Abstract

The effects of environmental nickel toxicity upon survival, growth, reproduction, fecundity, and lifespan of nematodes C*aenorhabditis elegans*, P*ristionchus pacificus*, and C*aenorhabditis briggsae*

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Nickel (II) is a common component of many natural products and man-made devices. Due to its frequent use in everyday life, relatively large amounts of nickel are being released into the environment. Nickel, however, is a known carcinogen; therefore, an assay must be developed in order to understand the toxic effects of this heavy metal on living organisms. Nematodes are a model animal species that have been used in sediment and water testing of contaminated environmental samples. Nematodes lend themselves perfectly to liquid and sediment assays due to their easy recovery, handling, distinction between larva and adults, and short life cycle. In this study, we used three nematode species, *Caenorhabditis elegans*, *Pristionchus pacificus*, and *Caenorhabditis briggsae*, in order to determine the effects of nickel on the survival, growth, reproduction, fecundity, and lifespan of these model biotic organisms. During preliminary testing, *C. briggsae* displayed highly variable results during both sediment and liquid assays. Thus, the majority of testing in this project was performed on *C. elegans* and *P. pacificus*. We found that *C. elegans* is best suited for environmental assays where there is a large portion of dissolved organic carbon in the sediment. However, *P. pacificus*, displayed no preference for any of the soil physio-chemical characteristics during these assays. The element Nickel (II) can either

bind to substrates within a sediment, or can freely move through aqueous solution. In this study, we showed that nickel bound in sediment is highly lethal to the P0 generation of nematodes; however, the effects of nickel bound in sediment were not readily apparent on the F1 generation of both *C. elegans* and *P. pacificus*. Using liquid assays, we also determined that aqueous nickel was not detrimental to the P0 generation for all three nematode species. Interestingly, higher dosages of aqueous nickel were shown to be detrimental to the F1 generation for *C. elegans*., whereas *P. pacificus* showed no decline in the number of F1 progeny recovered. Finally, our results show that nickel bound in sediment also has an effect on longevity. Using *C. elegans* strain JK574: *Cel-fog-2 (q71) LGV* in a longevity trial, it was determined that higher dosages of bound nickel can decrease the number of days needed to reach a 50% recovery rate by 7-8 times. Overall, the results of these experiments show that both *C. elegans* and *P. pacificus* can be used as bio-indicators of nickel contaminated water and sediment samples. By developing this quick, efficient, and reliable assay, other laboratories will be able to determine the amount of nickel that can be detrimental in various ecological samples and what the toxic effects of this metal will be on living organisms.

The effects of environmental nickel toxicity upon survival, growth, reproduction, fecundity, and lifespan of nematodes *Caenorhabditis elegans*, *Pristionchus pacificus*, and *Caenorhabditis briggsae*

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LIST OF ABBREVIATIONS

USGS	United States Geological Survey
SR	Spring River
SJ	St. Joseph River
P30	Pond 30
DOW	Dow Creek
RR2	Raisin River site 2
STM	South Tributary of Mill Creek
RR3	Raisin River site 3
WB	West Bearskin Lake
ORP	oxidation-reduction potential
TOC	total organic carbon
AVS	acid-volatile sulfide
CEC	cation-exchange capacity
log KD	distribution coefficient for TR-Ni / PW-Ni
TR–Ni	total recoverable nickel
PW-Ni	pore water nickel
DOC	dissolved organic carbon

CHAPTER 1: INTRODUCTION

For the last century, mankind has changed the face of the Earth in more ways than the average person would believe or understand. Mankind has become one of the most successful species with our abilities to change the environment to suit our needs, our ability to get minerals and other vital resources from the earth to aid in our ever expanding territory, and our ability to learn and take the knowledge that we obtain and put it to use in a way that benefits our species. For everything that we have done to change the environment for the better, we have manufactured a scenario where our species is being exposed to detrimental materials at a level that would not naturally occur. One group of these detrimental materials is the toxic metals or "heavy metals". According to the Occupational Safety and Health Administration (OSHA), a toxic metal is an individual metal or metal compound that has an adverse effect on a human's health (Heavy Metals 2009). OSHA provides a list of common heavy metals that are known to be toxic to humans such as arsenic, cadmium, lead, and mercury. These metals are being released into the environment and have potentially harmful or lethal effects (Rudolf and Cervinka 2010).

Studies conducted at a lead smelting plant have shown that the levels of heavy metals being released into the immediate surrounding environment are significant (Bisessar 1982). It was shown that these metals are at levels anywhere from 2 to 100 times higher than what was recorded at a control site 1000 m away from the smelter. It has also been shown that these metals do not leave the sediment or water column as quickly as once thought (Everhart et al 2006). From studies like this, humans have been able to determine what levels of heavy metals are being released into the environment, and it is important for humans to know how much of these heavy metals are being released into the environment because of their dangerous properties. Most of these metals have been studied and shown to affect different organ systems,

mainly the nervous system, and can lead to deaths from poisoning or cancer (Heavy Metals 2009).

In more recent years, OSHA has added additional metals to the list of toxic metals. These metals include cobalt, copper, iron, and nickel. These metals are important because they are commonly found in many items that are constantly in use by the average person. Copper is used in electrical wiring and plumbing, iron is commonly found in many metal alloys that we use in construction and automobiles, cobalt is used to give a blue color to ceramics and glass but it can also be used in industrial settings like in leveling devices and thickness gauges (Cobalt Compounds 2007), and nickel is used in everything from alloys, batteries, coins, and many other items that humans use on a regular basis (Nickel Compounds 2007). The one pressing issue that everyone should be asking, but a few are, is how much of these heavy metals are being released into the environment, how much are humans coming into contact with, and what levels are humans actually pulling into their bodies? United States Geological Survey (USGS) is especially interested in determining the levels of nickel being released into the environment.

How much nickel is being released into the environment is an especially important question, because nickel is used in alloys, batteries, coins, spark plugs, catalysts, and a lot of other common devices and tools that humans use every day (Nickel Compounds 2007). An especially ominous thought is that the cell phones that almost every American owns has nickel components in it, the cars they drive have nickel components, the coins that are used for currency contain nickel, and almost every electrical device contains some nickel. Then think about how humans dispose of these different electrical devices. In recent years, humans have done a better job in recycling electronic devices yet for decades before this practice began, humans would just toss unused items into junk yards, dump sites, or toss them into rivers or lakes. That means for

decades nickel was potentially being released into the water table which could get back into crops through irrigation, drinking water from wells or large bodies of water, or even fish and wildlife through ingestion. Evidence for nickel release from production and dump sites has been recorded. In a review of sources of mutagens in sediment samples, White and Claxton stated that a majority of the metal toxins released into the environment come from metal mining, processing and smelting of metals, and fabricated metal products (2004).

The question of why should we study nickel is obvious based upon the toxic effects of nickel to human health. Nickel toxicity can cause skin problems as well as cardiovascular and other systemic effects (Denkhaus and Salnikow 2001). It has also been shown to have effects at the cellular level on DNA repair which can induce cellular apoptosis (Kezhou et al 2010). Nickel toxicity has also been shown to have effects on plants as well, indicating that nickel would not only be limited to animals but would be an entire ecosystem toxicological problem (Ghasemi et al 2009) and nickel toxicity has been linked in rye grass to improper uptake of certain nutrients with allowing other elements, like iron, to be increased (Khalid, B. and J. Tinsley 1980). It has been shown that there are a variety of bacteria that need nickel for use in specific enzymes (Hausinger 1997), it has been debated what levels of nickel are required (Denkhaus and Salnikow 2001). The effects from nickel toxicity can also be used to study the effects of other heavy metals within the same group as nickel, and also give more insight into how toxic metals will be absorbed by the environment, by animals, and by humans. To understand nickel, which is becoming an increasing problem on its own, would give more information into what other metals would react in a similar way, or in a much more lethal manner.

With these heavy metals being released into the environment, scientists need an assay that can not only accurately evaluate how much metal is toxic to humans, but what the effects of different metals would do to the human body as well as what sediments or water samples retain or enhance the effects of metals on humans. Model organisms can be extremely beneficial in these types of environmental toxicological assays.

As Nathan Cobb stated in his article in the United States Department of Agriculture Yearbook,

> "In short, if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable, and if, as disembodied spirits, we could then investigate it, we should find its mountains, hills, vales, rivers, lakes, and oceans represented by a film of nematodes. The location of towns would be decipherable, since for every massing of human beings there would be a corresponding massing of certain nematodes. Trees would still stand in ghostly rows representing our streets and highways. The location of the various plants and animals would still be decipherable, and, had we sufficient knowledge, in many cases even their species could be determined by an examination of their erstwhile nematode parasites."

This is why the use of *Caenorhabditis elegans* is a viable option for these sorts of environmental assessments (Cobb 1914). *C. elegans* is a cosmopolitan nematode that has become a standard work horse in the laboratory. This is due to their short life cycle, easy maintenance, and the known cell fate map or cell lineage (Wood 1988). *C. elegans* are also an ideal model organism to use in any sort of toxicity experiment due to the fact that one can make many comparisons between the model organism and humans (Maglich et al 2001). Additionally, *C. elegans* are an ideal test organism because of their ability to test full development of the organism within liquid and substrate environments. Nematodes can easily be observed throughout their developmental cycle, they can be used to see the effects on adults, pre-zygotic and post-zygotic embryos, and along the various larval stages (Figure 1-1) (Altun 2009). *C. elegans* also give us a model

organism where the entire genome is already known and allows for scientists to control many of the variables, like food and temperature, that can affect nematode growth, development, and reproduction.

Free living nematodes have been used in previous experiments looking at high levels of metals including copper, lead, and cadmium to name a few (Haight et al 1982) (Korthals et al 1996) (Biessar 1981). Major issues with these types of study are that they are done in free soil where recovery of the individual animals would give one a broader understanding of the population but not of the effect on the individual or its progeny and how the toxins in the soil affect these numbers directly. . *C. elegans* have been used as toxicological test organisms in previous studies of copper (Donkin and Dusenbery 1993), and various other metals (Mutwakil et al. 1996). All of these studies looked at using *C. elegans* in various environments, giving evidence and credibility to using them in various sediment and liquid environments in future studies.

In addition to using *C. elegans*, it has been proposed to use a secondary and tertiary nematode, *Pristionchus pacificus* and *Caenorhabditis briggsae*, within our trials. Both *P. pacificus* and *C. briggsae* are good candidates for additional assay organisms because they both share many of the characteristics that makes *C. elegans* so vital to research. *P. pacificus* gives a secondary organism that has a different life history from that of *C. elegans* (Sommer 2006) while *C. briggsae* gives another nematode that has a similar life cycle to that of *C. elegans*. *P. pacificus* has a longer life cycle of 4 days at 20 °C (Figure 1-2.)(Sommer 2006) and in a natural environment it is associated with scarab beetles and the Colorado potato beetle (Herrmann et. al 2006).

Scientists use model organisms to make many inferences about potential effects that could be seen in humans. Whether it is from using *Drosophila melangaster* and understanding *Hox* genes, using *C. elegans* and understanding asymmetric cell division and how it leads to determining cell fates (Gönczy and Rose 2005) or programmed cell death within germline when forming sperm and oocytes (Gumienny et al. 1999), model organisms have led to many breakthroughs in understanding development as a universal topic. In using model organisms, we have a baseline to determine the potential effects that could occur within humans. In the assay described below, *C. elegans* could give use a variety of information about the toxicological effects of nickel on humans. By looking at the recovery of larva, development of larva to adults, and production of the next generation of larva, we can determine what effects nickel would have on *C. elegans.* These results will determine what effect nickel is having on survivability as well as development. This experiment has its genesis in other sediment assays that had highly reproducible results (ASTM 2008; ISO 2010; Hoss et al. 2011; Hoss et al. 2012).

This study will also give a base from which future experiments can be completed looking at the effects of nickel on *C. elegans* and their offspring. We can determine if the *C. elegans* are dying before making it to adulthood and the level of nickel that is lethal to the organism, and if adults are seen but larva are not being produced then the nickel could be affecting the genomic DNA of the developing embryo. This information could then be used for potential experiments looking at gene expression, RNA expression, and interruptions within the genomic pathways of *C. elegans* and their correlation into humans.

