mutants as described in similar studies in the literature (Figure 5, Table 1, Table 2). Previous research would suggest that about 20 mutants would be expected to result per 100 F1s for the EMS only groups (or 20%). This is not surprising as an average of 46 mutants per 200 F1s (or 23%) were present during the screen trials of this project and is a $30^{2}/_{3}$-fold increase in mutants as compared to the control group. The control group (N2 strain exposed to PBS) only showed an average of 1.5 mutants per 200 F1's analyzed and indicates only few spontaneous mutations in this strain under laboratory conditions (Figure 7, Table 1).

The screens of the nickel exposed group resulted in low numbers of mutants and indicated that by itself, nickel was indeed a mild mutagen at the concentration tested (Figure 5, Tables 1, Table 2). The trials of this project found a consistent number of 5 mutants per 200 F1s and is a $3^{1}/_{3}$-fold difference when compared to the control group at an average of 1.5 mutants per 200 F1s in the control.

Despite the results of the EMS only group and nickel only group following a similar pattern as was hypothesized, when EMS and nickel were combined and exposed to the *C. elegans* worms, the results showed decrease in the number of mutants which was not hypothesized (Figure 5, Table 1, Table 2). If nickel works indirectly as an enabler of other mutagens, then the number of mutants that was hypothesized for this group would be much higher than the EMS only group. The number found during the trials of this project found an average of 33.2 mutants per 200 F1s which is a 22.133-fold increase in mutants as compared to the control group but is only 72.3% of (or a 27.8% decrease as compared to) the EMS only

group. In addition, the results for each treatment were highly variable with the number of mutants found per experiment being as low as 4 mutants and as high as 43 (per 200 F1s).

The low numbers for the EMS and nickel treated group indicated that either nickel is not acting indirectly as an enabler of other toxins or that something else may be occurring to prevent the hypothesized results from being observed. Despite this, the mutant phenotypes that were found have been described in the literature (Figure 6, Table 3).

Mutants were categorized into a small set of known phenotypes. The overall mutant distribution shows that the three most common phenotypes obtained were Pvuls (47.61%), Egl (33.06%) and Dpys (7.48%) and together comprise 88.15% of the mutants obtained. The remaining 11.85% of the mutants comprise the other scored morphological phenotypes (Figure 8, Table 3).

