

Abstract

Assessing cellular and genomic damage from environmental nickel using a GFP containing strain of *Caenorhabditis elegans*

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Nickel is a naturally found mineral that has become widely used for many electronic devices. As use and subsequent discarding of nickel containing products continues exposure to nickel increases. Nickel can cause external superficial symptoms but if it enters the body the potential exists for genomic damage to occur which could lead to mutation and cancer. Nickel can act by generation of reactive oxygen species, interacting with DNA and altering chromatin wrapping. By utilizing a strain of *C. elegans* with a *ced-1::gfp* fusion protein, that detects apoptotic cells in the germ line, the deleterious effects of nickel can be analyzed. Analyses of varying concentrations of a water-soluble and an insoluble form of nickel have been done. Insoluble nickel is held to be more hazardous because while soluble easily enters and exits the cell insoluble nickel can remain in the cell for extended periods allowing for much greater damage. The results of this study were inconclusive about the effects of soluble versus insoluble nickel. Previous testing using the *C. elegans* germline to assess the effects of nickel have used 12 hour exposures (Kezhou et al. 2010). Tests completed in this study exposed animals to lower concentrations of nickel for their full development. A rise in cell deaths is seen as nickel concentration increases which was quantified with *ced-1::gfp* and Syto12. Analysis using a strain with resistance to heavy metals had no significant increase in engulfments when exposed

to nickel, which shows nickel exposure to have been the cause of increased engulfments seen in the wild-type. A lack of increased engulfments in a strain with a mutation to *cep-1*, the *C. elegans* homolog of p53, indicated damage from nickel is recognized by the p53 damage pathway.

Assessing cellular and genomic damage from environmental Nickel using a GFP containing
strain of *Caenorhabditis elegans*

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Introduction

Nickel

Nickel is the 24th most commonly found element in the Earth's crust (Kasprzak and Sunderman et al. 2003). Initial studies of the effects of Nickel analyzed workers at Nickel refineries and smelting plants (Sunderman 1981). The effects of Nickel upon living organisms are of concern due to the prevalence of Nickel in the manufacturing of electronics, coins, batteries and other products

(EPA). The creation and improper disposal of these items, as well as the burning of fossil fuels, causes air, ground and water pollution (Kasprzak and Sunderman et al. 2003).

Nickel is most harmful as part of a compound, though the metallic form can cause skin irritation (Denkhaus and Salnikow 2002). The toxicity

of nickel is based in part on the solubility of the compound with less soluble compounds having more harmful effects (Kasprzak et al. 2003). Particle size also influences the effects of Nickel exposure. Nanoparticles have a diameter of 1 to 100 nanometers. Their small size means they have massive amounts of surface area per unit of volume, making them more reactive, and can

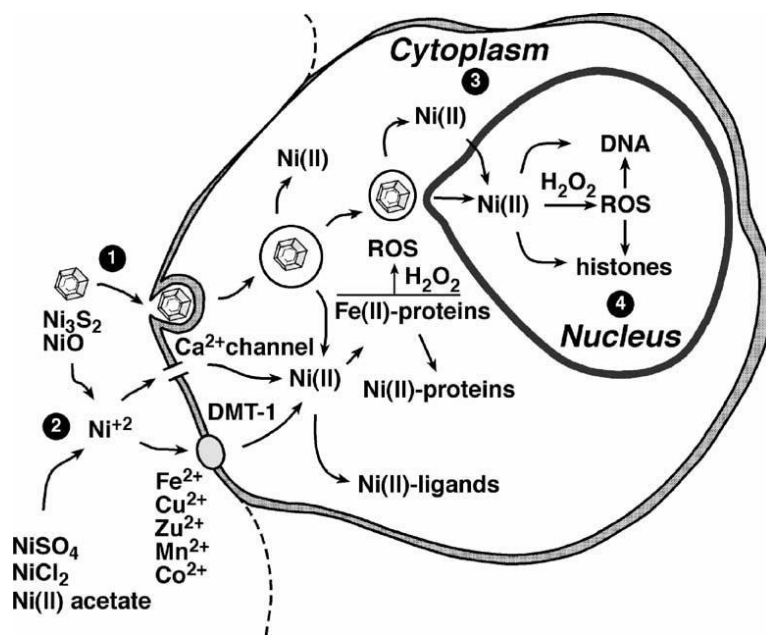


Figure 1. Image showing routes for soluble and insoluble forms of nickel to enter a cell. Note the localization of phagocytized insoluble nickel near the nucleus. Image from Kasprzak and F. W. Sunderman et al. 2003

pass more easily through biological barriers than larger particles (Oberdörster et al. 2005). This study uses NiCl₂, a soluble compound, and powdered NiO, an insoluble form with an average particle diameter of roughly 800 nm. This powdered form has a lot of surface area but less than nanoparticles. Primary routes of human exposure are inhalation, ingestion and direct skin contact. Nickel exposure can lead to dermatitis, lung fibrosis as well as increasing risk of lung, nasal or other cancers (Sunderman 1981, Denkhaus and Salnikow 2002, Kasprzak 2003, Lin and Costa 1994). Cellular uptake of soluble nickel occurs via diffusion and ion transport through calcium and iron channels (DMT-1), whereas insoluble nickel enters by phagocytosis (Figure 1, Kasprzak et al. 2003). Soluble nickel also exits the cell more readily than phagocytized insoluble nickel (Kasprzak et al. 2003). Vacuoles containing this phagocytized nickel congregate near the nucleus of the cell where acidic conditions solubilize the nickel compounds over an extended period (Lin and Costa 1994). Dissolving these insoluble particles can lead to very high concentrations of nickel in the cell (Cangul et al. 2002). Proximity means the, now soluble, nickel can enter the nucleus and cause genomic damage indirectly by generation of reactive oxygen species, which can bind to DNA or other cellular components. Nickel can also affect it directly by intercalating with the DNA, cross-linking DNA, base changing, increasing DNA methylation, which can cause gene inactivation, and altering chromatin binding by attaching to histones, which can silence gene expression (Kasprzak et al. 2003, Nackerdien et al. 1991, Costa et. Al 2003, Cangul et al. 2002)). Such effects can be mutagenic or carcinogenic unless detected and repaired by the cell.

Recently testing was done by Chandler Douglas on how Nickel exposure affects survivorship and brood counts of *Caenorhabditis elegans* and *Pristionchus pacificus*, another species of Nematode (Rudel et al. unpublished). In his work he noted that fecundity decreased in

worms exposed to Nickel, which may, among other things, indicate higher levels of cell death in the germ line.

Caenorhabditis elegans

Caenorhabditis elegans is a ground dwelling roundworm commonly used for toxicological assays. They have a rapid, well documented life cycle, and are easily cultured in a laboratory setting (Figure 2). Their short life cycle means they can be evaluated throughout development. They can also be frozen for long term storage. They occur as hermaphrodites (XX) and Males (XO). Males appear due to nondisjunction during meiosis.

C. elegans serves as a useful model organism because many of its molecular pathways have been mapped, using easily acquired mutants, and are homologous to other organisms. The *C. elegans* genome has been fully sequenced which allows for analysis of gene expression and generation of transgenes containing GFP. Additionally, due to the level of study on *C. elegans* powerful tools are available, such as Wormbook, Wormbase, WORMATLAS and the Caenorhabditis Genetics Center at the University of Minnesota for strain acquisition, as well as the expertise of many researchers in institutions around the world. These factors, and the presence of nematodes in every environment, make *C. elegans* useful for analyzing the effects of soil contaminants on humans.