Current studies including this one are being done by the United States Geological Survey on sediments obtained from test sites in Midwest water sheds in the United States, Figure 1-3, using the invertebrate species *Hyalella azteca, Gammarus pseudolimnaeus, Chironomus dilutes,*

Chironomus riparius, *Lumbriculus variegates*, *Tubifex tubifex*, *Lampsilis siliquoidea*, and *Hexagenia* sp (Bresser et al. 2011). All of the organisms, including *C. elegans*, were chosen based on ecological importance and representation of larger and numerous fauna. They are all being used in conjunction to determine how dangerous metals are to the environment so we can look at effects on the entire ecosystem. Caveats with most of the chosen animals are that they require very large tanks to run the trials and these research facilities are not always at the disposal of most field researchers, they have a longer developmental cycle compared to *C. elegans*, and they are lacking a completely sequenced genome. All of the chosen organisms are useful and will provide data that will go towards showing a more global perspective on effects of the nickel upon animals, *C. elegans* offer a member species of a phyla that comprises an extremely large percentage of the biomass of many ecosystems. They have reproductive life cycles of 4 days under laboratory conditions, and they can be used in small field labs to give a quick and accurate initial assessment of heavy metal toxicity.

Nickel is a transitional metal that can exist in either a soluble form, which can easily be dissolved in water, or in an insoluble form, traditionally seen as a metal alloy or bound in solid forms like Nickel Oxide or Nickel Carbonate (Nickel 2012). When nickel is dissolved it can be transmitted over vast areas due to the fact that it will remain in the water until it binds to another substrate and forms a precipitate. Conversely, bound nickel in an insoluble form will remain in a given area and can therefore be transmitted to the animals or plants in that location by ingestion or skin contact. Determining the toxicity of these different forms of Ni(II) is important because each form posses a different toxicogical issue and response from the plants and animals that it comes in contact with.. It is also important to determine the effects of insoluble and soluble nickel because nickel has a variety of point sources for release into the environment and this

information will be needed to properly identify major areas of concern. The added benefit is that the nickel assay could lead to studies using other heavy metals and looking at their toxicological effect on the environment.

OBJECTIVES

To help elucidate what effect nickel has on the survival, growth, reproduction, fecundity, and lifespan of nematodes, I had four main objectives.

1. Determine what sediments are essential for allowing nematodes to survive and reproduce
    a. Use a variety of sediments with different characteristics (see Tables 1-1 – 1-4)
    b. Include nematode species *Caenorhabditis elegans* laboratory strain N2, *Pristionchus pacificus* laboratory strain PS312, and *Caenorhabditis briggsae* laboratory strain AF16 for assays.
2. Determine what effect Nickel (II) has on the survivability, reproductive capabilities, and growth of the P_0 generation as well as the generation of the F_1 generation.
    a. Utilize two sediments WB-0 and SR-0 spiked with increasing levels of Nickel (II) to determine the detrimental effects that increasing levels will have on the
    b. Includes nematode species *Caenorhabditis elegans* laboratory strain N2 and *Pristionchus pacificus* laboratory strain PS312 for assays.
3. Determine whether the effect that bound Nickel (II) on the nematodes were due to the bound nickel within soil or the aqueous nickel present in pore water throughout the sediment.
    a. Comparable to the sediment assay in length, setup, and recovery of the animals
    b. Includes nematode species *Caenorhabditis elegans* laboratory strain N2, *Pristionchus pacificus* laboratory strain PS312, and *Caenorhabditis briggsae* laboratory strain AF16 for assays.
4. Determine what effect Nickel (II) has on the life span of *C. elegans*.

a. Use of a late stage L4 *C. elegans* strain JK574: *Cel-fog-2 (q71) LGV I* over a 22 day period

    i. Strain JK574: *Cel-fog-2 (q71) LGV* chosen for assay because the mutation prevents sperm production and therefore renders nematode sterile unless mated with a male.

    ii. Determines survivorship when C. elegans only goals are to move through the substrate, feed, and search for a mate.

    iii. Under normal laboratory conditions a hermaphrodite nematode can survive up to three weeks and therefore determined the time period for this experiment.

Materials and Methods

1.1. Nematode strains, handling, and synchronization.

Caenorhabditis elegans laboratory strain N2, *C. elegans* strain JK574: *Cel-fog-2 (q71) LGV, Pristionchus pacificus* laboratory strain PS312, and *Caenorhabditis briggsae* laboratory strain AF16 were used in this study. *Cel-fog-2* is a gonochoristic strain and must be maintained through matings. Animals were maintained at 20°C on either Nematode Growth Media plates or K media plates seeded with E*scherichia coli* strain OP50 using standard culture techniques (Brenner 1974). K media replaces the $CaCl_2$ with KCl. Divalent Ca^{2+} cations are a potential complication to studying the effects of divalent heavy metal ions.

Animals were age/development synchronized for all tests. Mixed staged cultures were grown on OP50 bacterial seeded 100mm K media plates. Fully-grown cultures were washed off the plates using M9 Buffer and placed into 15mL conical tubes. Nematodes were centrifuged at 800 g for 10 minutes at 4°C. The supernatant was drawn off the pellet and 10 ml of fresh M9 added to the conical tube. The nematode/egg pellet was suspended, centrifuged, and washed with M9 twice more for a total of three washes. The cleansed pellet was suspended in 10 ml of a basic hypochlorite solution and agitated for approximately 5 minutes. The solution was monitored during this time by pipetting samples to an unseeded plate and observing the corpses under the stereo dissecting scope. The surviving eggs were centrifuged at 1200 rpm after most adult carcasses were dissolved and the pellet washed 3X with fresh M9 buffer. Washed eggs were removed from the conical tube and placed in batches on unseeded K media plates to hatch for 24 hours. *C. elegans* hatch and developmentally arrest as L1 larvae without food, *P. pacificus* as J2 larvae.

1.2. Sediments and hard water.

Sediments were collected from sites in the Midwest, characterized, and spiked with nickel as reported in Besser et al. 2011 (see Tables 1-1 – 1-4). Sediments were stored at 4°C in lidded glass jars. Hard water was used for both the sediments and water tests.

Hard water was produced to exacting specifications at the United States Geological Survey, Columbia Environmental Research Center, Columbia, MO, USA as reported in Besser et al. 2011. Prior to use in tests water was titrated to a PH of 7.5. For the nickel-spiked hard water and distilled water tests, $NiCl_2$ solutions of 50, 100, 200, 400, and 800μg Ni(II)/L. Top nickel concentration was chosen specifically to exceed the level of Ni(II) worms were likely to be exposed to in the pore water of the sediments of interest.

1.3. Sediment tests.

Sediment preparation. To minimize exposure of worms to soluble Ni(II) and to imitate the natural flows of water, prior to use sediments were overlaid with excess hard water. This water was exchanged twice daily for a week. The last water exchange was collected and sent to the USGS for characterization of the levels of soluble Ni(II) in the pore water (Besser, Brumbaugh et al. 2011).

Food preparation. This test used OP50 as a food source. 500mL flasks of OP50 were inoculated with single colonies from an isolation plate. The bacteria were allowed to grow one day at 37°C and stopped while still in growth phase. *E. coli* cultures were removed from the incubator and ampicillin was added to the culture. Cultures were allowed to continue for 12hrs to kill all cells. Cells were washed 3X to remove media, waste, and the antibiotic then suspended in hard water [OP50; aqueous test: 1,000 50 formazin absorption units (FAU; according to ISO 7027); sediment and soil test: 12,000 600 FAU]. 2 μl of cholesterol in Ethanol were added per ml

of *E. coli* suspension.

Sediment measurement. To dispense an equal volume of sediment for each test, the average weight of 1 mL of sediment, for every sediment, was determined. 20 ml of sediment was placed in a conical tube and massed. The weight of the empty conical tube was subtracted from the total mass and the net sediment mass in grams divided by 20. 1 ml of sediment was determined for test replicates by weight.

Test set up. For each sediment, in a single trial, six replicates were established. For every test at least two trials were conducted. The number of trials is limited by the size of the sediment samples available. Within individual wells of a 12-well tissue culture plate, 1 ml of sediment and 0.5 ml of OP50 suspension were added. The sediment and food were mixed using a toothpick. To each well 10 synchronized freshly-hatched larvae were added. 12-well plates with fully set up trials were sealed with parafilm and placed on a rotating platform at 20°C. Tests involving *C. elegans* and *C. briggsae* were left in the incubator for 96 hours (four days) and tests involving *P. pacificus* for 120 hours (five days).

Adult and progeny recovery. At the end of the test duration animals are recovered from the sediment for analysis. To each well 4 ml of a silicate suspension solution is added (1 part Ludox TM-50 colloidal silica : 2 parts H_20). The contents of the well are mixed and transferred to a 15 ml conical tube. The tube is centrifuges at 800 G for 10 minutes. The sediment pellets at the base of the tube and adult nematodes and larvae remain suspended in solution. The suspension is drawn off and placed in 100mm petri dishes. The pellet is suspended twice more in the silicate suspension. The suspension is scanned under the stereo dissecting microscope for nematodes. Adults and recovered larvae are counted and placed into a new tube. Only live animals were included in our counts. Dead larvae, likely P0 animals, were recovered from many

sediments. We count these as P0 based upon the size and developmental stage being beyond what is attainable by hatched F1 progeny. In most of these wells, few or no progeny were collected. Not all P0 corpses were recovered in our washes.

Fixation of animals. Collected animals were fixed by placing them in a 3% Rose Bengal solution (weight per volume) and baking them at 80°C for 10 minutes. Fixed animals assume a rod-like shape allowing easier measurement without altering length and width measurements. Fixed animals are placed on agar pads on microscope slides and a cover glass added for microscopy and further analysis.

1.4. Water tests.

Water test were set up analogous to the sediment tests. For these tests, food preparation and the setup in the 12-well tissue culture plates were identical with the exception of using 1 ml of a hard water sample or distilled water sample substituted for the sediment. At the end of the test duration an additional volume of sterile distilled H_20 was added to each well and nematodes were transferred to empty 100 mm petri dishes and directly counted. Counted nematodes were transferred to a new collection tube and fixed with Rose Bengal as described in the "Sediment tests" section for analysis under the compound microscope.

1.5. Microscopy.

Fixed nematodes were analyzed on a Nikon Microphot FX compound microscope with DIC optics. Images were taken using a Nikon DS-Qi1Mc digital camera and NIS-Elements: Basic Research software. Length and width measurements were taken using 200X images with the NIS-Elements software. Length was determined by tracing a curve from tip of head through to the tip of the tail along the dorsal-ventral midline of the animal using the gut as a reference. Width measurements were taken at the vulva, a line was drawn on the ventral side on the animal

between the anterior periphery of the vulva and posterior periphery of the vulva. Using this line, NIS elements produced a perpendicular line bisecting the vulva from which width measurements were taken. Concurrently animals were scored for the presence of embryos in the uterus. Life-stage was confirmed based upon vulva and gonad morphology in addition to size and fertility.

1.6. Fecundity index.

For each sediment and hard water treatment, a quantified fecundity number was generated specific to every well for all trials. This number is computed by dividing the total recovered F1 progeny from the well by the total recovered live P0 parental adult animals.

1.7. Longevity assay.

To assess the effects of nickel upon adult lifespan, we used the *C. elegans fog-2* (q71) mutant strain. The *Ce-fog-2* hermaphrodite is essentially female: It does not make its own sperm, but is fertile if mated into by a male. Additionally, *fog-2* mutant animals have a normal lifespan. Using these mutants we can set up sediment tests that can run for several weeks. As no progeny are produced, only animals that were initially added into the test well are recovered. Hence, there is no ambiguity about whether a recovered adult is from the parental generation or a fully-grown daughter, granddaughter, great-granddaughter, etc… of the parental generation. For the longevity test, the WB nickel spiked sediment series was used: WB-0, WB-2, WB-3, and WB-5. The setup of the test was similar to the initial setup of the *C. elegans* four-day tests.; 22 test wells for each sediment were established (enough to cover 3 weeks). Next, *fog-2* L4 female larvae were added to each well. Due to different dates of the trials slightly different numbers of animals were added to the WB-0 and WB-5 than the WB-3 and WB-4, ~55 and ~75 animals respectively. Once a day for 22 days, at the same time each day, adults were recovered from a single well for each sediment treatment. For comparative purposes, recovered animals were converted to an index by generating a recovery ratio, i.e. dividing the number of recovered animals by the average number

of animals initially added. As the tests were significantly longer and the number of nematodes added greater than for a four-day test, an additional 200µl of food was added to each remaining test well every five days.

1.*8. Statistical analysis.*

Statistics were done using IBM statistical program SPSS Statistics version 20. A comparison of means was performed under a One-Way ANOVA statistic using the post hoc of Tukey with significance of $p \leq 0.05$ and while assuming homogeneity of variance among the populations. In each figure, an open circle indicates that the point is an outlier less than three times the height of the box where a star or asterisk is indicating an extreme outlier that is greater than three times the height of the box.