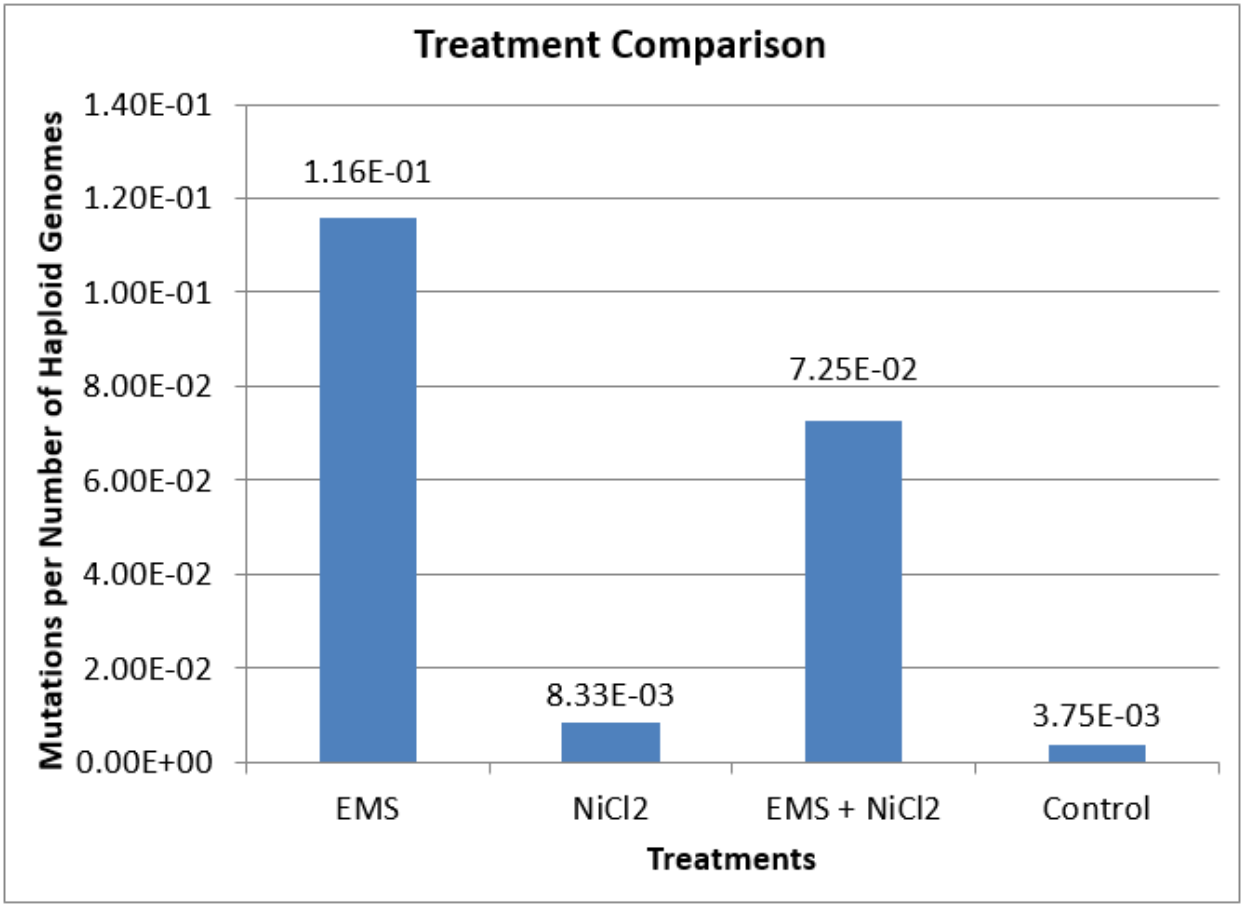

Figure 7: The bar chart and the table showing the treatment comparison of the number of mutants per number of haploid genomes are shown including the control group exposed to standard PBS solution for the N2 strain. Values indicates the number of mutants/generation/haploid genome. The t-test values for a two-tailed and two-sample unequal variance t-test for the different comparisons are as follows with a 0.05 significance value: EMS and $NiCl_2$ was 0.020160783, EMS and EMS+$NiCl_2$ was 0.504989533, EMS and Control was 0.013302901, $NiCl_2$ and EMS+$NiCl_2$ was 0.024123353, $NiCl_2$ and Control was 0.291837701, and EMS+$NiCl_2$ and Control was 0.01299077.

Combined Data

	EMS	**$NiCl_2$**	**EMS + $NiCl_2$**	**Control**
Round 1	N= 400 M= 40	N= 800 M= 5	N= 400 M= 24	N= 800 M= 2
Round 2	N= 400 M= 47	N= 800 M= 5	N= 400 M= 36	N= 800 M= 3
Round 3	N= 800 M= 102	N= 800 M= 10	N= 800 M= 73	N=800 M=4
Round 4	N= 800 M= 89		N= 800 M= 41	
Total M/N	278/2400= **1.16E-01**	20/2400= **8.33E-03**	174/2400= **7.25E-02**	9/800= **3.75E-03**

Table 1: The bar chart and the table showing the treatment comparison of the number of mutants per number of haploid genomes are shown for the N2 strain. N is the number of haploid genomes that were screened and M is the number of mutants that were found for that round and treatment.