C.
elegans has a
 fixed cell
 lineage; each
 hermaphrodite
 produces 1090
 somatic cells
 (Meier et al.
 2000).
 However,
 during
 development

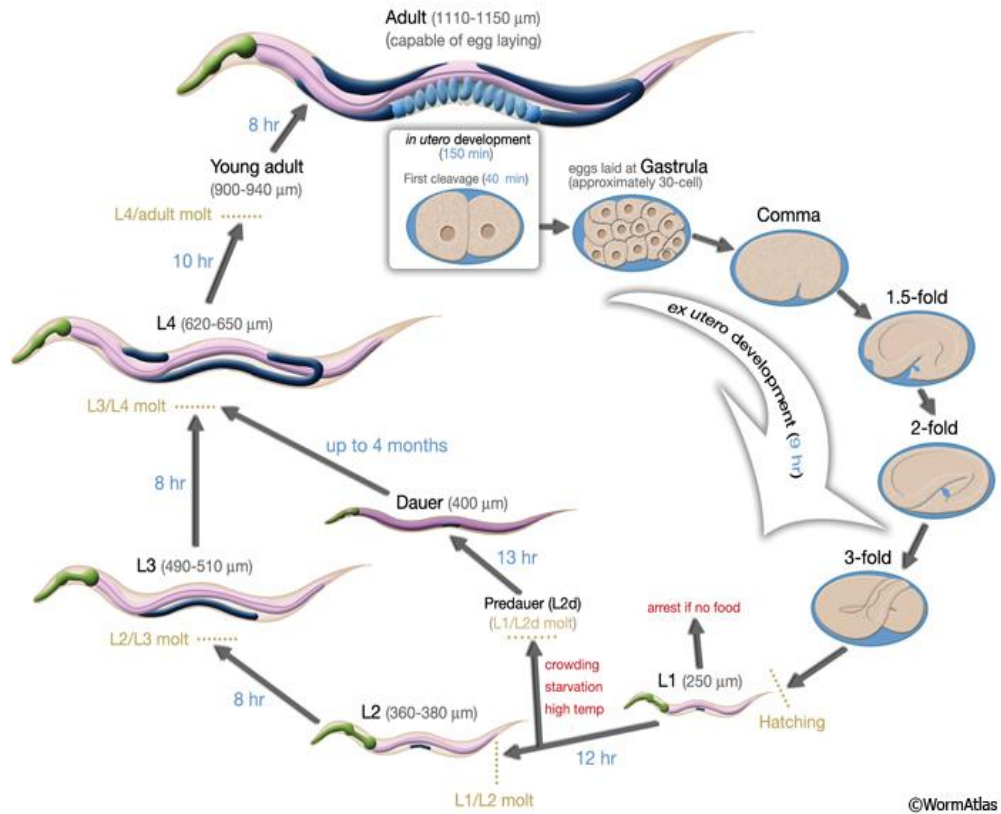


Figure 2. Complete developmental cycle for *C. elegans* hermaphrodite at 22°C. Gonadal arm development appears in dark blue. At 20°C 60 hours pass between L1 hatching and initial egg deposition. Taken from WormAtlas.org.

131 of these
 cells are
 removed by
 apoptosis. After this point programmed cell death generally will not occur in somatic cells, as the worm lacks the ability to regrow lost or damage somatic cells (Gartner et al. 2000). Once fully grown, cell death only happens to germ cells in the gonadal arms.

C. elegans hermaphrodites have two rotationally symmetrical gonadal arms, while males only have one. Hermaphrodite gonadal arms begin dorsal of the vulva, extend half the body length then reflex and return to the vulva (Fig 3.) The gonadal arm is enclosed by a layer of sheath cells to separate the developing oocytes from the rest of the body. Hermaphrodites produce roughly 300 sperm during early adulthood, which get stored in the spermatheca,

afterwards only oocytes are produced. This means a hermaphrodite cannot continue producing offspring after it has used all its sperm, unless inseminated by a male. Additionally, male sperm are larger and outcompete the hermaphrodite's sperm (Lamunyon and Ward 1999). The gonadal arms are enclosed by a layer of sheath cells to separate the germ cells from the rest of the body and direct them as they undergo development.

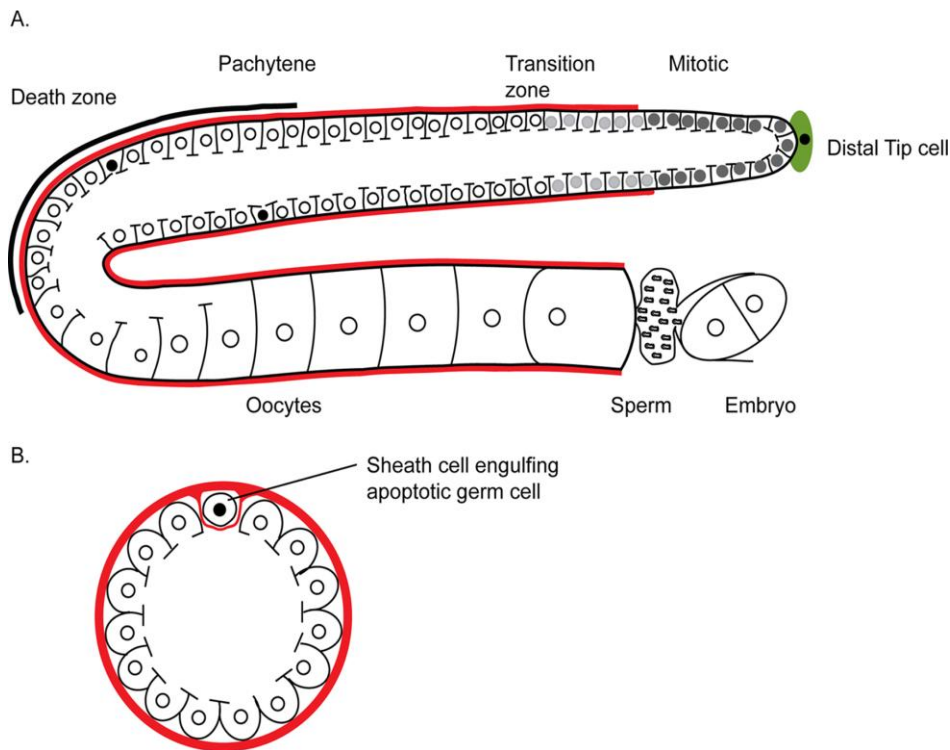


Figure 3. This diagram provides a schematic of the shape and setup of the gonadal arm. The distal tip cell provides a niche for stem cells. Sperm are stored in the spermatheca next to the vulva. Taken from Gartner et al. 2008

Oocytes are constantly generated by germline stem cells in the distal portion of the adult hermaphrodite gonad (Fig. 3). These germ cells are not entirely enclosed by membranes; they share a common cytoplasm (Gartner et al. 2000). They exit the mitotic region and

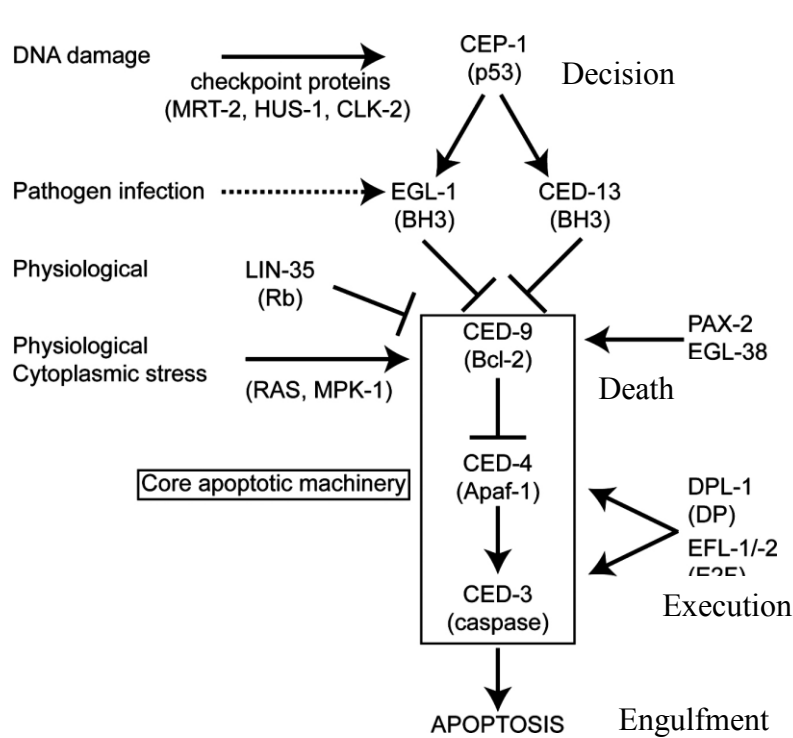
enter the meiotic zone around the bend of the arms. In this region, assessment of their genomic stability and general suitability occurs. If the checkpoint is passed the oocyte will continue through the gonadal arm be fertilized and deposited. If the oocytes are unsuitable or programmed to die, as in the case with nurse cells which get destroyed to provide their nutrients

to other cells, the cell cycle stops and they undergo apoptosis then engulfment by the surrounding sheath cells.