Best-fit models for P0 recovery as a function of nickel dosage in Ni(II)-spiked sediment series were generated using Microsoft Excel 2008 for the Mac version 12.3.4. Based upon best-fit equations, an LD50 dosage (50% lethality of added P0 animals over test duration) for substrate bound Ni(II) was estimated for the WB and SR Ni(II)-spiked sediment series. Each formula was intersected by a line through y=5 for *C. elegans* and y=4 for *P. pacificus* in the WB spiked series while a line at y=3 was chosen for *P. pacificus* in the SR spiked series. In order to achieve an exponential decline (i.e. a curve that did not cross the y=0 axis) each P0 recovery data point was adjusted by 1 (n+1) and consequentially to figure the LD50 amount of nickel using the exponential curve, the y-intercept was raised by 1 as well. LD50 ranges given in the results section of the manuscript are based off binomial and trinomial curves, as these models consistently gave high R^2 values. LD50 for WB and SR are seen in Table 1-5.

Best-fit models were generated for *Cel-fog-2* longevity survivorship as a function of days in sediment and used to estimate the time when the population size reached 50% of the initial

animals added. The average life span day is based upon the intersection of the formula described for each sediment and a recovery ratio of y=0.5. Average estimated days to recovery of 50% are seen in Table 1-6.

Table 1-1. Physico-chemical characteristics of sediments.

Nickel distribution coefficient, Kd = TR-Ni/pore-water Ni										
Treatment	**pH**	**ORP (mv)**	**TOC (%)**	**AVS (umol/g)**	**Particle Size (%)**			**CEC (meq/ 100 g)**	**log Kd (TR/ PW)**	**TR-Ni (µg/g)**
					Clay	Silt	Sand			
SR-0	7.03	-169	0.40	0.94	6.9	13.6	79.5	5.5	3.56	8.9
SJ-0	7.28	-186	1.9	3.78	7.9	10.3	81.8	11.3	3.979	8
P30-0	6.87	-168	1.8	12.37	24.2	66.0	9.8	19.0	4.248	14
DOW-0	6.90	-155	1.2	1.04	6.0	7.0	87.0	6.4	3.794	6
RR2-0	6.98	-188	3.5	6.06	8.1	19.8	72.1	14.5	4.164	12
STM-0	7.14	-189	8.1	24.70	8.4	37.5	54.1	29.1	4.349	18
RR3-0	7.02	-179	7.2	7.98	5.9	19.5	74.6	29.3	3.857	9
WB-0	6.63	-87	10.40	38	24.7	68.3	7.0	44.1	4.56	59.7

Modified from Bresser et. al 2011

Table 1-2. Major constituents of sediment pore waters.

All values in mg/L.											
Treatment	**DOC**	**Cl-**	**F-**	**NO_3^-**	**SO_4^{2-}**	**Ca**	**Fe**	**K**	**Mg**	**Mn**	**Na**
SR-0	7	16.7	0.4	<.08	<.08	118	9.0	5.6	8.7	10.0	10
SJ-0	32	40	0.9	7.7	18.2	237	10	4.9	67	10	22
P30-0	21	20	<.08	6.6	<.08	182	46	7.0	52	6	22
DOW-0	51	79	<.08	<.08	<.08	271	35	7.2	61	8	26
RR2-0	21	19	0.9	7.2	<.08	189	21	4.4	47	5	10
STM-0	47	85	<.08	<.08	<.08	348	35	10.0	70	4	26
RR3-0	32	27	<.08	5	<.08	192	29	4.3	43	7	16
WB-0	30	18.8	0.4	<.08	80	26	18.0	2.0	6.9	2.9	6.6

Modified from Bresser et. al 2011

Table 1-3. Total-recoverable element concentrations in Task-3 sediments measured by - ICPOES.

[All values µg/g dry weight. All samples were below detection limits for Be (<0.5), B (<200), Cd(<2), Co (<5), Cu (<10), Mo (<50), Pb (<50).]

Sample	Al	Ba	Ca	Cr	Fe	K	Mg	Mn	Na	Sr	V	Zn
SR-0	12,556	-	1,481	-	7,753	1,598	488	215	-	-	-	54.9
SJ-0	7,212	72	6,731	14	22,260	962	1,875	529	<1000	16	19	39
P30-0	28,947	158	34,450	33	16,268	2,392	3,206	292	<1000	86	48	46
DOW-0	6,965	39	3,682	10	6,468	995	1,692	119	995	17	16	40
RR2-0	7,000	70	56,500	15	10,700	1,500	7,000	425	<1000	50	20	45
STM-0	10,784	172	85,784	15	24,755	2,745	12,892	637	<1000	162	28	64
RR3-0	5,238	95	54,762	10	14,429	476	7,000	905	<1000	110	14	43
WB-0	24,707	-	4,546	-	51,317	5,957	5,368	678	-	-	-	141.1

Modified from Bresser et. al 2011

Table 1-4. Physico-chemical characteristics of WB and SR Ni spiked sediments.

Nickel distribution coefficient, Kd = TR-Ni/pore-water Ni										
Treatment	**pH**	**ORP (mV)**	**TOC (%)**	**AVS (µmol/g)**	**Particle Size (%)**			**CEC (meq/ 100 g)**	**log Kd (TR/PW)**	**TR – Ni (mg/kg)**
					Clay	Silt	Sand			
WB-0	6.63	-87	10.40	38	24.7	68.3	7.0	44.1	4.56	59.7
WB-1	6.62	-79	11.20	25	21.1	66.1	12.8	42.5	4.37	156
WB-2	6.65	-85	9.70	26	20.6	66.5	12.9	37.4	4.24	369
WB-3	6.65	-89	11.20	26	24.8	63.6	11.6	44.7	4.20	1040
WB-4	6.62	-86	10.50	18	21.0	63.5	15.5	39.7	4.13	2680
WB-5	6.65	-80	10.20	12	23.5	66.3	10.2	37.4	3.92	7660
SR-0	7.03	-169	0.40	0.94	6.9	13.6	79.5	5.5	3.56	8.9
SR-1	6.96	-171	0.60	0.90	7.9	15.7	76.4	6.4	3.56	56.6
SR-2	7.03	-165	0.40	0.77	7.1	12.7	80.2	5.0	3.55	122
SR-3	7.03	-162	0.40	0.70	8.4	13.9	77.7	6.3	3.55	213
SR-4	7.06	-156	0.30	0.51	8.1	12.2	79.7	5.0	3.57	411
SR-5	7.03	-152	0.40	0.46	7.9	14.2	77.9	5.4	3.57	941

Modified from Bresser et. al 2011

Table 1-5 . Trendlines for scatter plots of recovered P0 adults against nickel to determine LD50 nickel concentration for WB and SR sediment series.

For WB *C. elegans*			
Formula	Best-fit curve	Nickel (µg/g)	R^2
Binomial	$Y=5*10^{-07}x^2 - 0.0049x + 10.426$	1272.60	0.9754
Trinomial	$Y=2*10^{-10}x^3 – 1*10^{-06}x^2 - 0.0014x + 9.6338$	1813.16	0.9965
Log	$Y=-2.314\ln(x) + 20.796$	921.75	0.8291
Exp (n+1)	$Y=8.8914e^{-3E-04x}$	1311.08	0.7106
For WB *P. pacificus*			
Formula	Best-fit curve	Nickel (µg/g)	R^2
Binomial	$Y=4*10^{-07}x^2 - 0.004x + 7.9846$	1122.05	0.9326
Trinomial	$Y=-1*10^{-11}x^3 + 5*10^{-07}x^2 - 0.0042x + 8.0417$	1104.27	0.9328
Log	$Y=-1.919\ln(x) + 16.753$	769.43	0.8935
Exp (n+1)	$Y=6.8283e^{-3E-04x}$	1038.79	0.7016
For SR *P. pacificus*			
Formula	Best-fit curve	Nickel (µg/g)	R^2
Binomial	$Y=2*10^{-05}x^2 - 0.0224x + 6.9736$	221.00	0.965
Trinomial	$Y=2*10^{-08}x^3 – 2*10^{-05}x^2 - 0.0132x + 6.572$	215.43	0.9756
Log	$Y=-1.563\ln(x) + 11.008$	167.92	0.8093
Exp (n+1)	$Y=6.5431e^{-0.002x}$	246.06	0.7625

Table 1-6. Trendlines for longevity trial using *Cel-fog-2*.

Sediment	Trinomial best-fit curve	Day	R^2
WB-0	$Y= 0.0001x^3 – 0.0056x^2 + 0.0347x + 0.8407$	14.052	0.7985
WB-2	$Y = -0.0003x^3 + 0.0108x^2 – 0.1324x + 1.1398$	18.172	0.7651
WB-3	$Y = 0.0001x^3 – 0.0014x^2 – 0.0633x + 1.0282$	7.751	0.8995
WB-5	$Y = -0.0004x^3 + 0.0158x^2 – 0.2272x + 1.0489$	2.991	0.9702

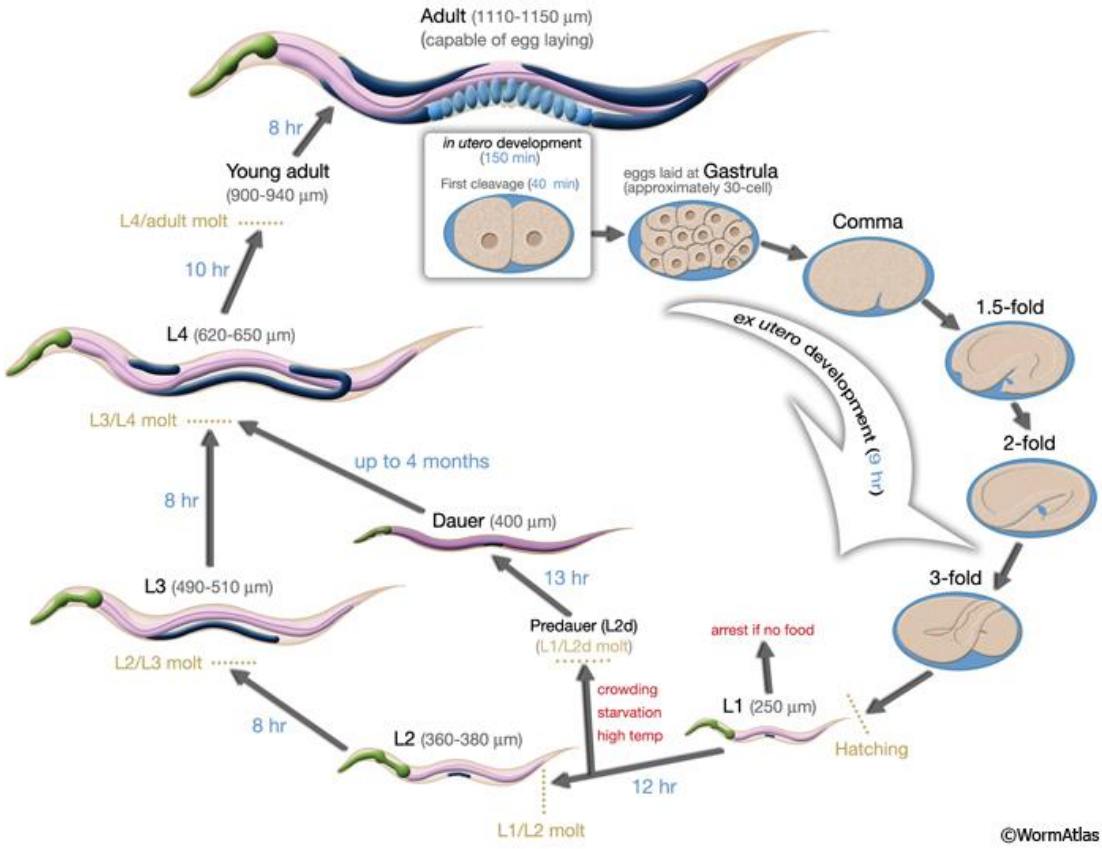


Figure 1-1. Life Cycle of the *Caenorhabditis elegans* at 22°C (Worm Atlas).