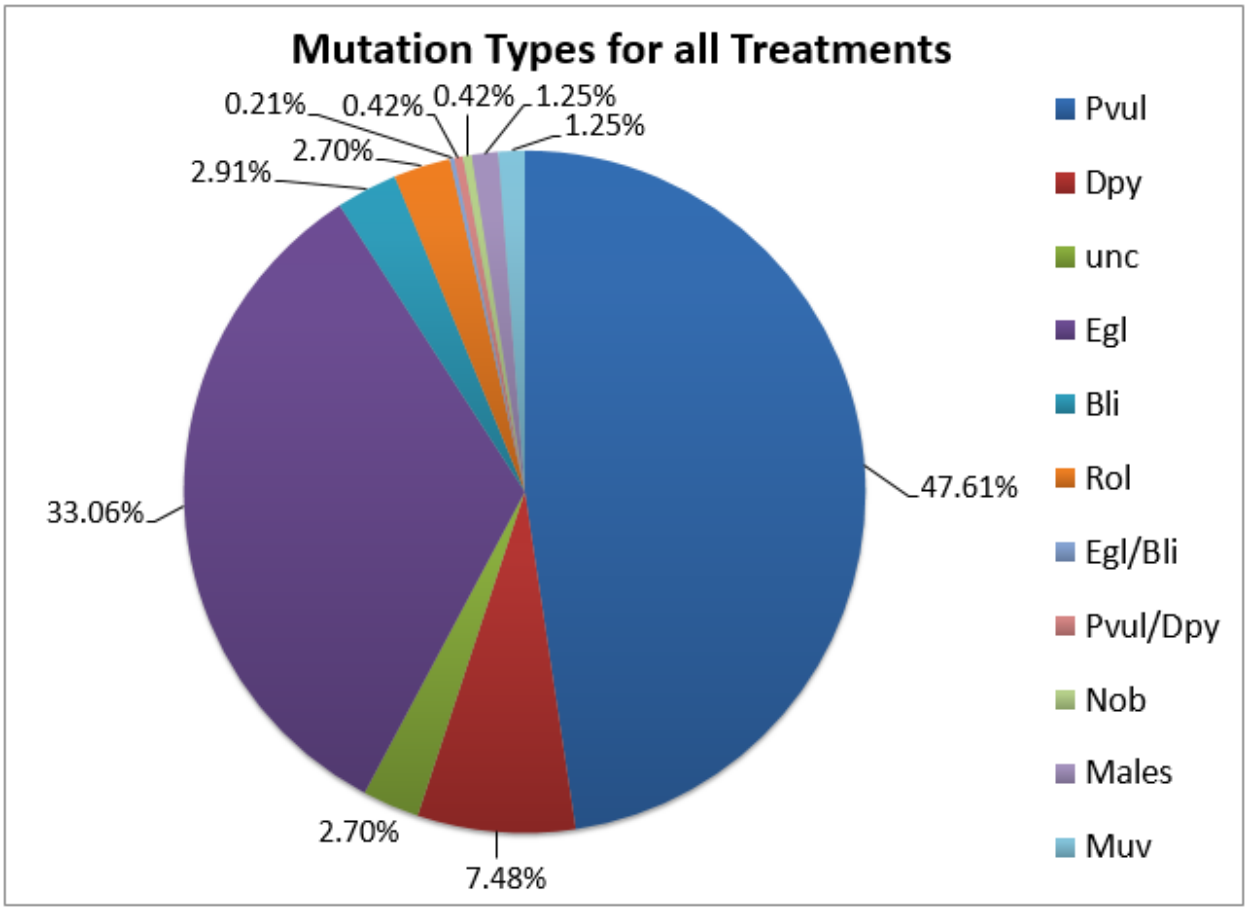

Figure 8: The pie chart showing the breakdown of mutant phenotypes is shown for the N2 strain. Individual phenotypes are described as follows: Pvul (protruding vulva) are worms that develop incomplete vulval development resulting in a protrusion at the vulval site; Dpy (dumpy) are worms that are shorter than wild-type; Unc (uncoordinated) are worms that show deviations in movement from wild-type; Egl (egg-laying defective) are worms that do not lay eggs and also known as bag of worms, i.e. they do not have proper vulva, egg-laying muscles, or enervation, this results in the eggs remaining and potentially hatching inside the mother; Bli (blisters) are worms showing fluid-filled blisters on the cuticle of the worm; Rol (roller) are worms in which the cuticle of the animal contains a twist causing the animal to move in tight circles; Egl/Bli (egg-laying defective and blister double mutant) are worms that show both Egl and Bli characteristics. Pvul/Dpy (protruding vulva and dumpy double mutant) are worms that show both

Pvul and Dpy characteristics. Nob (no back end) are worms with poorly developed posterior regions. Males are male worms and although is a normal phenotype is rare and is generally produced through stress in the laboratory. Muv (multi-vulva) are worms that exhibit multiple vulval protrusions.

Table 2. The breakdown of mutant phenotypes found for each round of treatments for the N2 strain is shown below.