Cell Death pathway

The mechanisms of Programmed Cell Death (PCD) were originally elucidated by Robert Horvitz and John Sulston (Sulston and Horvitz 1977, Sulston et al. 1983). While mapping the lineages of all the cells in *C. elegans*, Sulston noticed that certain cells always undergo PCD (131 of 1090 cells generated), which provided reliable subjects for studying the apoptotic processes. The factors involved during apoptosis in *C. elegans* are highly conserved in other animals.

The process PCD, in *C. elegans*, is made up of four steps: Decision, Death, Engulfment



and Degradation (Fig. 4).

During the decision/specification phase factors, like CEP-1, C. Elegans P-53 like protein, a homolog of the human p53 tumor suppressor gene, detect DNA damage or other problems and make the choice to kill the cell (Derry et al. 2001). CEP-1 is required for DNA damage induced apoptosis; (Schumacher 2001). p53

Figure 4. Diagram of the *C. elegans* cell death pathway and human homologs. Multiple factors can influence CED-9 but afterwards the cell fate is largely decided. Modified from Gartner et al. 2008

checks the genome for damage and can initiate repair, stop the cell cycle or initiate apoptosis; p53 is mutated in over 50% of all human cancers (Elmore 2007). During the death phase, EGL-1 bind to CED-9 (CELL Death abnormality 9, Bcl-2 in humans) so it can no longer inhibit CED-4 (Apaf-1 in humans), from activating the CED-3 caspase (Lettre et al. 2004, Meier et al. 2000, Conradt and Xue 2005). The caspase triggers cascade of cellular degradation and DNA fragmentation. The final step involves a neighboring cell engulfing the apoptotic cell due to “eat me” signals on the cell surface (Conradt and Xue 2005). CED-1 is a cell surface receptor that detects an unknown factor expressed by apoptotic cells (Reddien and Horvitz 2004). When this factor is detected CED-1 expression increases and a neighboring cell begins engulfing the dying cell. This increased expression of *ced-1* and its localization to the cell surface provides an easy means for generation of a GFP fusion protein to track cell engulfments (Gartner et al 2008). Fusion proteins are made by inserting the sequence for another protein onto the end of the sequence for a gene of interest. These two proteins get transcribed as a single mRNA then translated as a single peptide. In humans the engulfment of dying cells is done by macrophages. In *C. elegans* this process is carried out by neighboring cells, such as sheath cells in the gonadal arms. Finally, after the cell has been engulfed its degradation is completed by the engulfing cell.

Objectives

1. Determine if nickel increases germline cell death

Syto12 staining and a *ced-1::gfp* fusion protein detecting dying cells were used to quantify the number of cell deaths occurring.

2. Determine whether insoluble nickel results in more cell death than soluble nickel.

Comparisons were made between the deaths caused by NiCl_2 (soluble) and NiO (insoluble).

3. Determine if inhibiting the activity of nickel leads to no increase in cell death.

A strain of worms resistant to heavy metal toxicity, from increased cellular histidine, provided a means to show whether nickel exposure caused increased cell deaths.

4. If nickel is causing cell deaths via genomic damage then increased cell deaths should be abrogated by knocking out the pathway that detects such damage.

Used a *cep-1* (p53) mutant strain to analyze cell deaths from nickel exposure without the inclusion of deaths related to genomic integrity.

Methods

Stock Maintenance

All worm stocks are maintained at 20°C on 60mm NGM agar plates with the OP50 strain of *Escherichia coli* for food until needed for experimentation (Brenner et. Al 1974). Stocks are maintained as hermaphrodite cultures unless needed for genetic crosses.

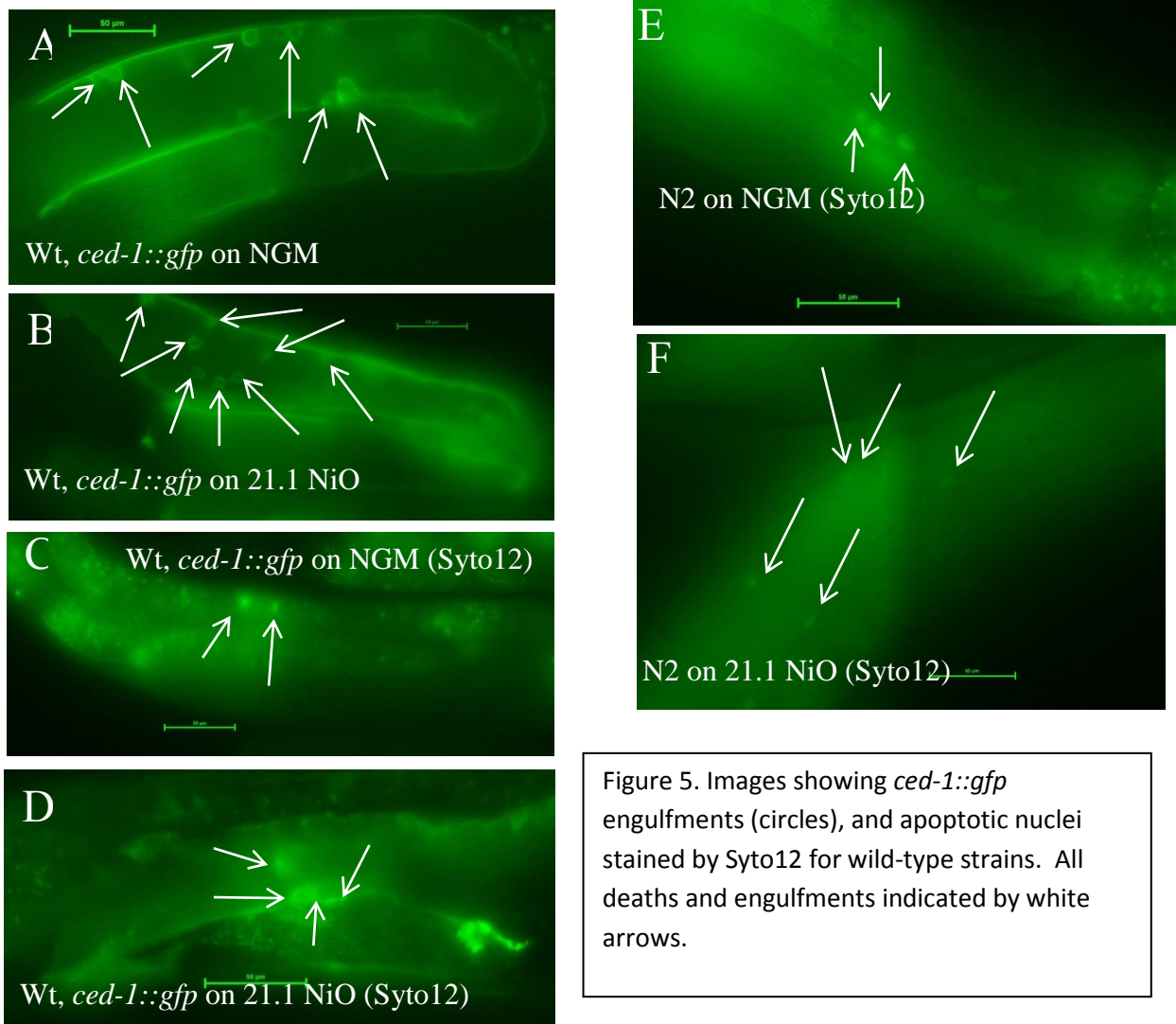
Worm stocks

Three strains of GFP worms were acquired from Dr. Brett Keiper at the Brody School of Medicine. These strains were MD701 (*bcIs39 V [Plim-7::ced-1::gfp; lin-15(+)] V*), KX110 (*ced-9(n1653) mab-5(mv114) III, bcIs39 V [Plim-7::ced-1::gfp; lin-15(+)] V*), and KX84 (*ced-3(n2452), bcIs39 V [Plim-7::ced-1::gfp; lin-15(+)] V*). A *cep-1* mutant, XY1054 (*cep-1(1g12501) I*) was received from the Caenorhabditis Genetics Center (CGC). Additionally, the WU970 (*haly-1(am132) X*) strain, which has a modified histidine ammonia lyase gene, was received from Drs. John Murphy and Kerry Kornfeld at Washington University in Saint Louis.