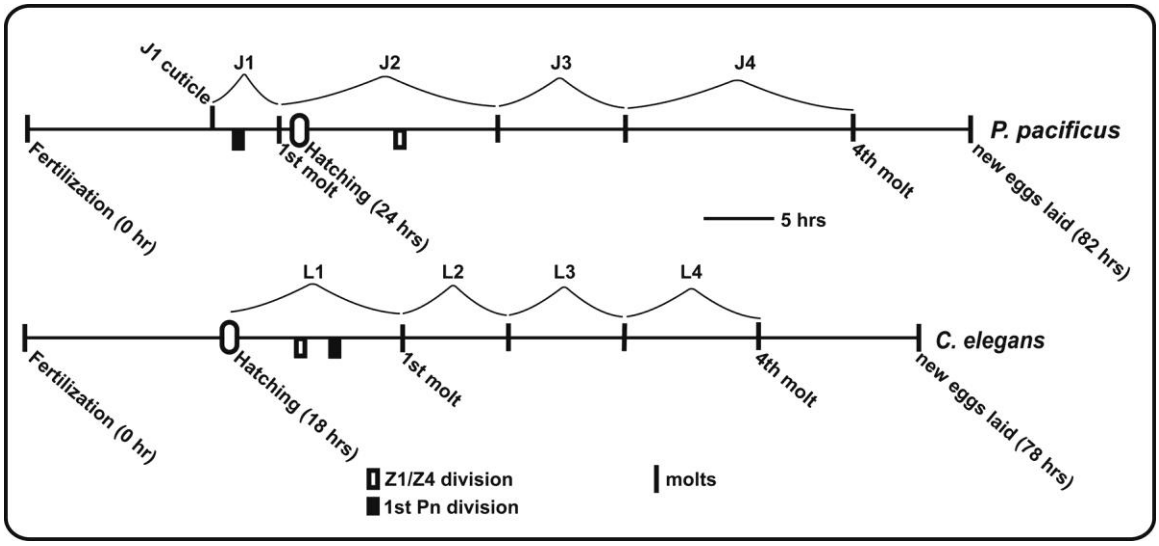


Figure 1-2. Comparable life cycle of *P. pacificus and C. elegans* (Sommer 2006).

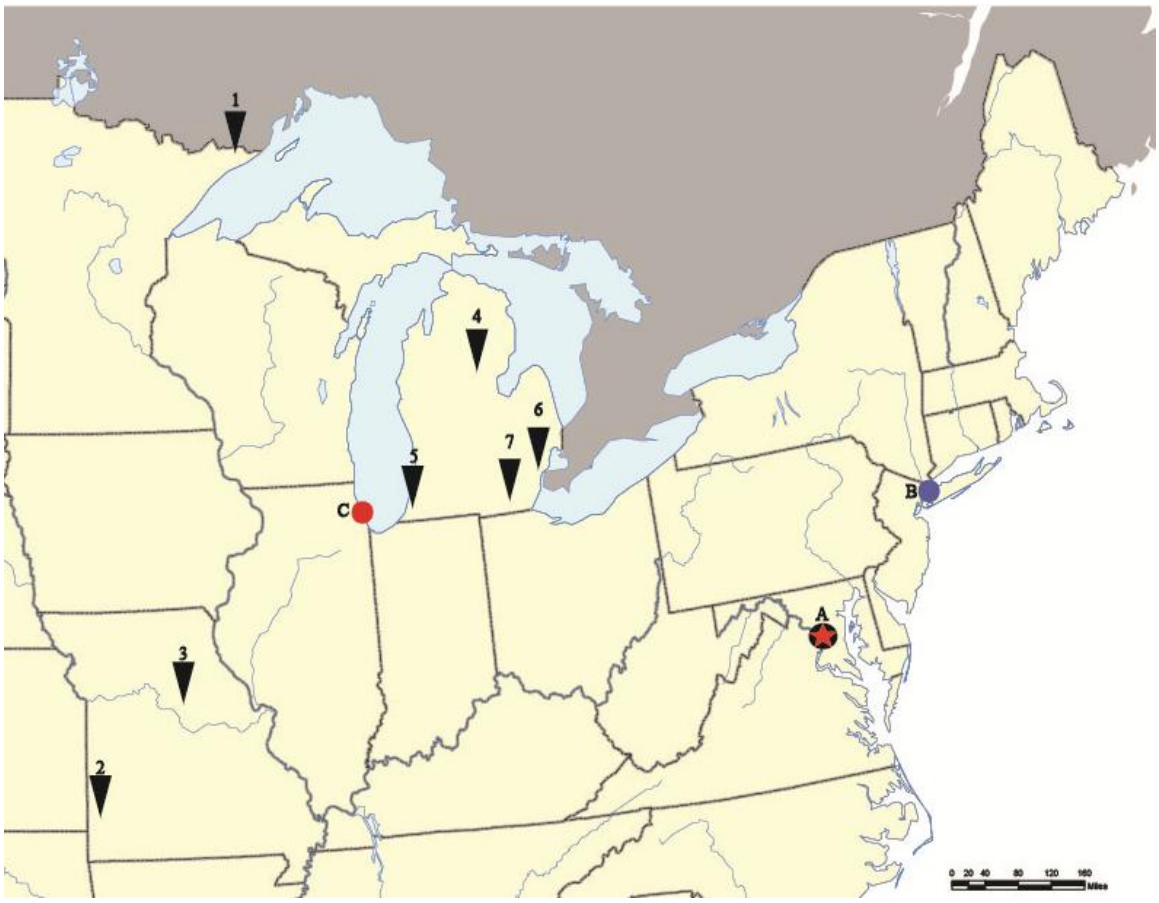
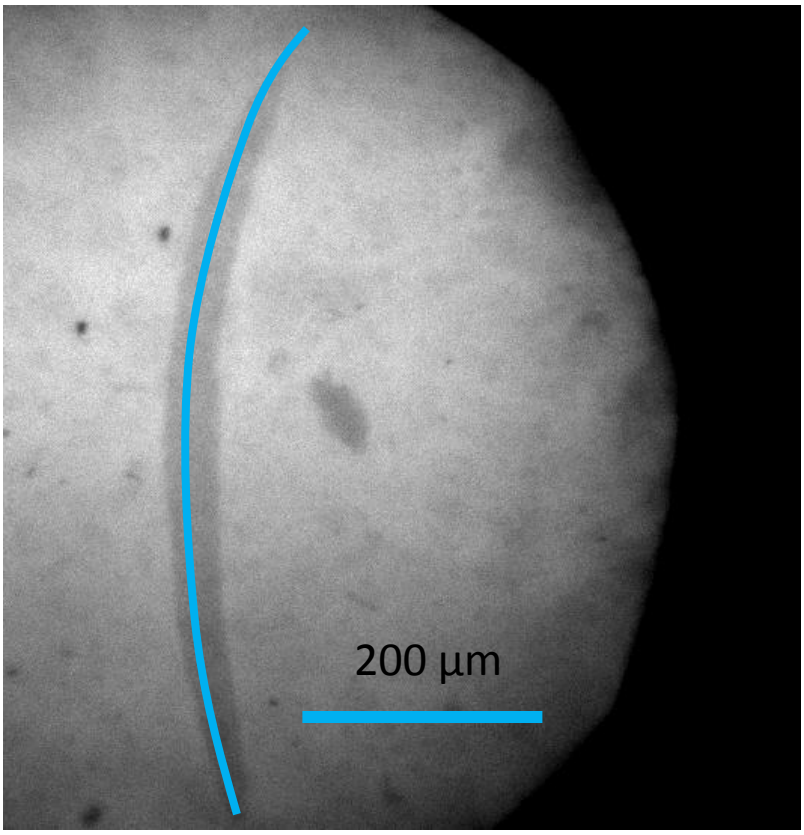


Figure 1-3. Locations of sample sites from the Midwest of United States.
(1) WB – West Bearskin Lake Minnesota, USA. (2) SR - Spring River Jasper County, Missouri, USA. (3) P30 - Pond 30 USGS Facility Columbia, Missouri, USA. (4) DOW - Dow Creek Michigan, USA. (5) STJ - St. Joseph River, Michigan, USA. (6) STM - South Tributary of Mill Creek, Michigan, USA. (7) RR2 and RR3 - Raisin River Sites 2 and 3, Michigan, USA. (A) Star – Position of Washington DC, USA. (B) Blue Circle – Position of New York City. New York, USA. (C) Red circle - Position of Chicago. IL, USA.

A



B

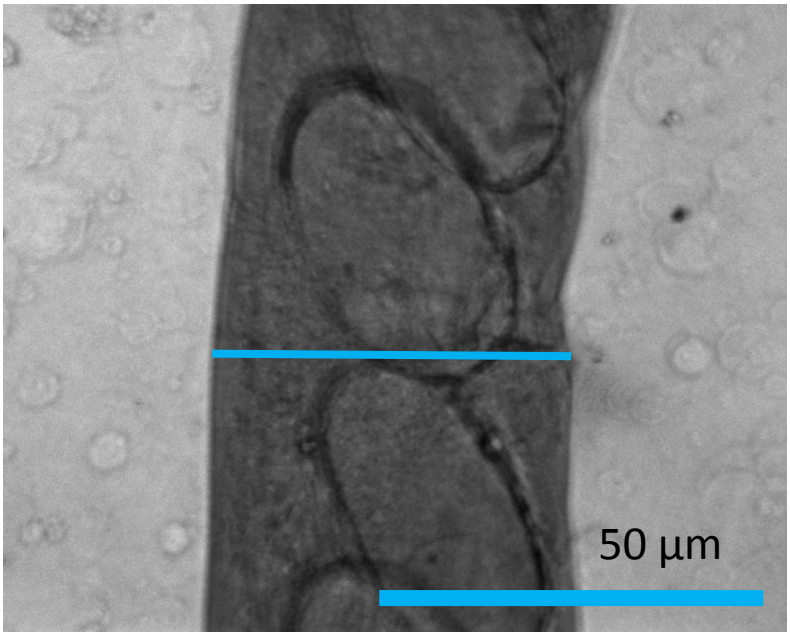


Figure 1-4. Images of measurements of adult N2. (A) Picture of a length measurement on an adult N2 at 2.5X magnification, (B) Picture of a width measurement on an adult N2 at 100X magnification.

CHAPTER 2: ANALYSIS OF RECOVERY, SURVIVAL, AND REPRODUCTION OF 3 NEMATODE SPECIES IN 8 CONTROL SEDIMENT SAMPLES

INTRODUCTION

With the need of the USGS for an assay organism that can be used in sediment trials from various locations where Nickel (II) may be a problem, it is nematodes that appear most frequently within the microfauna on a world wide scale. Nematodes have different life histories and requirements of the sediment that are needed for the species to be successful. This leads to the question, what sediments, and sediment characteristics, would the nematodes *C. elegans*, *C. briggsae*, and *P. pacificus* be successful in and lend themselves to a sediment analysis? Out of this initial question, we can ask in addition if the sediment will have any impact on the development, survival, or recovery of the P0 generation as well as the F1 generation? It is from these question that we can determine where *C. elegans* can be used in comparison to *C. briggsae* and *P. pacificus*, and aid us in laying the foundation for a possible nematode assay library. This library would be useful to not only the USGS but multiple government organizations around the world to determine the impact of heavy metals and other toxins released into the environment.

MATERIALS AND METHODS

This assay was conducted using the above described sediments from Tables 1-1, 1-2, and 1-3 and the nematode species *Caenorhabditis elegans* laboratory strain N2, *Pristionchus pacificus* laboratory strain PS312, and *Caenorhabditis briggsae* laboratory strain AF16. Sediment, weighed out to 1 mL, was placed into 6 wells. Into each of those wells, 10 larva, L1s for *C. elegans* and *C. briggsae* or J2s for *P. pacificus,* were inserted. Antibiotically killed *E. coli* strain OP50 was added to each well at 0.5 mL. Once the test was setup, it was allowed to run for 96 hours for *C. elegans* and *C. briggsae* and 120 hours for *P. pacificus*. Recovery was done by adding 3 mLs of a silica solution (2 parts water to 1 part silica suspension) to the wells and mixing the sediment up. The sediment solution mixture was removed and placed into 15 mL conical tubes upon which 5 mLs of additional silica solution was added. The conical tube was spun down at 800 gs to allow the nematodes to be removed from the sediment and into the overlying silica solution. This sediment was washed two more times with 8 mLs of silica solution to ensure all nematodes were removed.

RESULTS AND CONCLUSIONS

From the sediment assay with *C. elegans*, 0 adults were recovered from the SR-0 sediments, and increased in recovery to WB-0 where a mean of 9.42 adults were recovered. *P. pacificus* had recovery profile that varied across all the trials to a point where a mean of 5.67 adults were recovered from SR-0 sediments while STM-0 had a mean recovery of 1.17. All of the P0 individuals recovered had length and width measurements within standard laboratory values as well as being 100% gravid (Figure 2-1). This lead us to conclude that *C. elegans* are best suited to sediments in which there is a large percentage of organic carbon due to the fact that we saw an increase in recovery of the P0 individuals as the organic carbon increased from SR-0 to WB-0. *P. pacificus* appears to be a much broader generalist due to its overall successful recovery from all 8 control sediments as well as being recovered from sediments in which *C. elegans* had little to no recovery. *P. pacificus* did not appear to be directly correlated to one or two physio-chemical traits of the sediments based upon the criteria in Tables 1-1, 1-2, and 1-3. With all P0 individuals being recovered as gravid adults with no statistical differences between the length and width measurements when compared to laboratory numbers, then it stands to reason that the sediments are having no impact on development from larva/juveniles to adults and that the trace amounts of nickel present are not having any impact as well.