	EMS Only			**$NiCl_2$ Only**			**EMS and $NiCl_2$**			**Control**		
Round	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Number of F1's Used	200	200	400	200	200	400	200	200	400	400	400	400
Pvul	21	35	48	3	0	3	11	15	27	1	3	1
Dpy	9	4	8	0	0	0	2	1	4	0	0	0
Unc	5	1	2	0	0	0	0	0	2	0	0	0
Egl	4	6	37	2	5	7	6	10	37	1	0	3
Bli	0	1	3	0	0	0	3	1	1	0	0	0
Rol	1	0	4	0	0	0	0	3	1	0	0	0
Nob	0	0	0	0	0	0	0	1	1	0	0	0
Muv	0	0	0	0	0	0	0	0	0	0	0	0
Males	0	0	0	0	0	0	0	5	0	0	0	0
Egl/Bli	0	0	0	0	0	0	1	0	0	0	0	0
Pvul/Dpy	0	0	0	0	0	0	1	0	0	0	0	0
Dpy/Egl	0	0	0	0	0	0	0	0	0	0	0	0
Total	**40**	**47**	**102**	**5**	**5**	**10**	**24**	**36**	**73**	**2**	**3**	**4**
Average mutants out of 200 F1 Animals	**46.0**			**5.0**			**33.2**			**1.5**		

Mutation Type	EMS	$NiCl_2$	EMS + $NiCl_2$	Control	Total
Pvul	149	6	69	5	229
Dpy	24	0	12	0	36
Unc	10	0	3	0	13
Egl	76	14	65	4	159
Bli	6	0	8	0	14
Rol	8	0	5	0	13
Egl/Bli	0	0	1	0	1
Pvul/Dpy	0	0	2	0	2
Nob	0	0	2	0	2
Males	0	0	6	0	6
Muv	5	0	1	0	6
Total	278	20	174	9	481

Table 3: The table showing the mutant phenotypes is shown for the N2 strain.

KX84 ((*ced-3*(n2452) strain

The decrease in mutants for the EMS and nickel treatment as compared to EMS only for the N2 strain suggests that perhaps the premature worm gametes were harmed so severely from being exposed to both solutions, that they underwent apoptosis before developing into worms. In order to explore this possibility the treatments and screens were repeated with the KX84 strain. Without a functional ced-3 gene, it is hypothesized that a clearer relationship between the treatments would be revealed indicating either an additive or synergistic relationship.

The screens of the EMS exposed KX84 group found higher levels of mutations as compared to the EMS exposed N2 strain (Figure 9, Table 4). The number of mutants for this group was 59.8333 (per 200 F1s) as compared to 46 for the EMS exposed N2 strain (a 1.3-fold increase). This indicates that potential mutant gametes may have undergone apoptosis before development in the N2 screen.

The screens of the nickel exposed KX84 group resulted in lower average numbers of mutants as compared to the KX84 EMS and EMS plus nickel groups as well but much higher than the nickel exposed N2 group (Figure 9, Table 4). The average number of mutants for this group was 15.33 (per 200 F1s) as compared to the average number of 5 mutants found for the nickel exposed N2 strains (a 3.066-fold increase). This further indicates that nickel is a mutagenic agent by itself. Given the 3.066-fold increase between nickel exposed KX84 and N2 strains compared to the 1.3-fold increase between EMS exposed KX84 and N2 strains, it seems nickel may have toxicity effects in the developing germ cells that trigger apoptosis through mechanisms that do not involve DNA damage.

The results of the EMS plus nickel exposed KX84 group result in a substantially higher number of mutants per generation per haploid genome than in the EMS plus nickel N2 group (Figure 9, Table 4). Putatively agreeing with the hypothesis that nickel can act synergistically with other genotoxic agents. Furthermore, the average number of mutants was 76.67 (per 200 F1s using the KX84 strain) as compared to 33.2 for the EMS plus nickel using the N2 strain (a 2.3 fold increase). The results indicate that the synergistic effect may have been masked by premature nematodes undergoing apoptosis before development into worms.

The distribution of the mutants differed as compared to the N2 strain although the top three most common mutants remained the top most common and together being 97.2% of the mutants found (Figure 10, Table 5, Table 6). Egl mutants became the most common mutant found (70.16%) in this treatment as compared to the N2 strain (33.06%). Pvuls (22.38%) and Dpy (4.66%) mutants were the next most common mutant in this treatment found as compared to the N2 strain as compared to 47.61% for Pvul and 7.48% for Dpy. While Pvul mutants had the

largest percentage of mutants in the N2 strain treatments, Egl mutants became most common in these treatments with the KX84 strain.