Gonadal death experiments

Gravid adults and eggs were washed, using M9 buffer, into 15 mL conical vials. Tubes were centrifuged at 800 g for 10 minutes, to pellet the worms and eggs, then supernatant was removed. 1 mL of bleaching solution (five parts Sodium hypochlorite, two parts 1M Sodium Hydroxide, three parts water) was added to the 15 mL tubes which then get mixed vigorously until only eggs remain. Adult bodies dissolve then three cycles of washing with M9 buffer and centrifuging assure that remaining bleach solution is removed and intact eggs remain. After the final centrifugation the excess M9 is removed and the eggs are transferred onto an unseeded plate.

The next day, 50, or more, L1 larvae were removed from the unseeded plate and placed onto an experiment plate seeded with OP50. The worms grew for four days at 20 °C, or 3 days at 25°C in the case of the Kx110 strain. 25°C causes the mutated CED-9 protein to misfold and lose functionality. Finally, the gonadal cell deaths were counted using fluorescent microscopy on a compound scope (Fig. 5).



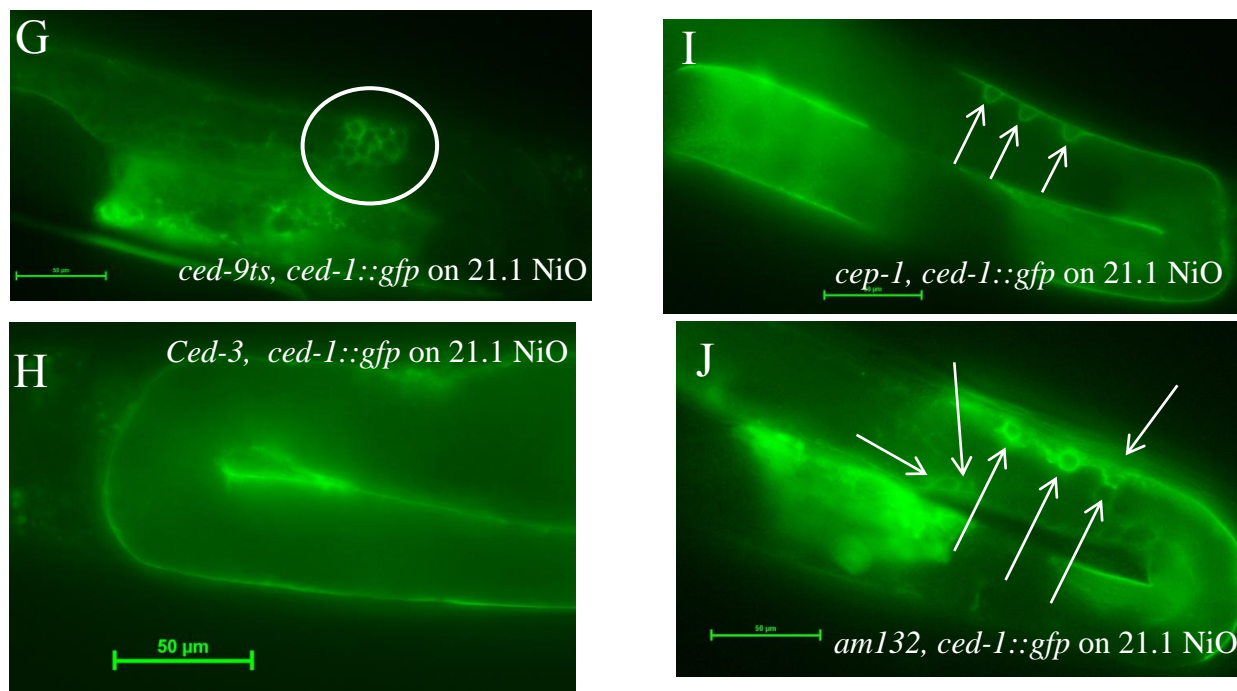


Figure 5 continued. Images with *ced-1::gfp* engulfments labeled for mutant strains

Strain construction

When attempting to crossbreed strains heavily populated, but not starved, plates were placed at 37°C for 30 to 45 minutes, to cause the nondisjunction that generates males. Portions of agar from these plates were then placed onto large plates and placed at 20°C. Two to three days later males were searched for and moved to fresh plates with hermaphrodites to maintain a male stock, as 50% of mated offspring are males. When attempting crosses, eight or more males, of one strain, were placed on a plate with two or three L4 hermaphrodites from another strain. After 10-12 hours at 20°C the males were removed and each P0 hermaphrodite was put on a separate plate. The F1 generation will be heterozygous for both mutations provided mating occurred, which can be seen by the presence of males (Fig. 6). Eight to ten F1 L4

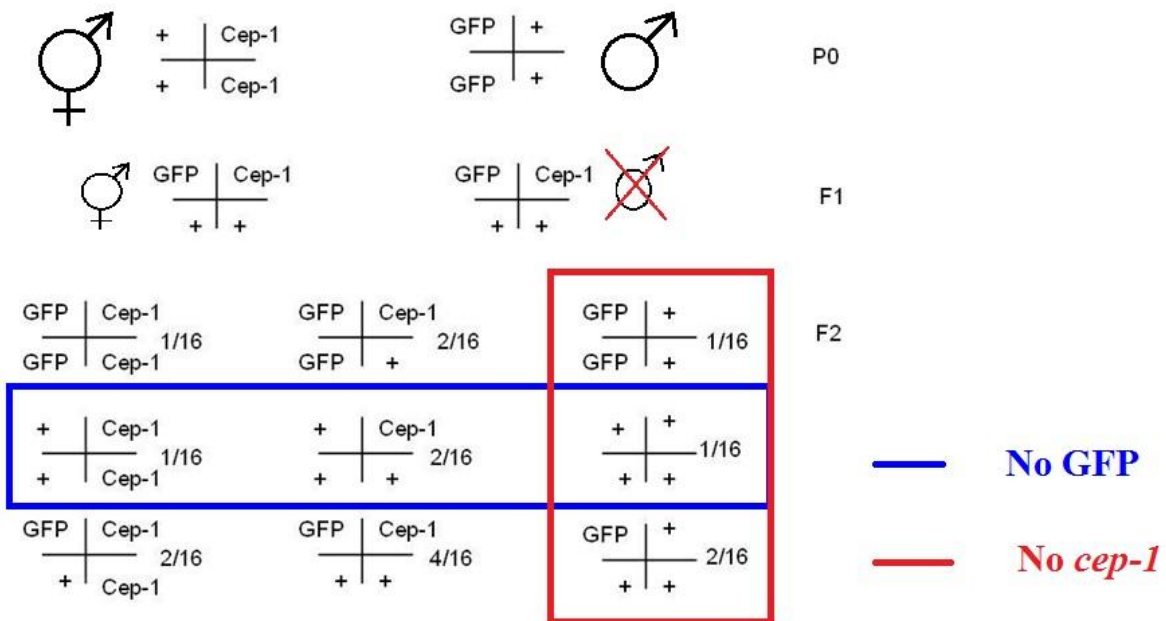


Figure 6. Diagram showing what proportions of each generation should have with desired genes. Boxes in lower portion show F2s lacking either GFP or *cep-1* mutation.

hermaphrodites were placed on separate plates and allowed to deposit eggs. It is important to pick L4s as they will not have been mated with by males.