From the sediment assay looking at the recovery of L1 and J2 F1 larva, *C. elegans* showed a recovery of 0 mean larva for SR-0 sediments increasing in number up to the WB-0 sediment series where a mean of 170.75 larva were recovered. *P. pacificus* J2 F1 larva were recovered the lowest in sediment SR-0 with a mean of 5.67 and largest recovery in the sediment RR3-0 with a mean of 38.83. All F1 individuals recovered had length and width measurements within standard laboratory values (Figure 2-2). With the recovery of the F1 individuals from the

8 control sediments, we saw a similar trend compared to the P0 individuals. *C. elegans* once again had the highest recovery of F1 individuals from the sediments with the highest percentage of dissolve organic carbon and little to no recovery from the sediments with reduced organic carbon. *C. elegans* are once again being having the most individuals successfully recovered from increasing organic carbon sediments. *P. pacificus* once again proved to b a generalist with respect to the F1 generation. Their individuals were recovered from across all 8 control sediments once again proving that *P. pacificus* recovery of the F1 individuals is not directly correlated to one or two physio-chemical traits of the sediments based upon the criteria in Tables 1-1, 1-2, and 1-3.

A fecundity index profile showed that for *C. elegans* that the number of F1 larva to P0 adults showed that the number of F1 larva recovered was about 2-10 per adult for all of the sediments except SR-0 that had an index of 0 and WB-0 that had an index around 25 F1 per adult. *P. pacificus* had a fecundity index that of 1-3 for F1 larva per adult for SR-0 and WB-0 and around 5-7 F1 larva per adult for the other 6 sediments (Figure 2-3). The fecundity index for both *C. elegans* and *P. pacificus* gave a better insight into what is truly going on with respect to the P0 and F1 generations. When we looked at *C. elegans* and their fecundity index, we saw that once again that organic carbon is the determining factor when it comes to survival and recovery of the individuals. We saw that for most of the mid range levels of organic carbon , DOW-0 through RR3-0, that there was no statistical difference between the sediments but when the highest amount of organic carbon (WB-0) was compared to the lowest amount of organic carbon (SR-0) a very strong statistical difference was seen. From this information, it aided us in concluding that for *C. elegans* to be used in a sediment assay, they would lend themselves most suited to sediments with a large percentage of organic carbon. *P. pacificus* also showed an

interesting result in relation to their fecundity index. It appeared that the recovery and survival of the nematode was truly general. The number of F1 individuals produced compared to P0 individuals was pretty uniform across all 8 trials and there were significant differences between the trials. It is assumed that these statistical differences are most likely due to the recovery of the P0 individuals from Figure 2-1 and not the F1 individuals from Figure 2-2 because we saw similar recoveries of F1 individuals across all 8 sediments.

Caenorhabditis briggsae showed highly inconsistent recovery from all 8 sediments over 3 trials due to human testing error and the fact that the sediments by the time these trials were complete were old and may have issues that never arose during the testing with *C. elegans* or *P. pacificus*. Due to these errors, the recovery profiles for this nematode was not included in the figures but included in Table 2-1 as well as Appendix.

Table 2-1. Recovery of *C. briggsae* from 3 trials in 8 control sediments.

	Trial 1		Trial 2		Trial 3	
Sediment	**P0**	**F1**	**P0**	**F1**	**P0**	**F1**
SR-0	1.0	1.5	0.5	0.0	0.5	1.3
SJ-0	4.2	6.8	3.3	3.0	8.2	0.0
P30-0	3.5	1.8	0.3	0.0	4.7	2.5
DOW-0	2.2	1.8	5.0	5.3	2.7	1.3
RR2-0	6.7	0.0	4.5	3.2	8.7	2.3
STM-0	3.8	1.3	3.7	2.3	1.0	6.7
RR3-0	2.5	2.3	4.0	13.8	7.3	1.2
WB-0	7.5	30.2	5.3	20.7	0.0	0.0

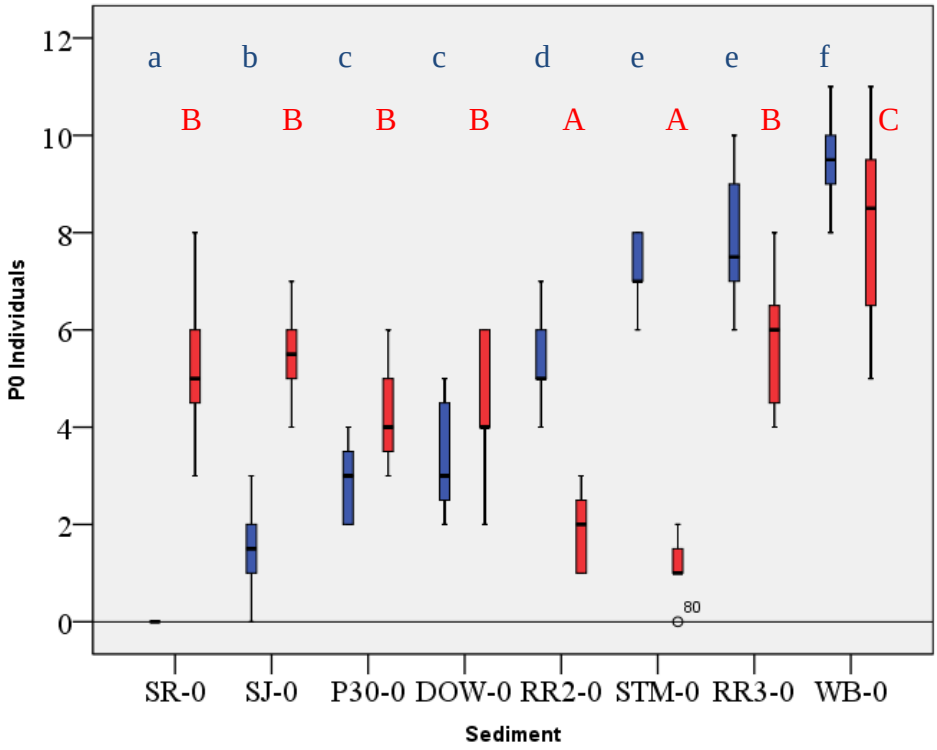


Figure 2-1. Recovery of adult P_0 animals from 8 control sediments.
Blue – *C. elegans* Red – *P. pacificus*

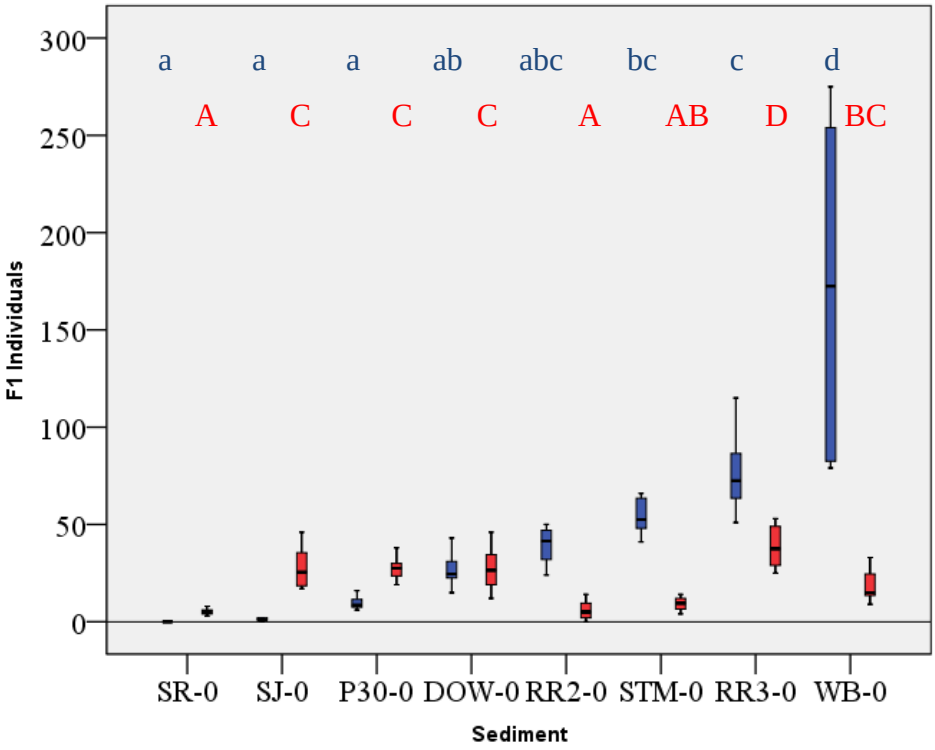


Figure 2-2. Recovery of L1/J2 F_1 larva from 8 control sediments.
Blue – *C. elegans* Red – *P. pacificus*

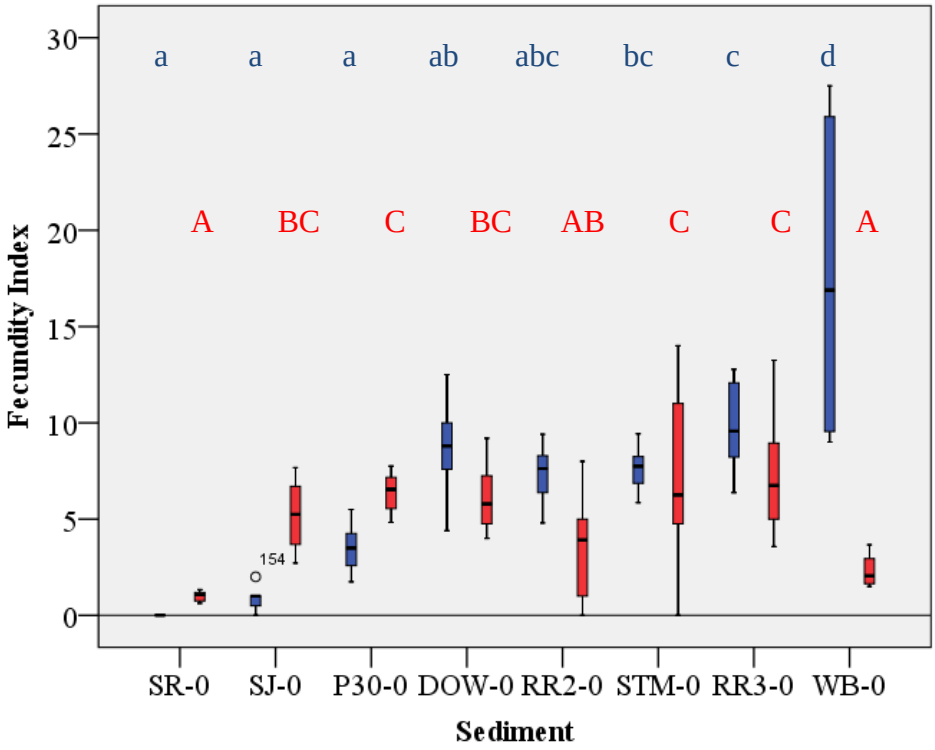


Figure 2-3. Fecundity index as a function of sediment.
Blue – *C. elegans* Red – *P. pacificus*

CHAPTER 3: ANALYSIS OF RECOVERY, SURVIVAL, AND REPRODUCTION OF 2 NEMATODE SPECIES IN 2 NICKEL SPIKED SEDIMENT SERIES

INTRODUCTION

Based upon the results from the previous assay looking at the 8 control sediments with only trace amounts of nickel, 2 sediments were chosen by the USGS to have a spiked series created to answer the question, what effect would Nickel (II) bound in the sediment have on the recovery, survivability, fecundity, and development of the nematodes? The sediments chosen were SR and WB. These two sediments were chosen based on the recovery of *C. elegans* as well as *P. pacificus.* This was chosen because *C. elegans* had their lowest recovery in SR-0 and highest recovery in WB-0, while *P. pacificus* had its best recovery from WB-0 and had a recovery rate from the SR-0 sediment that was much higher than that of *C. elegans*. Using these sediments, we could see the effect of nickel upon survival of larva to adults, and then the effects on reproduction. As previously described, nickel is a mutagen responsible for a variety of diseases ranging from simple skin rashes to cancers of the respiratory tract and it is important to determine what effect the nickel is having at various doses so we can infer the nickels response in the environment. The sediments were spiked and the results of the spiked sediment series can be seen in Table 1-4. This trial was conducted under the same parameters as the above mentioned sediment assay.

MATERIALS AND METHODS

This assay was conducted using the above described sediments from Tables 1-4 and the nematode species *Caenorhabditis elegans* laboratory strain N2 and *Pristionchus pacificus* laboratory strain PS312. Sediment, weighed out to 1 mL, was placed into 6 wells. Into each of those wells, 10 larva, L1s for *C. elegans* or J2s for *P. pacificus*, were inserted. Antibiotically killed *E. coli* strain OP50 was added to each well at 0.5 mL. Once the test was setup, it was allowed to run for 96 hours for *C. elegans* and 120 hours for *P. pacificus*. Recovery was done by adding 3 mLs of a silica solution (2 parts water to 1 part silica suspension) to the wells and mixing the sediment up. The sediment solution mixture was removed and placed into 15 mL conical tubes upon which 5 mLs of additional silica solution was added. The conical tube was spun down at 800 gs to allow the nematodes to be removed from the sediment and into the overlying silica solution. This sediment was washed two more times with 8 mLs of silica solution to ensure all nematodes were removed.