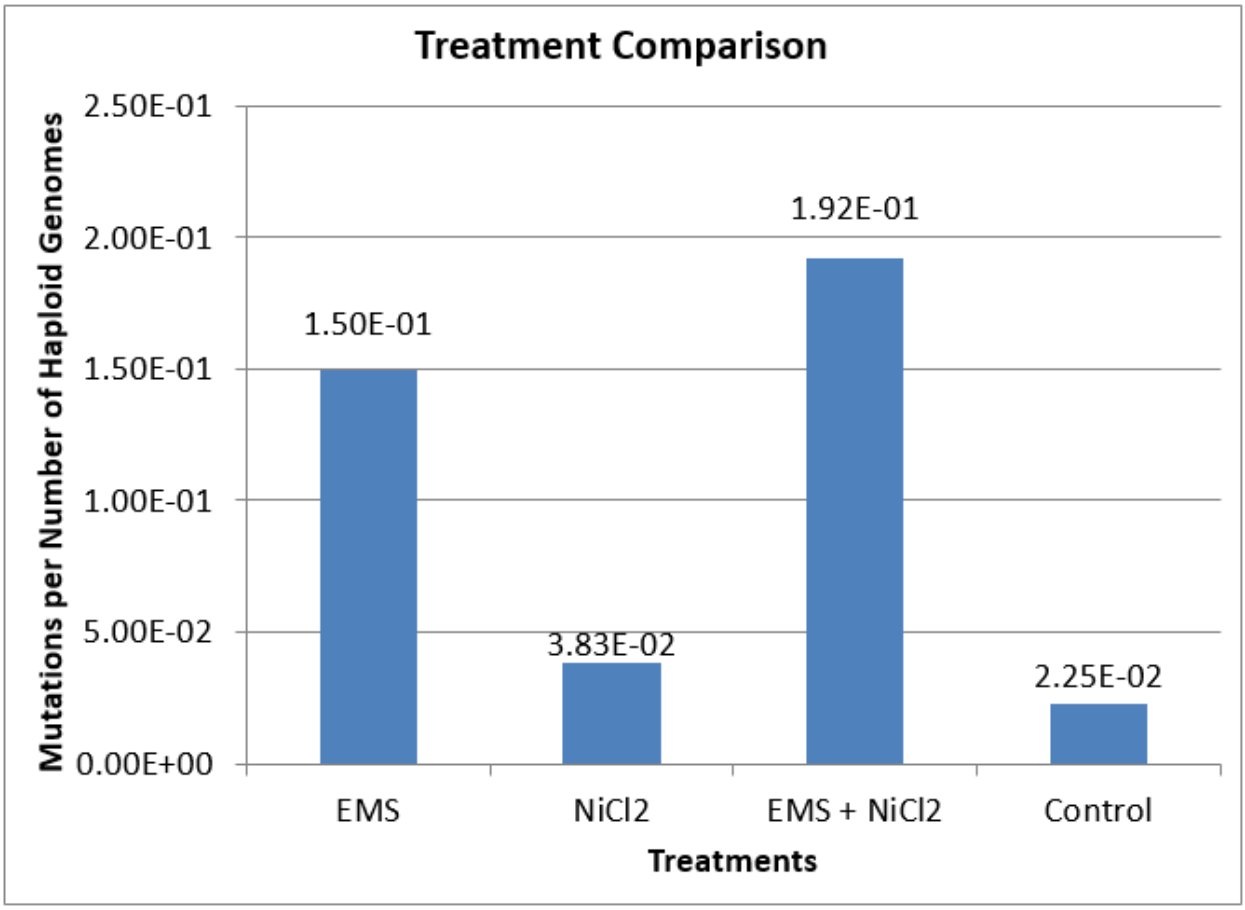

Figure 9: The bar chart and the table showing the treatment comparison of the number of mutants per number of haploid genomes are shown for the KX84 strain. The t-test values for a two-tailed and two-sample unequal variance t-test for the different comparisons are as follows with a 0.05 significance value: EMS and $NiCl_2$ was 0.082537702, EMS and EMS+$NiCl_2$ was 0.656896168, EMS and Control was 0.043098397, $NiCl_2$ and EMS+$NiCl_2$ was 0.048862798, $NiCl_2$ and Control was 0.472972969, and EMS+$NiCl_2$ and Control was 0.027977042.