The rest of this procedure involves checking adults in successive generations for each mutation then continuing their lines. An adult worm (first round with F2s) gets assessed for *ced-1::gfp* by looking at the gonadal arms under fluorescence. If GFP was present this worm was placed on a new plate and allowed to deposit all of its eggs. When the worm had laid all or most of its clutch the adult was removed and assessed for the second mutation. DNA was harvested from the adult worm using 2 mg/mL proteinase K in Single worm lysis buffer at 60°C for 45minutes. Deletion mutations were tracked using PCR, as smaller strands traverse further during gel electrophoresis, while point mutations required sequencing to ensure their presence. If the desired mutation was found the worm's progeny were allowed to grow to L4s then 5-20 of them were then scored for GFP and moved to new plates. This cycle of checking an adult for

GFP, letting it deposit its clutch, and then assessing it for the second mutation continued until the likelihood of it being homozygous for both traits seemed high (likely F6 or F7). At this point, multiple progeny (30+) were selected and assessed for GFP and the mutation. If both were found in all tested individuals then that group of worms would be given a new strain name, used for testing, and monitored to make sure the desired traits remained in later generations.

Syto12 staining

Adults were placed in 33 μ M SYTO 12 in M9 for 1 hour at 20°C and then then returned to the plate they were harvested from for 2 hours at 20°C. Syto12 stains nucleic acids in apoptotic nuclei. The gonadal arms of the worms were then assessed for localized Syto12, which appears as bright green dots.

Microscopy

At test completion worms, or excised gonadal arms, were moved into a drop of 30 mM Sodium Azide in M9, on 4% noble agar pads, before adding a cover slip. Slides were viewed under a GFP cube and either cell engulfments, visible as cell-sized circles in the gonadal arms, or Syto12 dots, were counted at 400x using a Nikon Microphot-FX scope with an HBO 100 W light source and GFP filter cube (Figure 5). To ensure accounting of all deaths and engulfments the fine focus must be used to view all focal planes of the gonadal arm. Images were taken using a Zeiss Axio Observer Z1 scope with HXP 120C fluorescence cube, and Nikon Digital Sight DS-U3 camera.

Soil Survival Analysis

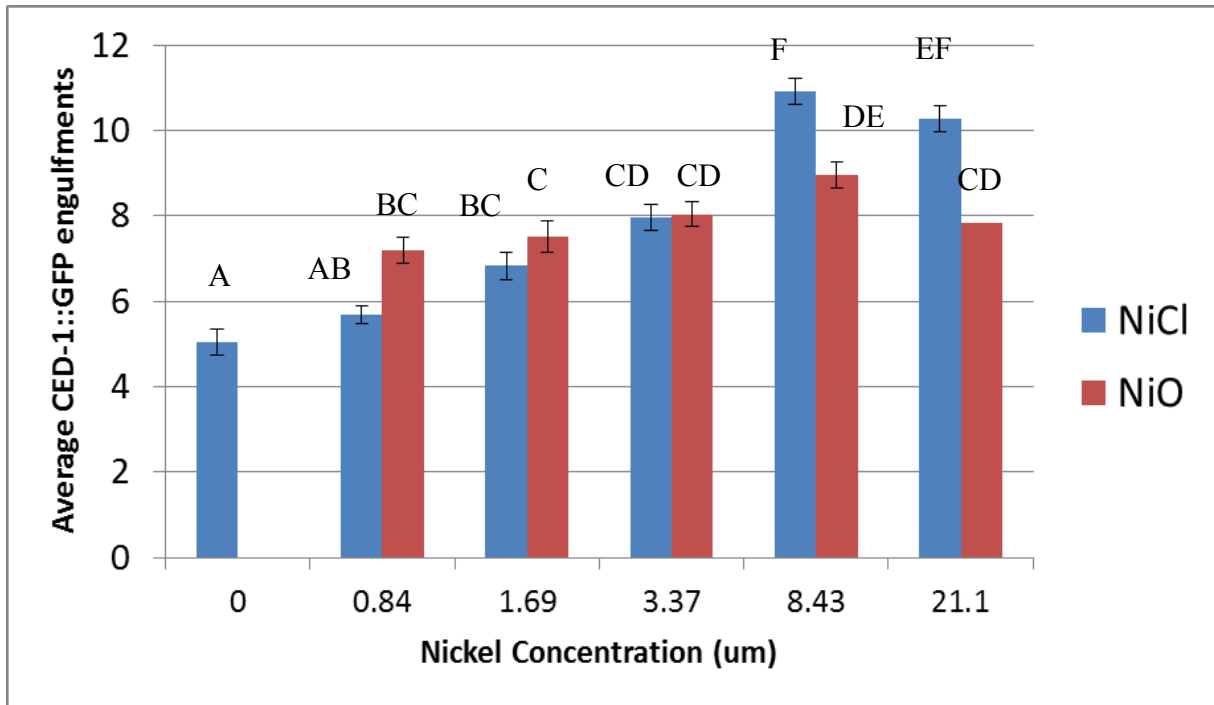
Tests have been done to assess the effect of varying food and sediment quantity on Nematode survival in traditional sediment tests. The protocol is an alteration of Hoss et. al 2009. In accordance with the experiments conducted by Chandler Douglas all tests are done with 10 synchronized L1 larvae and run for 96 hours at 20° C (Rudel et al. unpublished). Tests contain varying quantities of antibiotic killed OP50. The food added contains $\sim 10^{10}$ bacteria and 10 ug Cholesterol per mL.

First, the desired amount of sediment is spread across the bottom of each well in a 12-well plate. Next, Food and worms are added before placing the plate on a shaker table in a 20° C incubator. For each treatment six replicate wells are used per test.

After 96 hours the plates were removed and the contents of each well rinsed out into a 15 mL conical vial. The sediment samples were mixed with a silica based solution (2:1 of Water:Ludox) and centrifuged at 800g for 10 minutes in three successive washes. Adults and larvae float in the Ludox while the sediment pellets out. Supernatant was poured onto unseeded 100mm NGM plates and adults and larvae were harvested and counted. The adults were then measured. Length measurements following the gut tube and width at the anterior-posterior center position of the vulva are found using NIS Elements BR3.2 software with a Nikon Microphot-FX scope.

Statistical analysis

Statistical comparison was done using univariate analysis of variance in SPSS 19 with Tukey post-hoc analysis. All statistical significance was based around a value of 0.05.



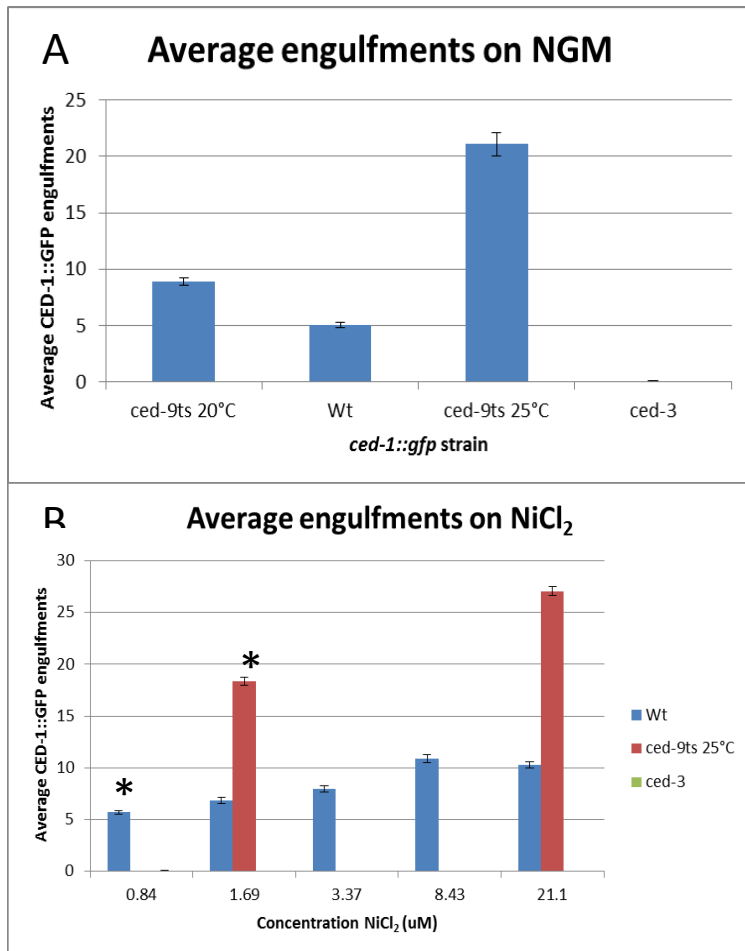
Results

Nickel induces germline apoptosis

To assess the effect(s) of nickel on living organisms, worms grew on agar plates laced with varying quantities of NiCl₂ and then visible engulfments, in the gonadal arms, were counted. The wild-type *ced-1::gfp* strain, MD701, showed increased levels of programmed cell