RESULTS AND CONCLUSIONS

C. elegans had around 10 P0 individuals recovered in the WB-0 and WB-1 sediments. Their recovery dropped to around 8.5 P0 individuals for WB-2 and 7.5 P0 individuals for WB-3. No P0 individuals were recovered in the WB-4 or WB-5 sediment. *P. pacificus* had around 8.5 and 8 P0 individuals recovered for WB-0 and WB-1. This dropped to around 5 P0 individuals recovered for WB-2 and WB-3 and finally no P0 individuals were recovered for the WB-4 and WB-5 sediments (Figure 3-1). For both nematodes, the recovery of the P0 individuals followed similar patterns described in Figure 2-1 for the control sediment and the first dosage level, WB-1. As the amount of nickel increased from WB-1 to WB-2 and WB-3, we saw that there was a gentle decline for *C. elegans* and 50% reduction for *P. pacificus*. When looking at the highest levels of bound nickel in sediments WB-4 and WB-5 we see a 0% recovery for both nematode species indicating that at these high levels of nickel it is acting as a lethal agent to the nematodes. For the P0 individuals, we see that the nickel bound in the WB spiked series is having an effect on survival and recovery of these nematodes in a dosage dependent manner and this is evident from Figure 3-1. The P0 generation saw all individuals being recovered as gravid and that there was no statistical difference from standard laboratory measurements for both species. This indicates that the bound nickel may only have an effect on survivability at this concentration levels and for this amount of time studied.

C. elegans had around 175 F1 individuals recovered in the WB-0 and WB-1 sediments. Their recovery dropped to around 130 F1 individuals for WB-2 and 100 F1 individuals for WB-3. No F1 individuals were recovered in the WB-4 or WB-5 sediment. *P. pacificus* had around 15 F1 individuals recovered for WB-0 and WB-1. This dropped to around 10 F1 individuals recovered for WB-2 and WB-3 and finally no F1 individuals were recovered for the WB-4 and

WB-5 sediments (Figure 3-2). When looking at the F1 generation for both animals, we once again see similar recoveries for the WB-0 and WB-1 sediments compared to the 8 control sediments seen in Figure 2-2. Once again we see that the overall recovery is higher for *C. elegans* compared to *P. pacificus* and that there is a general decrease in larva recovered as we go from WB-0 and WB-1 to WB-2 and WB-3, and finally seeing no larva in WB-4 and WB-5. The zero recovery from the WB-4 and WB-5 sediments is most likely due to their being no recovery of the P0 generation (Figure 3-2). The F1 generation also had no statistical differences in length and width measurements indicating that from this trial, we cannot determine if the nickel is acting as a mutagen or having any long term developmental effects on the larva. This also shows that the overall recovery of the F1 generation is declining but we cannot determine if it is due to an effect on the F1 generation or if it is due to the recovery of the P0 generation. It is from the fecundity index that we can determine the where the effect on the F1 generation lies.

The fecundity index showed that between 15 and 17 F1 larva were produced for every P0 *C. elegans* adult that was produced for the sediments from which the P0 generation survived. The fecundity index showed that between 2 and 3 F1 larva were produced for every P0 *P. pacificus* adult that was produced for the sediments from which the P0 generation survived (Figure 3-3). The fecundity indexes for both nematodes finally allowed for us to understand what effect the nickel was having on the nematodes in the WB spiked series. That effect was that the F1 generation was not directly affected by the bound nickel. This is evident by the fact that the mean number of F1 individuals per P0 individual recovered was not statistically different from each other within sediments where there were individuals being recovered. This led to the conclusion that the bound nickel is having an effect on the P0 generation of survival and subsequent recovery but not on the F1 generation over the course of our study. This however

doesn't mean that there isn't a long term effect on development on the F1 generation; it just indicates that we did not see one within the time frame of our study.

The SR sediment only had a recovery of the *P. pacificus* species. The SR sediment saw a mean recovery of 5.17 P0 individuals for SR-0 and 5.67 P0 individuals for SR-1. This dropped to 3.92 P0 individuals for SR-2 and 3.67 P0 individuals for SR-3. The SR-4 and SR-5 sediment saw 0 P0 individuals recovered (Figure 3-4). *C. elegans* showed no recovery of individuals from the SR spiked series. This is consistent with our evidence from our 8 control sediments and the effects of the nickel in this sediment will be dependent on *P. pacificus*. The P0 generation once again showed a similar recovery profile for both the SR-0 and SR-1 sediments that had the two lowest nickel levels. The number of individuals recovered decreased in the SR-2 and SR-3 sediments before flat-lining in the SR-4 and SR-5 sediments in a similar manner to the WB spiked series. For the P0 individuals, we see that the nickel bound in the SR spiked series is having an effect on survival and recovery of these nematodes in a dosage dependent manner and this is evident from Figure 3-4. The P0 generation saw all individuals being recovered as gravid and that there was no statistical difference from standard laboratory measurements for the species. This indicates that the bound nickel may only have an effect on survivability at these concentration levels and for this amount of time studied.

The SR sediment saw a mean recovery of 5.00 F1 individuals for SR-0 and 5.33 F1 individuals for SR-1. This dropped to 3.58 F1 individuals for SR-2 and 3.92 F1 individuals for SR-3. The SR-4 and SR-5 sediment saw 0 F1 individuals recovered (Figure 3-5). Once again we see a similar decline in the recovery of the F1 individuals. SR-0 and SR-1 showed a similar recovery profile compared to Figure 2-2 out of the 8 control sediments, and we see that the numbers decline overall from SR-0 through SR-3 before flatling and having 0 recovery in SR-4

and SR-5. Once again it is believed that the zero recovery for the SR-4 and SR-5 sediment is due to zero P0 individuals being recovered so no F1 larva should be produced. Similar to the WB spiked series, we see that there is no statistical difference between length and width measurements taken compared to that of laboratory measurements and the nickel may be having an effect on the survivability of the larva, but that is left to be determined by the fecundity index.

The fecundity index had a recovery around 1 F1 larva were produced for every P0 *P. pacificus* adult that was produced for the sediments from which the P0 generation survived (Figure 3-6). The fecundity index once again tells the story of what is happening within the SR spiked sediment series. We see that for all of the sediments in which we had a recovery, there was a similar recovery profile in terms of the number of F1 individuals per P0 individual recovered. This indicates also that the nickel is having a direct impact on survival and recovery of the P0 generation, but the survivability and recovery of the F1 generation is not directly impacted. This once again does not indicate that nickel is not having an effect on the F1 generation; it simply means that one is not directly apparent from the length and time of the experiment.

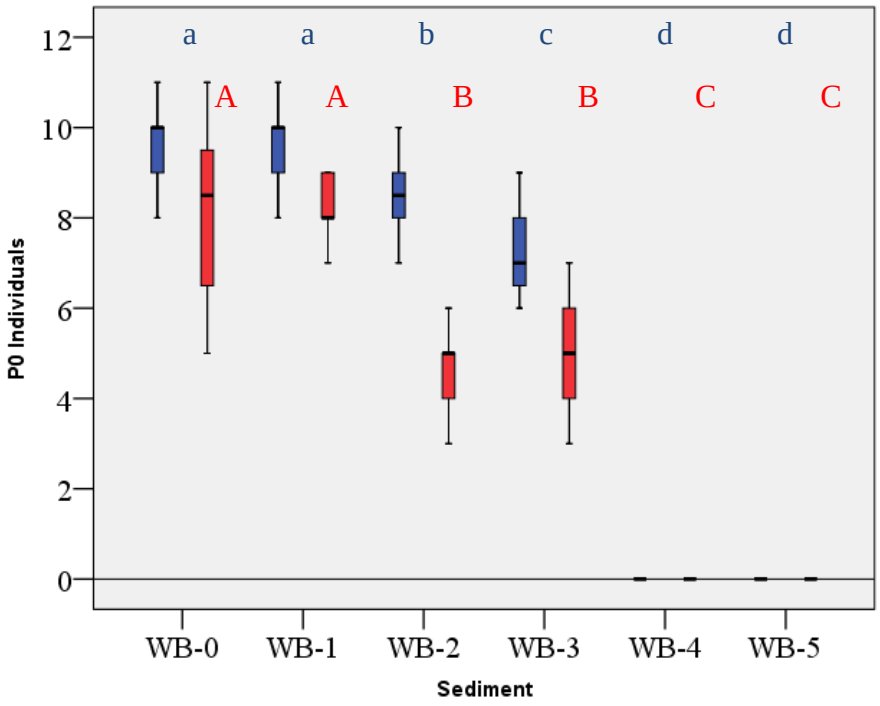
The nickel itself has led to an interesting result in determination of the nickel concentration needed for an LD50. From the scatter plots, Figures 3-1B and 3-4 B, and Table 1-5, an estimate of the dosage of nickel that leads to a LD50 can be approximated. This estimation was made based upon a binomial, trinomial, logarithmic, and exponential (n+1) trendlines. Table 3-1 shows the amount of nickel estimated for the LD50 and where it fit among the spiked sediment series. For determining the concentration needed to reach a LD50 for the WB series, y was equal to 5 for *C. elegans* and 4 for *P. pacificus*. For determining the concentration needed to reach a LD50 for the SR series, y was equal to 3 for *P. pacificus*. These numbers were chosen

based off of the closest whole number rounded up from the recovery of the P0 generation in the WB-0 or SR-0 sediments. Most of the estimated LD50 nickel amounts for both the WB and SR series fell between the WB-3 and WB-4 data or the SR-3 and SR-4 data. This is consistent with the data where we saw a 0 recovery after WB-3 and SR-3 respectfully. This means that the nickel is showing a titration effect where the nickel is having a strong effect on the survival of the nematodes. This begs the question that if the nickel is having an effect, is it because of the bound nickel within the sediment or the nickel in liquid suspension?

Table 3-1 . LD50 nickel estimate comparison to the nickel levels of spiked sediment.

For WB *C. elegans*			
Formula	Nickel (µg/g)	Sediment	Nickel Sediment (µg/g)
Binomial	1272.60	WB-3 WB-4	1040 - 2680
Trinomial	1813.16	WB-3 WB-4	1040 - 2680
Log	921.75	WB-2 WB-3	369 - 1040
Exp (n+1)	1311.08	WB-3 WB-4	1040 - 2680
For WB *P. pacificus*			
Formula	Nickel (µg/g)	Sediment	Nickel Sediment (µg/g)
Binomial	1122.05	WB-3 WB-4	1040 - 2680
Trinomial	1104.27	WB-3 WB-4	1040 - 2680
Log	769.43	WB-2 WB-3	369 - 1040
Exp (n+1)	1038.79	WB-2 WB-3	369 - 1040
For SR *P. pacificus*			
Formula	Nickel (µg/g)	Sediment	Nickel Sediment (µg/g)
Binomial	221.00	SR-3 SR-4	213 - 411
Trinomial	215.43	SR-3 SR-4	213 – 411
Log	167.92	SR-2 SR-3	122 - 213
Exp (n+1)	246.06	SR-3 SR-4	213 – 411