Combined Data

	EMS	**$NiCl_2$**	**EMS + $NiCl_2$**	**Control**
Round 1	N= 800 M= 122	N= 800 M= 27	N= 800 M= 181	N= 800 M= 22
Round 2	N= 800 M= 129	N= 800 M= 31	N= 800 M= 143	N= 800 M= 13
Round 3	N= 800 M= 108	N= 800 M= 34	N= 800 M= 136	N= 800 M= 19
Total	359/2400=	92/2400=	460/2400=	54/2400=
M/N	**1.50E-01**	**3.83E-02**	**1.92E-01**	**2.25E-02**

Table 4: The bar chart and the table showing the treatment comparison of the number of mutants per number of haploid genomes are shown for the KX84 strain. N is the number of haploid genomes that were screened and M is the number of mutants that were found for that round and treatment.

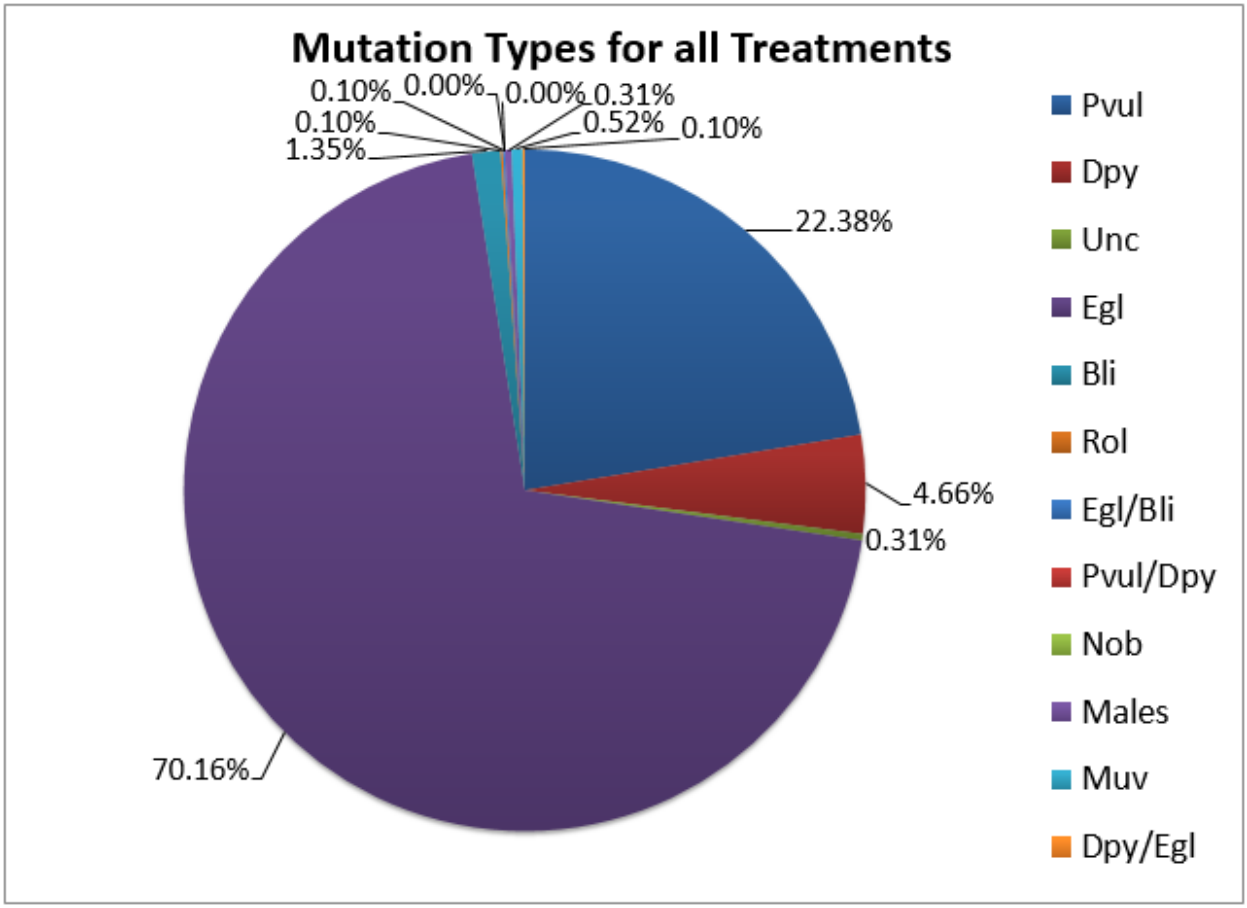

Figure 10: The pie chart showing the mutant phenotypes is shown for the KX84 strain. Individual phenotypes are described as follows: Pvul (protruding vulva) are worms that develop incomplete vulval development resulting in a protrusion at the vulval site; Dpy (dumpy) are worms that are shorter than wild-type; Unc (uncoordinated) are worms that show deviations in movement from wild-type; Egl (egg-laying defective) are worms that do not lay eggs and also known as bag of worms, i.e. they do not have proper vulva, egg-laying muscles, or enervation, this results in the eggs remaining and potentially hatching inside the mother; Bli (blisters) are worms showing fluid-filled blisters on the cuticle of the worm; Rol (roller) are worms in which the cuticle of the animal contains a twist causing the animal to move in tight circles; Egl/Bli (egg-laying defective and blister double mutant) are worms that show both Egl and Bli characteristics. Pvul/Dpy (protruding vulva and dumpy double mutant) are worms that show both

Pvul and Dpy characteristics. Nob (no back end) are worms with poorly developed posterior regions. Males are male worms and although is a normal phenotype is rare and is generally produced through stress in the laboratory. Muv (multi-vulva) are worms that exhibit multiple vulval protrusions