Ni Type/Conc. (um)	Average deaths	St. Error	N
NGM	5.05	0.3	153
0.84 Cl	5.69	0.21	353
0.84 O	7.19	0.3	150
1.69 Cl	6.83	0.32	150
1.69 O	7.51	0.3	150
3.37 Cl	7.97	0.31	153
3.37 O	8.04	0.37	100
8.43 Cl	10.91	0.32	150
8.43 O	8.96	0.3	150
21.1 Cl	10.29	0.31	152
21.1 O	7.85	0.3	150

Figure 7. Data for MD701 shows a significant increase in engulfments with exposure to Nickel Chloride and Nickel Oxide. Letters denote groupings based on Tukey post-hoc analysis.



Strain/ Conc. NiCl ₂ (uM)	Avg. Deaths	St. Error	N
ced-9ts 20°C NGM	8.9	0.35	151
Wt NGM	5.05	0.25	153
ced-9ts 25°C NGM	21.08	1	150
ced-3 NGM	0.052	0.02	155
Wt 0.84	5.69	0.2	353
ced-3 0.84	0.049	0.02	205
Wt 1.69	6.83	0.32	150
ced-9ts 25°C 1.69	18.37	0.4	92
Wt 3.37	7.97	0.28	153
ced-3 3.37	0.019	0.01	154
Wt 8.43	10.91	0.38	150
ced-3 8.43	0.02	0.01	200
Wt 21.1	10.28	0.33	152
ced-9ts 25°C 21.1	27.08	0.41	99
ced-3 21.1	0.02	0.01	152

Figure 8. Removal of *ced-3* or *ced-9* results in strong effect on engulfments. A shows data for strains on media without nickel. All significantly different from each other. B shows data for strains on Nickel. All mutant strains showed statistically significant difference from wild-type at each treatment level. *ced-3* showed no significant difference in engulfments at any treatment level. * indicates lack of statistically significant difference from no nickel for wild-type and *ced-9ts*.

death as the level of Nickel increased, with the exception 0.84 μM NiCl₂ (Figure 7). Initial testing doubled the concentration of NiCl₂ between levels until 3.37 μM was reached. This

concentration was the largest added to sediment samples during previous experiments in this lab (Rudel et.

unpublished). Above this level concentrations increased by 2.5x between treatments and caused a statistically significant increase in engulfments from 3.37 μ M to 8.43 μ M. Finally, the effect of NiCl₂ decreased between 8.43 μ M and 21.1 μ M, though not significantly.

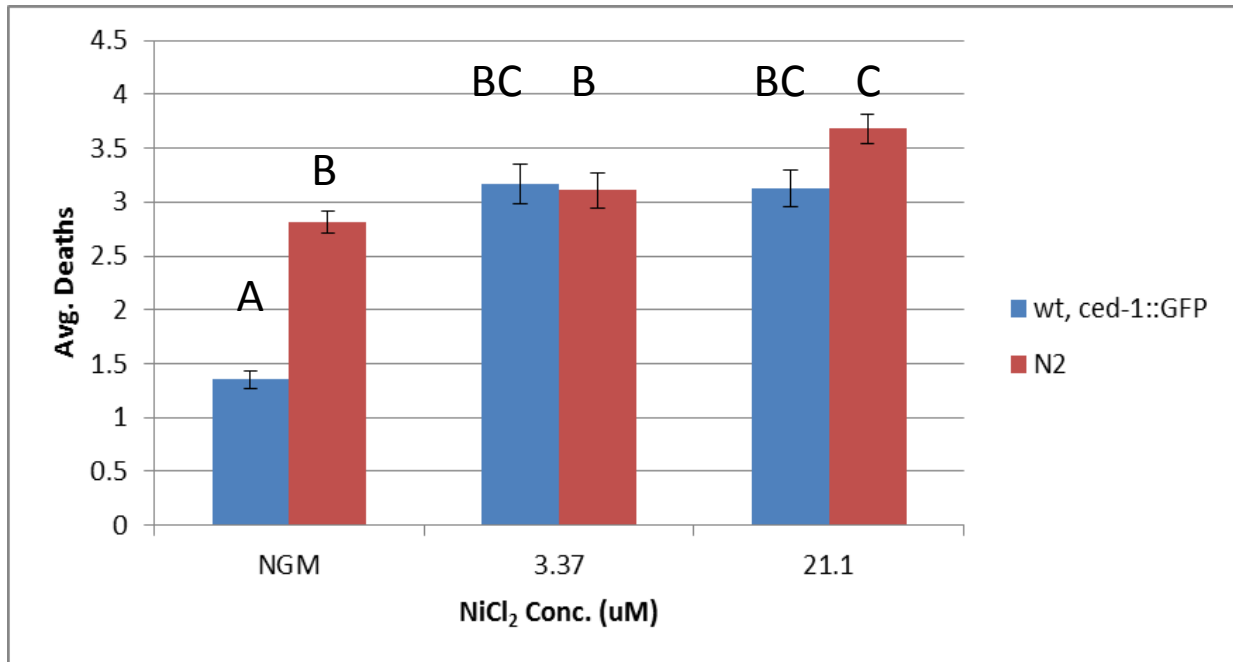


Figure 9. Analysis of changes in cell death via Syto12 staining for N2 and wt, *ced-1::gfp* strains. Groups generated by Tukey post-hoc analysis.

Strain/Conc. NiCl ₂ (uM)	Avg. Deaths	St. Error	N
wt, <i>ced-1::gfp</i> NGM	1.35	0.08	147
N2 NGM	2.81	0.1	318
wt, <i>ced-1::gfp</i> 3.37	3.17	0.18	125
N2 3.37	3.11	0.16	139
wt, <i>ced-1::gfp</i> 21.1	3.13	0.17	136
N2 21.1	3.68	0.14	128

To confirm the validity of the circles seen as programmed cell deaths, a strain lacking Caspase-3 (KX84) and a strain with a temperature sensitive mutation to an element inhibiting programmed cell death (Bcl-2 in KX110), that had been generated and validated by the Keiper lab, were analyzed after growing on plates with nickel (Contreras et al 2011). The strain lacking Caspase-3 served as a negative control because no programmed cell death should occur without the primary effector caspase. In this strain circles occurred once in every 20 to 50 animals and no significant increase in engulfments was seen with the addition of Nickel (Figure 8). On the other hand, when Switched from growing at 20°C to 25°C, the *ced-9ts* strain showed a significantly increased numbers of circles. To observe the increase in circles required removal of the gonadal arms by decapitating worms in 0.25mM levamisole in PBS, so the gonadal arms could be observed without the gut tube affecting visibility. Based on these results, no circles without the Caspase and many more without Bcl-2, we conclude the circles visualized were in fact cell engulfments and indicated cell deaths.

Confirmation by Syto12

To further confirm results seen from counting GFP engulfments the effects of nickel exposure were tested using Syto12 staining. For both N2 and *ced-1::gfp* wild-type a significant increase in cell deaths was seen when exposed to nickel (Fig 9). This confirmed that the circles seen in the gonadal arms of *ced-1::gfp* strains were cell engulfments.