A



B

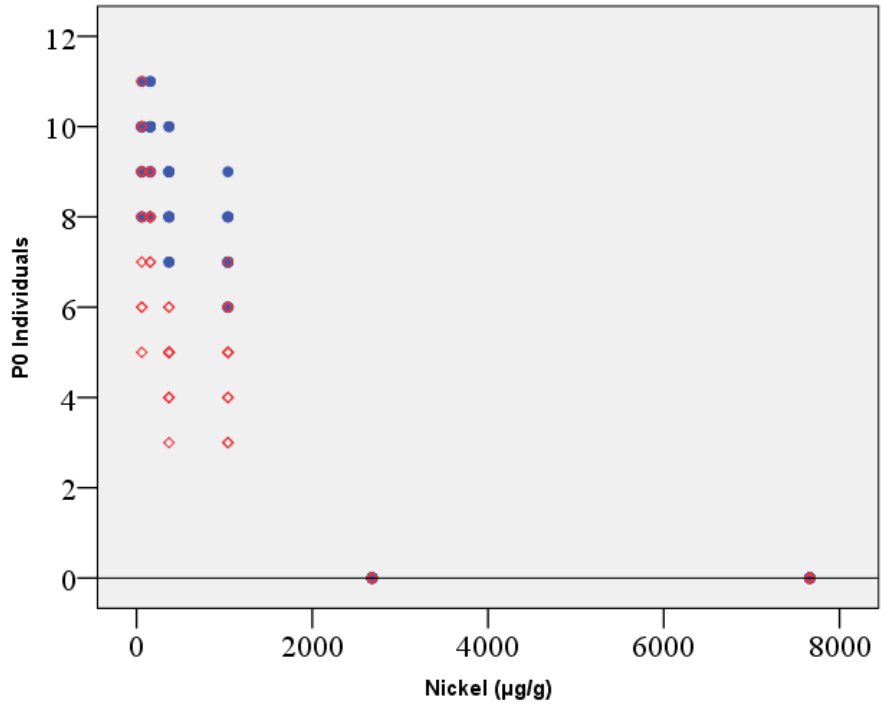
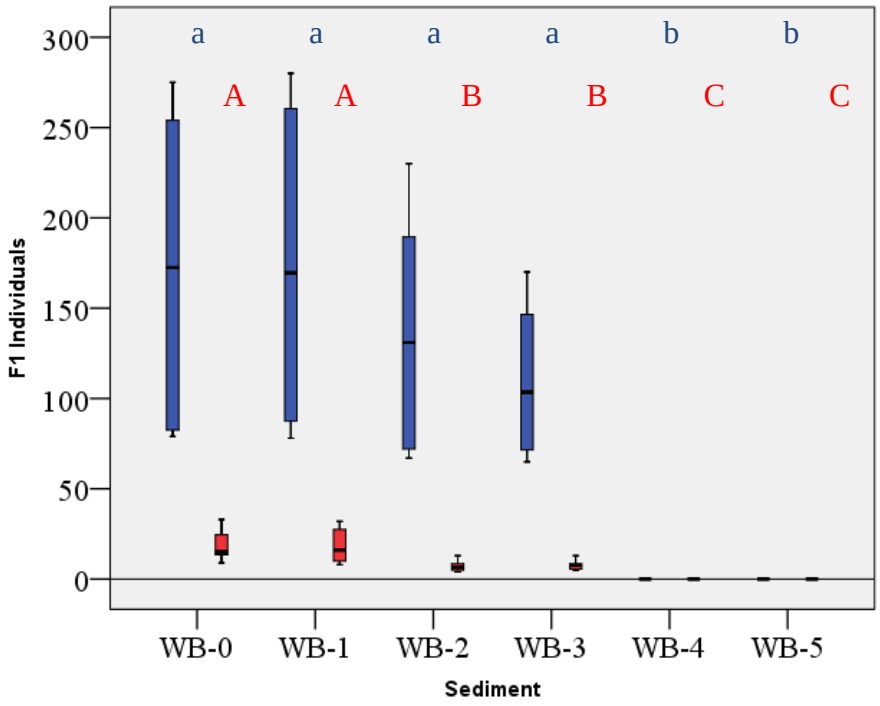


Figure 3-1. Recovery of adult P_0 animals from WB spiked sediment series. (A) Box and Whisker plot of P0 recovery, (B) Scatter plot of recovery of P0 based upon nickel.
Blue – *C. elegans* Red – *P. pacificus*

A



B

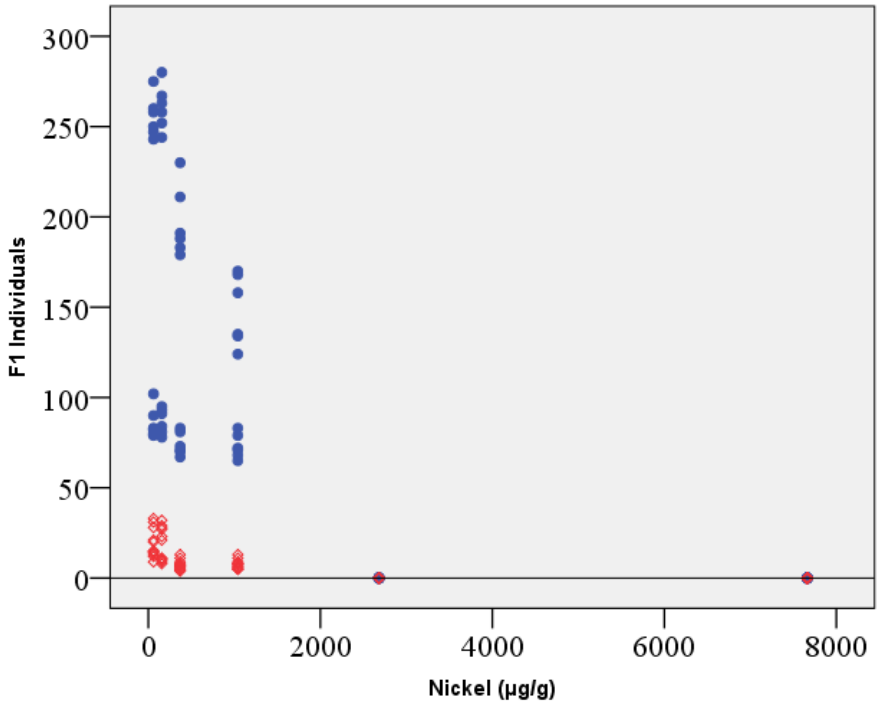


Figure 3-2. Recovery of L1/J2 F_1 Larva from WB spiked sediment series. (A) Box and Whisker plot of F1 recovery, (B) Scatter plot of recovery of F1 based upon nickel.
Blue – *C. elegans* Red – *P. pacificus*

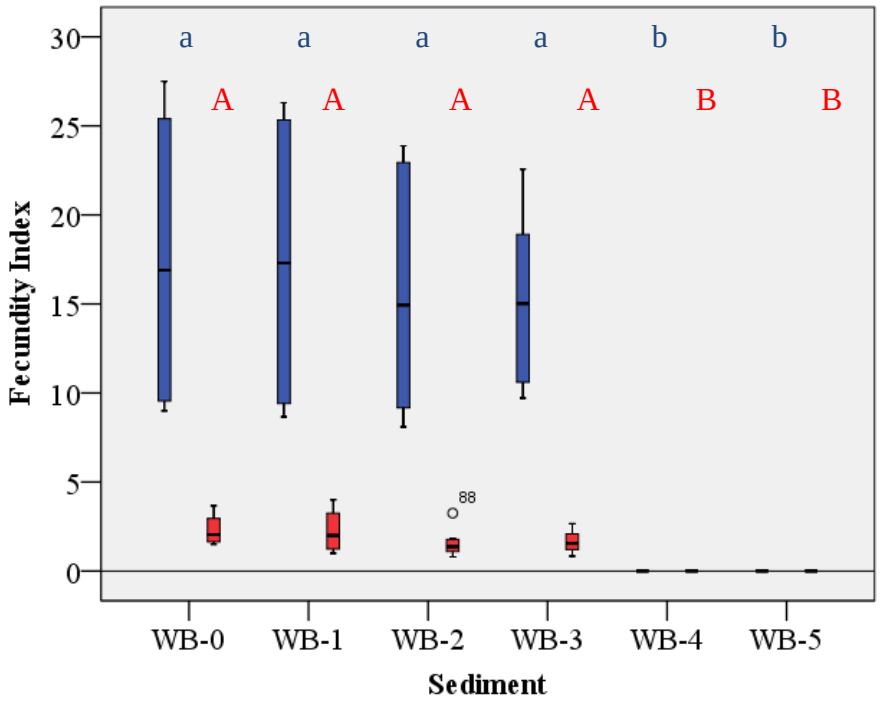
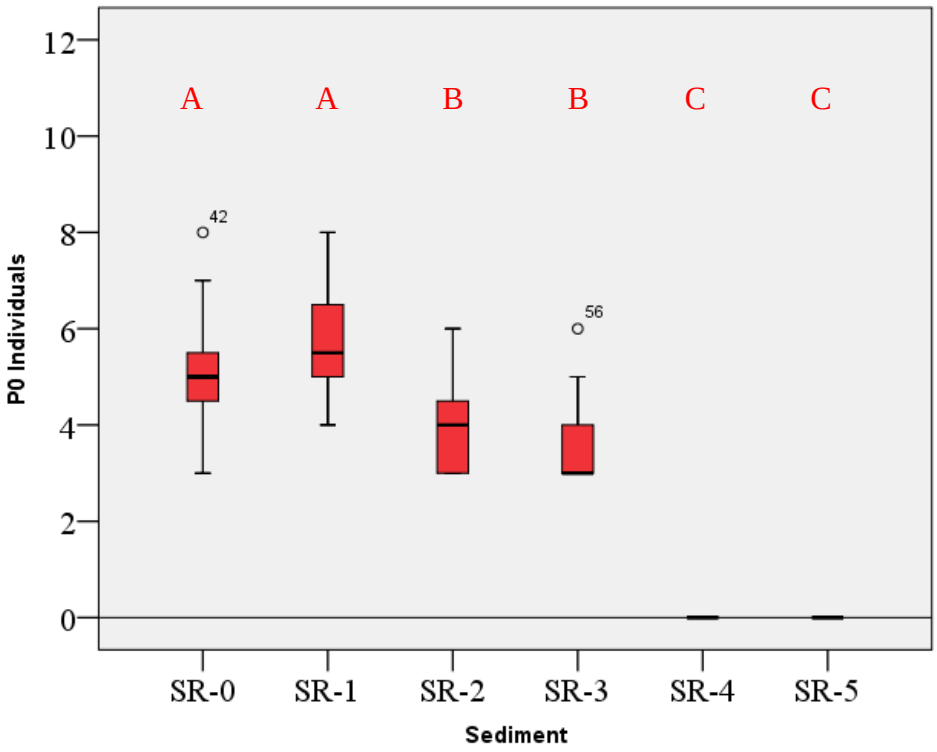


Figure 3-3. Fecundity index as a function of WB sediment.
Blue – *C. elegans* Red – *P. pacificus*

A



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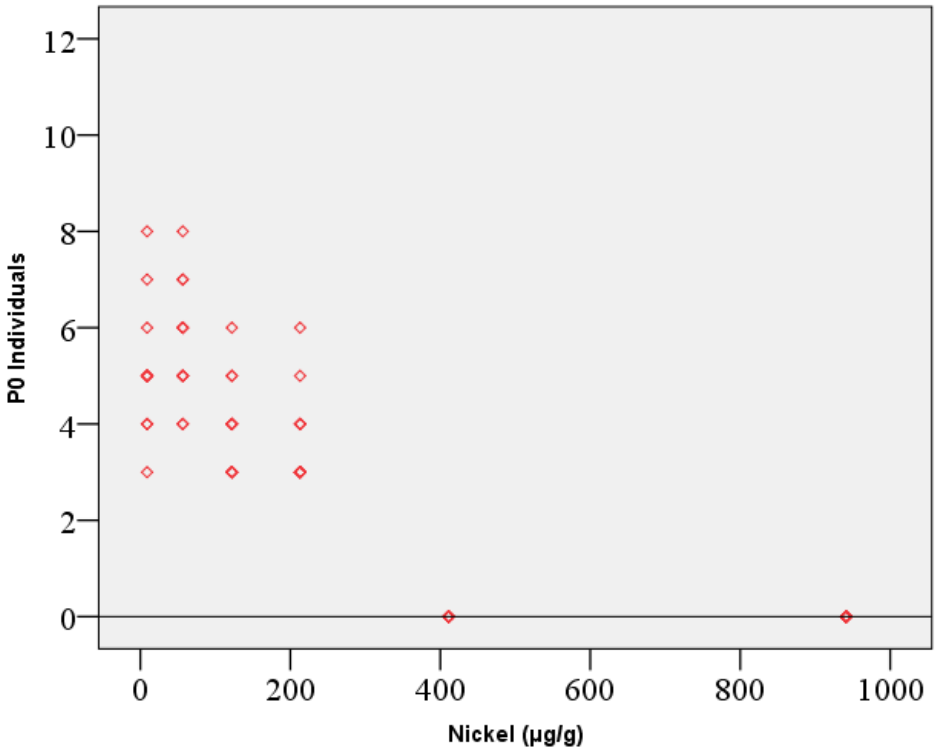
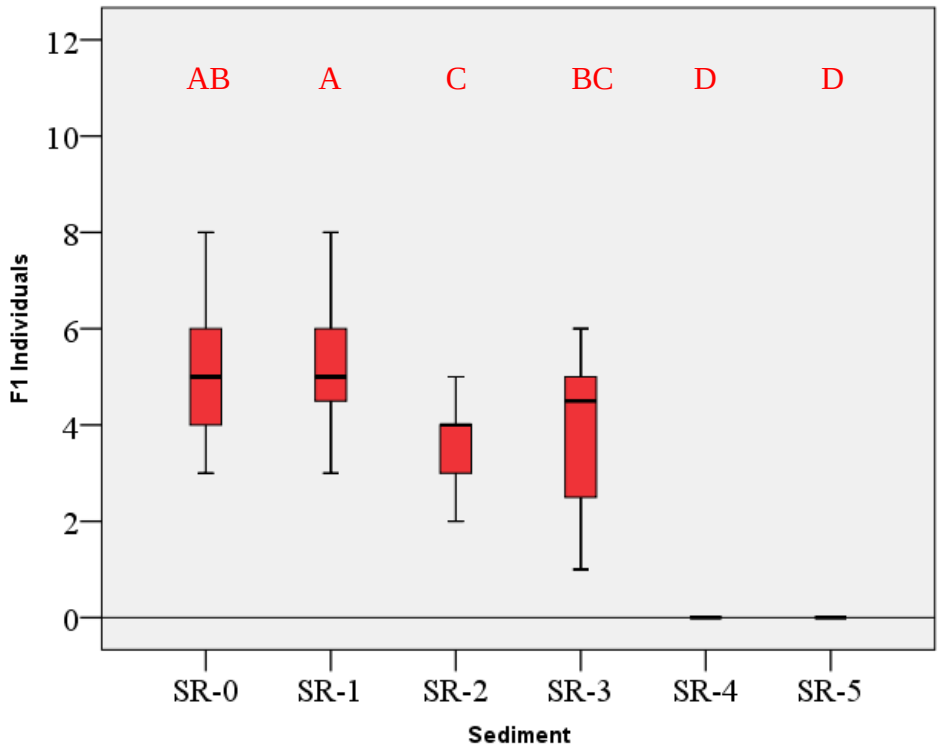


Figure 3-4. Recovery of adult P_0 animals from SR spiked sediment series. (A) Box and Whisker plot of P0 recovery, (B) Scatter plot of recovery of P0 based upon nickel.