Table 5. The total number of mutants found for each round of treatments is shown below for the KX84 strain. The number of F1s that were used is indicated in the parenthesis of each round.

	EMS Only			**$NiCl_2$ Only**			**EMS and $NiCl_2$**			**Control**		
Round	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Number of F1's Used	400	400	400	400	400	400	400	400	400	400	400	400
Pvul	26	32	22	4	1	4	45	42	34	1	1	4
Dpy	5	5	6	0	0	0	7	7	15	0	0	0
Unc	0	2	0	0	0	0	1	0	0	0	0	0
Egl	85	88	79	21	29	30	123	92	83	20	12	15
Bli	3	2	1	1	1	0	1	1	3	0	0	0
Rol	1	0	0	0	0	0	0	0	0	0	0	0
Nob	0	0	0	0	0	0	0	0	0	0	0	0
Muv	2	0	0	0	0	0	2	0	0	0	0	0
Males	0	0	0	1	0	0	2	0	0	0	0	0
Egl/Bli	0	0	0	0	0	0	0	0	1	0	0	0
Pvul/Dpy	0	0	0	0	0	0	0	0	0	0	0	0
Dpy/Egl	0	0	0	0	0	0	0	1	0	0	0	0
Total	**122**	**129**	**108**	**27**	**31**	**34**	**181**	**143**	**136**	**22**	**13**	**19**
Average per 200 F1's Used	**59.833**			**15.33**			**76.67**			**9.00**		

Mutation Type	EMS	$NiCl_2$	EMS + $NiCl_2$	Control	Total
Pvul	80	9	121	6	216
Dpy	16	0	29	0	45
Unc	2	0	1	0	3
Egl	252	80	298	47	677
Bli	6	2	5	0	13
Rol	1	0	0	0	1
Egl/Bli	0	0	1	0	1
Pvul/Dpy	0	0	0	0	0
Nob	0	0	0	0	0
Males	0	1	2	0	3
Muv	2	0	2	1	5
Dpy/Egl	0	0	1	0	1
Total	359	92	460	54	965

Table 6: The table showing the mutant phenotypes is shown for the KX84 strain.

Discussion

Nickel is mutagenic to animals. If the nickel works as an enhancer for other mutagens, then the *C. elegans* N2 group exposed to both $NiCl_2$ and EMS should show a synergistic increase of mutants as compared to the groups exposed to each one separately. Based on the results of the parallel screens of the N2 strain, the group exposed to both $NiCl_2$ and EMS didn't produce the synergistic increase of mutants as was hypothesized.