Insoluble versus Soluble nickel

To compare how solubility of nickel affects cell engulfment levels we repeated the same procedure as above. GFP wild-type, worms (MD701) were grown on plates laced with the same concentrations of NiO (insoluble) as had been used for NiCl₂ (soluble). Initial results for lower

levels of NiO showed greater effect than NiCl₂ (Fig. 7). However, as concentration increased the effect from NiO tapered off and on 8.43 and 21.1 μM the effect of NiO was less than that of NiCl₂. As previously seen with NiCl₂ treatments, fewer engulfments were seen in worms exposed to 21.1 μM NiO than 8.43 μM NiO. Further research with more concentrations will be done to better assess this relationship.

Cell deaths caused by Nickel

To ensure that the increase in engulfments happened as a result of nickel exposure, a

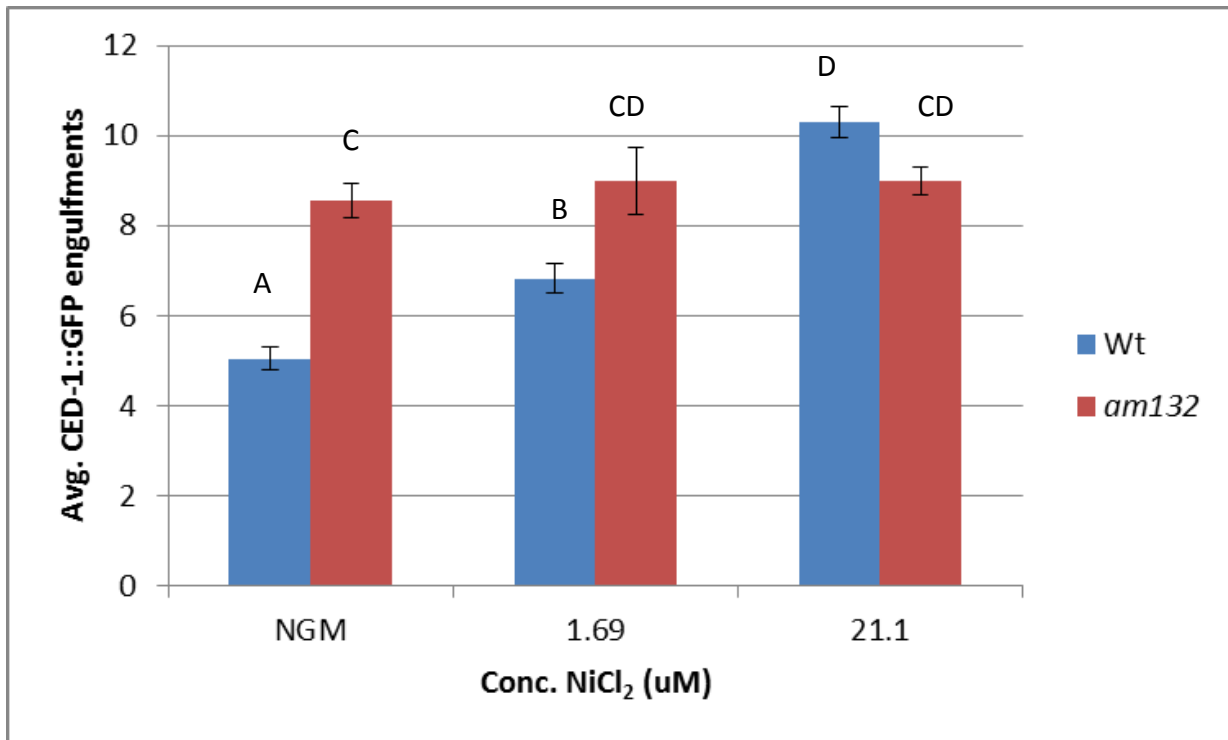


Figure 10. Mutation of an ammonia lyase increases basal engulfment levels. When exposed to nickel no significant increase in engulfments was seen. Letters denote groupings based on Tukey post-hoc analysis.

Strain/Conc.	Avg. Deaths	St. Error	N
ced-1::GFP NGM	5.05	0.42	153
am132 NGM	8.57	0.43	150
ced-1::GFP 1.69 CI	6.83	0.43	150
am132 1.69 CI	9	0.43	150
ced-1::GFP 21.1 CI	10.3	0.42	152
am132 21.1 CI	9	0.37	200

cross was done using a strain with a mutated histidine ammonia lyase (WU970) and wild-type *ced-1::gfp*. The altered lyase gene contained a point mutation that was proposed impart resistance to heavy metals (Murphy et al. 2011). When tested, the resulting strain showed higher levels of engulfments, than *ced-1::gfp* wild-type, on agar without nickel. However, it showed no increase in engulfments when exposed to nickel (Figure 10). This trend continued even on high nickel, when the wild-type had more engulfments than the nickel resistant strain. These data indicate that increases seen in engulfments for wild-type worms happened as a result of nickel exposure.

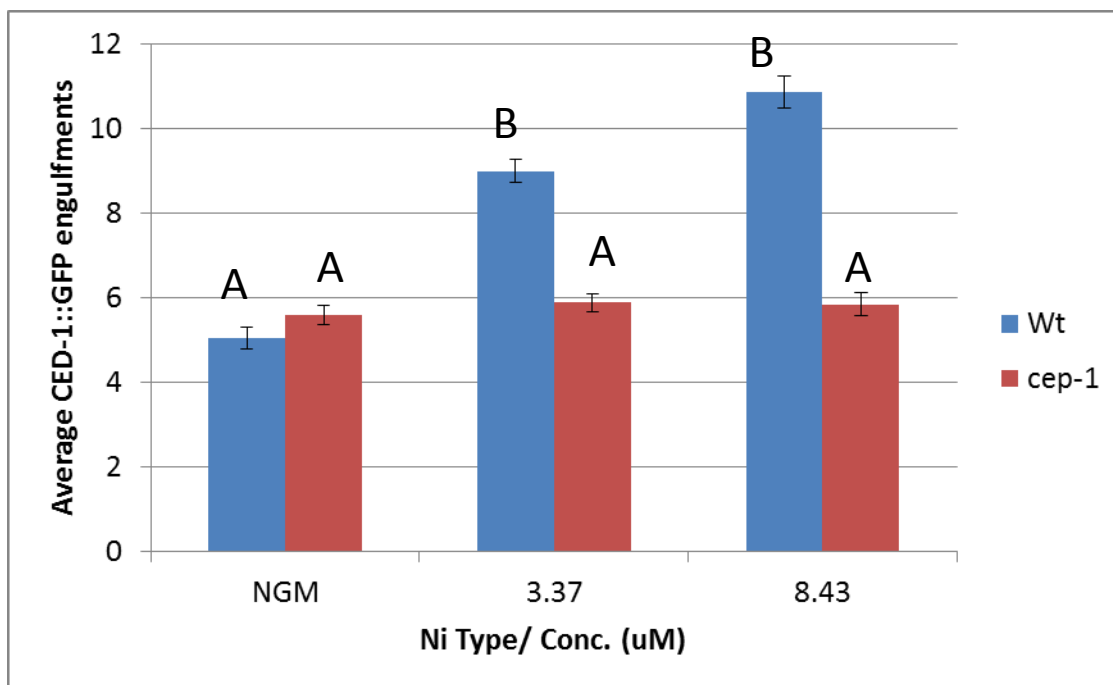
Cell deaths result of genotoxicity

In an effort to learn how nickel damage is assessed, XY1054 (*cep-1*) animals were crossed with wild-type *ced-1::gfp* to generate a strain without a functional genomic damage repair pathway. When subjected to the same tests as wild-type *ced-1::gfp* this strain showed no change in gonadal engulfments even when subjected to high levels of nickel (Figure 11). Engulfment counts remained the same for this strain on nickel as wild-type on NGM. This indicates that nickel's toxic effects are assessed by the p53 pathway, which disagrees with findings from other labs. Therefore, further testing using mutants for other parts of the DNA damage repair pathway will occur.