Nickel has a complex *modus operandi* for its toxicological effects. One possible cause for the decrease in mutants for the EMS plus $NiCl_2$ treated N2 strain is that the damaged gametes were so compromised by the exposure to either $NiCl_2$ or EMS, or both $NiCl_2$ and EMS that they undergo apoptosis and die prematurely before developing into a zygote that will mature into a worm that would then display a mutant phenotype. Recall the toxic effects of nickel triggers apoptosis potentially through multiple pathways, perhaps having a substantial effect through a non-genotoxic mechanism. If this is true, more phenotypically identifiable mutations may have occurred but they could not be discerned as the germ cells died. This possibility was explored when the screens were repeated using the KX84 strain of *C. elegans* in which apoptosis cannot occur. The results of this study indicate that nickel in particular may result in premature death of compromised germ cells via apoptosis. Further, the results of those screens showed a potential synergistic relationship of nickel with EMS as previously hypothesized. Thus, nickel putatively works synergistically with environmental genotoxic agents. However, additional repetitions of this experiment in both strains are necessary to further clarify this potentially synergistic action of nickel with the mutagen EMS.

It remains unclear if nickel is only additive with the effect of EMS in the treatment or truly synergistic. Additional studies could explore increased nickel concentrations to determine if

a higher concentration of nickel in conjunction with other genotoxic agents (like EMS) would result in a further enhanced synergistic effect.

The distribution of the mutant phenotypes change between the strains. In the wild type N2 strain, the different mutants phenotypes were more equally distributed in comparison to those from the KX84 strain. In the N2 strain, Pvul, Egl and Dpy phenotypes comprised almost 90% of the mutants (actually 88.15%), with the Pvuls phenotype being the most common. In the KX84 strain, Pvuls, Egl and Dpys made up 97% of the mutant phenotypes (actually 95.2%) with the Egl phenotype being the most common for the mutants. Perhaps this reflects a bias where mutations in particular phenotypic classes of genes are more likely to trigger apoptosis in the developing germ cells; this would result in a differing phenotypic distribution between the two strains. Furthermore, the size and number of the genes associated with the phenotypes found in this study probably had a factor in the types of mutants found. For instance dpy genes are large with an average coding sequence length (nt) of over 2,276 base pairs and are relatively numerous with over 24 dpy-associated genes indicating that they can easily be affected by mutagen exposure. Bli genes have only about 6-bli associated genes but have a large average coding sequence length of over 2,101 bp. Similarly, there are over 4 rol-associated genes with a large average coding sequence length of over 2,715 bp (WormBase, 2016)

The nickel concentration level used in this research project (25 µg/L) reflects environmental concentration levels that individuals may be exposed. In healthy adults, the normal human reference value for nickel is 1 to 3 µg/L in urine and 0.2 µg/L in serum. Minimal Risk Levels (MRLs) have been established for an intermediate-duration inhalation exposure of 15 to 364 days (0.0002 mg Ni/m3) and for chronic-duration inhalation exposure of 1 year of exposure or longer (0.00009 mg Ni/m3). These risk levels compare to concentration levels found

in the air, soil and freshwater in the environment. An estimated average nickel concentration in ambient air levels in the US is 2.22 ng/ m^3. Typical concentrations found in soil range from 4 to 80 ppm. Median nickel concentrations in freshwater sources including groundwater range from 0.05 to 6 µg/L. However these values vary depending on local anthropogenic activity and geology (ATSDR 2005). The United States Environmental Protection Agency has collected data to determine the no-observed-adverse-effect level (NOAEL), the lowest-observed-adverse-effect level (LOAEL) for soluble nickel as well as data about neonatal mortality. The Integrated Risk Information System reports a NOAEL of 5 mg/kg bw (or 100 ppm or 5 mg/kg/day) for soluble nickel salts for rats given nickel in food and water. It also reports a LOAEL of 35 mg/kg bw (1000 ppm) in one study and 50 mg/kg/day in another study for soluble nickel salts for rats given nickel in food and water resulting in decrease body weights. Finally, studies with rats found that the number of offspring decreased, offspring mortality increased and body weights of offspring decreased at 500 ppm or 5 mg Ni/L (or 0.43 mg/kg bw) dose level with a smaller effect at the 250 ppm level (IRIS 1994).

As people become exposed to different environmental toxins in combination with nickel due to anthropogenic activity, certain groups of individuals need to be more vigilant of exposure than others. Those who work directly with nickel and known toxic chemicals such as metal alloy industry workers, smokers and individuals who are pregnant or of child-bearing age in highly urbanized areas may be especially vulnerable while exposed to other toxins. Based on this research, these individuals should limit their exposure to nickel such as not wearing nickel jewelry while handling other potentially harmful substances. Although exposed individuals potentially may not visibly suffer from an abnormality directly, changes in the DNA may be passed on to future generations that could result in inherited developmental problems.

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