Discussion

Using *ced-1::gfp* to assess nickel exposure

Counting GFP engulfments provided a simple straightforward assay for the nonlethal effects of nickel. The wild-type *ced-1::gfp* strain showed that nickel exposure did increase engulfments. Although not extreme, the increases in engulfments showed statistical significance even on low nickel. Previous research by Wang and Wang (2008) showed that exposure to



Strain/ Conc.	Avg Deaths	St. error	N
Wt NGM	5.05	0.25	153
<i>cep-1</i> NGM	5.58	0.23	149
Wt 3.37	9	0.28	153
<i>cep-1</i> 3.37	5.88	0.20	152
Wt 8.43	10.91	0.38	149
<i>cep-1</i> 8.43	5.84	0.25	150

Figure 11. Removal of *cep-1* activity lead to nickel exposure not affecting the number of engulfments occurring. This indicates that nickel has genotoxic effects. Letters denote groupings based on Tukey post-hoc analysis.

NiSO₄, ranging in concentration from 2.5 μM to 200 μM, could shorten the lifespans, decrease brood size, cause movement defects, and alter chemotactic plasticity in exposed animals as well as their progeny. Their analysis, using a soluble nickel compound, showed that the nickel entered the eggs. Analysis, in this study, showed an increase in engulfments at low levels, of soluble and insoluble nickel, that tapered off at higher levels. It is possible that even higher levels of nickel, like the 75 μM and 200 μM concentrations used by Wang and Wang (2008) would show higher or lower levels of engulfments. Higher levels of cell death would be generally expected, especially considering that most previous testing used higher concentrations than this study and saw worms survive long enough to reproduce (Wang and Wang 2008, Brown 2004). For engulfment levels to decrease at levels above 21.1 μM raises the question that the worms may have been generating fewer oocytes due to stress from the nickel (Kezhou et al. 2010).

Mutating factors in the apoptotic pathway drastically changed the number of visible engulfments. Removal of caspase activity reduced engulfments to zero regardless of biochemical affront. On the other end of the spectrum, loss of function mutations of the apoptotic inhibitor *ced-9* caused high levels of engulfments to the point of overshadowing the toxic effects of the nickel (Fig. 8). A statistically significant increase in engulfments was seen for the *ced-9ts* strain on a higher concentration of nickel. However, anecdotal accounts of large data ranges in tests question the significance of this increase. Whereas tests, for other strains, saw maximum ranges of 10-20 engulfments between individuals, some tests for *ced-9ts* had ranges above 40; which shows this mutation has a strong capacity to influence initiation of apoptotic engulfments.

NiO versus NiCl₂

Comparisons between NiCl₂ and NiO at these levels remain inconclusive. Low levels of NiO and NiCl₂ initially indicated that NiO would have a greater effect but at higher levels the difference in effect dropped and eventually NiO showed less effect than NiCl₂. Kasprzak et al. 2003 states, “As a rule, insoluble compounds, such as NiS, NiO, and Ni₃S₂, are better carcinogens than soluble compounds, Ni(II) acetate, chloride or sulfate.” It is possible that the methodology of this experiment increased the effective toxicity of NiCl₂. Soluble forms of nickel are believed to be less toxic because they exit the cell just as readily as they enter. In this test the worms were constantly exposed to the nickel, so when some left more could enter. This may have allowed the soluble nickel a similar mode of effect to insoluble nickel. It is also possible that the large particle size of the NiO used in this study could have decreased its toxicity. To assess this possibility future testing will be done with NiO nanoparticles.

Effect of cellular histidine on nickel

Increasing cellular histidine can allow worms to withstand higher levels of heavy metals (Murphy et al. 2011). Analysis by Murphy et al. has previously shown that increasing cellular levels of histidine increases the number of worms growing to adulthood when exposed to zinc and nickel (2011). Their data showed that cellular levels of zinc and nickel were unaffected by increased histidine, from either dietary intake or mutation. They hypothesized that the histidine effectively chelated excess zinc and nickel, thereby reducing deleterious effects on the cell. In analysis of this mutant, baseline engulfment levels were higher than those of wild-type, but this is likely a result of the mutation; increasing the levels of histidine present, particularly in developing oocytes with shared cytoplasm, may have a toxic effect. When exposed to nickel no significant increase in engulfments was observed. At higher levels of nickel the average number of engulfments occurring in wild-type worms was greater than that of individuals with this

mutation. This data indicates that by increasing cellular levels of histidine ameliorates the effects of heavy metals.

Is p53 involved?

Removal of a DNA checkpoint protein removes the effect of nickel on gonadal engulfments. Previous analysis of nickel exposure on germline apoptosis in *C. elegans* indicated that increased deaths did not occur due to genomic damage (Kezhou 2010). In this study, *cep-1* mutants showed increased levels of apoptosis, as determined by acridine orange staining, when exposed to NiSO₄ for 12 hours. In the current study there was no increase in engulfments when exposed to nickel (Fig 10). A recent study using human lung epithelial cells also concluded that nickel causes genomic damage that is assessed by the p53 pathway (Ahamed et al. 2011). Their study found that nickel exposure lead to an increase in p53 and caspase expression, using QRT-PCR, as well as increased caspase activity in the exposed cells. A study by Nackerdien et al. noted that nickel and cobalt exposure lead to DNA damage in isolated chromatin by hydroxyl radicals (1991). In the study by Kehzou et al. their *cep-1* mutant showed higher levels of engulfments, even without nickel exposure, as did their *egl-1* mutant, compared to wild-type. Both *egl-1* and *cep-1* are pro-apoptotic factors. The data acquired in this study indicates that nickel does cause damage which is assessed by the *cep-1* pathway; as when *cep-1* was removed there was no increase in engulfments, with nickel exposure. To insure the credibility of this claim further testing will be done using mutants for other pro-apoptotic factors such as *hus-1*, *mpk-1*, *mek-1*, and *pmk-1*.

Future considerations

As manufacturing and use of electronics containing nickel continues environmental levels will increase. With the potential for nickel to harm organisms and environmental integrity it is important to understand the mechanisms that cause harm as well as the effects of increasing levels. This study indicates that nickel does harm living organisms and some portion of the damage done is genomic. It is also important to note that the damage affects not just the exposed organism but also its progeny. The potential for debilitating mutations to occur is increased in young and developing organisms. Therefore if people desire the safety of their children and grandchildren the effects of environmental toxins and the potential for damage to be passed down should be considered when designing policy or products that involve nickel. Proper disposal of items containing nickel compounds or other heavy metals could reduce toxic effects and improve both the survival and quality of life for generations of humanity to come.

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Appendix A: Image use approval

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Dr. Kasprzak,

Kazimierz S Kasprzak, F.William Sunderman Jr., Konstantin Salnikow, Nickel carcinogenesis, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Volume 533, Issues 1–2, 10 December 2003, Pages 67-97, ISSN 0027-5107, 10.1016/j.mrfmmm.2003.08.021.
<http://www.sciencedirect.com/science/article/pii/S002751070300209>

I would like to use Figure 1.

Hope you're well,
-Ian Huffnagle

From: Kasprzak, Kazimierz (NIH/NCI) [V] [kasprzak@mail.nih.gov]
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Subject: RE: Permission for image use

Dear Mr. Huffnagle:

Please give me full citation of the paper you are interested in (I published seven papers on nickel in 2003).

K. S. Kasprzak

From: Huffnagle, Ian Michael [HUFFNAGLEI10@students.ecu.edu]
Sent: Friday, February 15, 2013 11:21 PM
To: kasprkaz@mail.ncifcrf.gov
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Dear Sir,

My name is Ian Huffnagle. I am a Masters candidate in the Biology department at East Carolina

University. For my research I have looked at the effects of Nickel exposure on the levels of programmed cell death in the *C. elegans* germline. May I use figure 1 from your 2003 paper, Nickel carcinogenesis, in my thesis?

Thank you for your consideration,
-Ian Huffnagle