A



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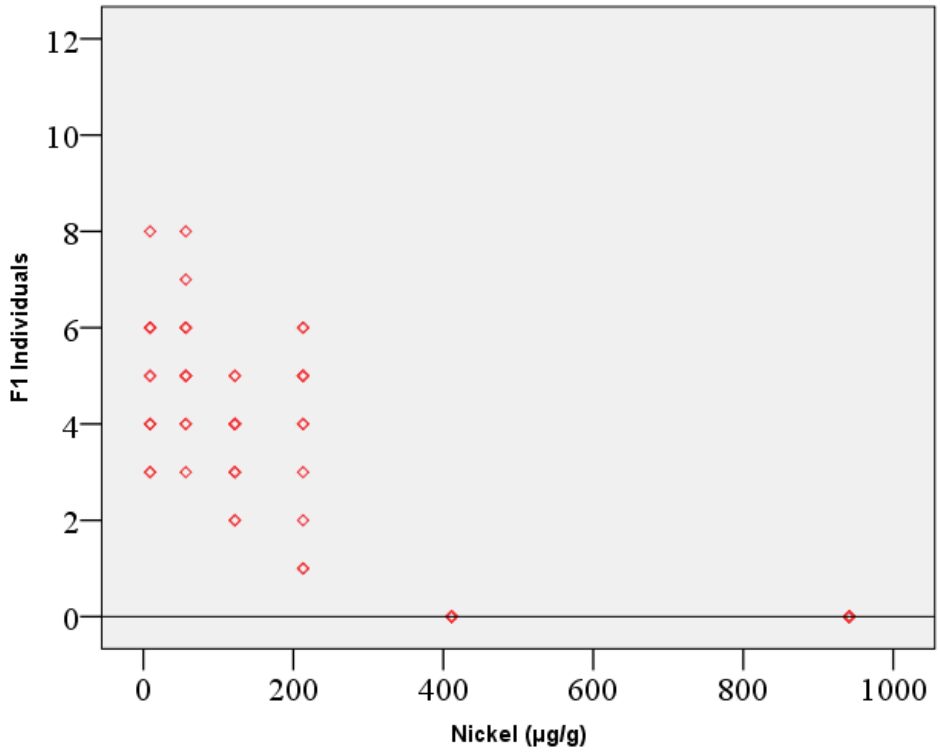

Figure 3-5. Recovery of L1/J2 F_1 Larva from SR spiked sediment series. (A) Box and Whisker plot of P0 recovery, (B) Scatter plot of recovery of P0 based upon nickel.

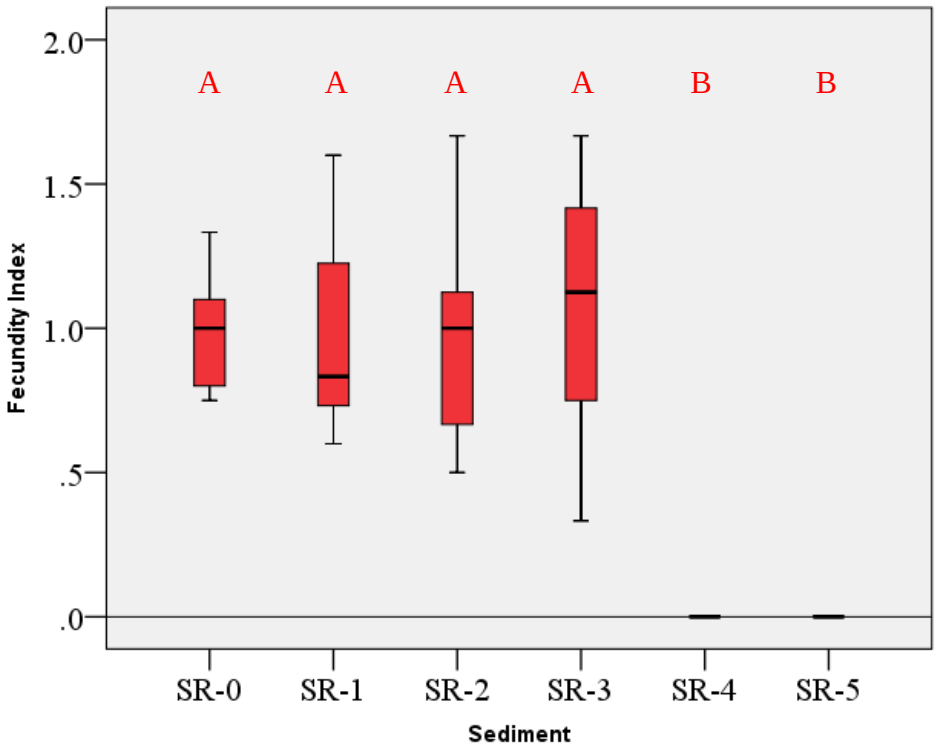

Figure 3-6. Fecundity index as a function of SR sediment

CHAPTER 4: ANALYSIS OF RECOVERY, SURVIVAL, AND REPRODUCTION OF 3 NEMATODE SPECIES IN AQUEOUS NICKEL WATER

INTRODUCTION

With nickel having the ability to go into solution or bound in a salt or substrate form, it must be determined what form of nickel is lethal and which is leading to the results we are seeing. Nickel has been shown in its aqueous state to be able to freely move though the environment similar to other heavy metals. This is a major risk because this means that it can be washed out into the water table and allowed to filter down through and be released and exposed to a greater part of the environment. If it were spread into a major water source, like a river, then it could be washed farther downstream leading to the potential exposure to a greater environmental risk, ecological risk, and human risk. When it is in its bound state, it is locked into the substrate generally as an oxide or sulfide compound. This water assay is crucial to determining whether or not the aqueous form or the bound form is the leading contributor to the results being seen and which we should be looking out for when trying to determine which is the greater environmental toxin. From our previous experiment with the spiked sediment, not all of the nickel was locked into the sediment. There was a slight aqueous layer that did contain an amount of nickel that was reported back to us from the USGS as being around 200 μg /L. It was from this information that we decided to look at the effects of aqueous nickel and we wanted to answer the question of what effect would aqueous nickel have on the recovery, survival, reproduction, and development of all three nematode species? From the information we received, two spiked water series, distilled water and hard water, were created with the concentrations of 0, 50, 100, 200, 400, and 800 μg /L.

The water assay was conducted under the same parameters as the sediment samples described above. The water sample was made with Hard Water, provided by the USGS, or distilled water. This assay was conducted using 3 of the nematode species: *Caenorhabditis elegans* laboratory strain N2, *Pristionchus pacificus* laboratory strain PS312, and *Caenorhabditis briggsae* laboratory strain AF16.

MATERIALS AND METHODS

This assay was completed using water, hard water pH of 7.5 or distilled water (di), that had contained dissolved $NiCl_2$. Concentrations were made based upon results given back to us from the USGS indicating that the amount of nickel in the over lying pore water was around 200 µg/L. *Caenorhabditis elegans* laboratory strain N2, *Pristionchus pacificus* laboratory strain PS312, and *Caenorhabditis briggsae* laboratory strain AF16. The Nickel (II) contaminated water was measured out to 1 mL and was placed into 6 wells for each concentration. Into each of those wells, 10 larva, L1s for *C. elegans* and *C. briggsae* or J2s for *P. pacificus*, were inserted. Antibiotically killed *E. coli* strain OP50 was added to each well at 0.5 mL. Once the test was setup, it was allowed to run for 96 hours for *C. elegans* and *C. briggsae* and 120 hours for *P. pacificus*. Recovery was done by simply removing all liquid including the P0 and F1 generation.

RESULTS AND CONCLUSION

For all of the hard water assays performed, all L1/J2 animals that were inserted were collected as P0 adults as seen in Figure 4-1. This was true for all three nematode species. This indicated that the aqueous nickel had zero effect on the nematode development from larva to adult. With 10 individuals being inserted and 10 individuals being recovered, the aqueous nickel didn't have an effect on survival. There was also no effect on length and width measurements for all of the species compared to that of laboratory numbers. All P0 individuals were recovered as gravid adults indicating that there was also no effect on development. The aqueous nickel result for the P0 individuals confirmed that the results that were seen in the spiked sediment series was most likely due to the bound nickel and that our assumption of the bound nickel being lethal to the individuals is supported.

The larva recovery was different between the three species as well. *P. pacificus* showed no reduction in larva from 0 μg/L to 800 μg/L staying around 200 F1 individuals recovered for the hard water spiked nickel trial. *C. elegans* and *C. briggsae* showed a similar recovery profile between both species and for both water trials. The largest amount of F1 individuals was recovered from the 0 μg/L trials with around 116 F1 individuals for *C. elegans* and 148 F1 individuals for *C. briggsae* for the hard water trial. The least amount of F1 individuals was recovered from the 800 μg/L trials with around 72 F1 individuals for *C. elegans* and 98 F1 individuals for *C. briggsae* for the hard water trial (Figure 4-2). It should be noted that *C. briggsae* did have a varied recovery response and because of this, the data was included and referenced but its results are suspect due to human testing error. The F1 individuals also showed that *P. pacificus* appears to be more resistant to the aqueous nickel compared to *C. elegans*. *C. elegans* did have a strong response in a dose dependent manner showing a decline in F1

individuals from 0 to 800 µg /L. This indicates that the aqueous nickel could be having an effect on the F1 generation, but this is only assumed without confirmation from the fecundity index.

The fecundity index showed that there was no difference between the F1 larva per P0 adult ratio for *P. pacificus*. There was a decrease in the amount of F1 larva per P0 adult ratio for both *C. elegans* and *C. briggsae* in a dosage dependent manner as seen in Figure 4-3

For all of the distilled water assays performed, all L1/J2 animals that were inserted were collected as P0 adults as seen in Figure 4-4. This was true for all three nematode species. This indicated that the aqueous nickel had zero effect on the nematode development from larva to adult just like in the hard water assay. With 10 individuals being inserted and 10 individuals being recovered, the aqueous nickel didn't have an effect on survival. There was also no effect on length and width measurements for all of the species compared to that of laboratory numbers. All P0 individuals were recovered as gravid adults indicating that there was also no effect on development. The distilled water and hard water aqueous nickel assay results for the P0 individuals confirmed that the results that were seen in the spiked sediment series was most likely due to the bound nickel and that our assumption of the bound nickel being lethal to the individuals is supported.

The larva recovery was different between the three species as well. *P. pacificus* showed no reduction in larva from 0 µg/L to 800 µg/L staying around 195 F1 individuals recovered for the distilled water spiked nickel trial. *C. elegans* and *C. briggsae* showed a similar recovery profile between both species and for both water trials. The largest amount of F1 individuals was recovered from the 0 µg/L trials with around 118 F1 individuals for *C. elegans* and 77 F1 individuals for *C. briggsae* for the distilled water trial. The least amount of F1 individuals was recovered from the 800 µg/L trials with around 74 F1 individuals for *C. elegans* and 52 F1

individuals for *C. briggsae* for the distilled water trial (Figure 4-5). The F1 individuals from the distilled water assay showed similar results compared to the hard water trials. *P. pacificus* appears to be more resistant to the aqueous nickel compared to *C. elegans*. *C. elegans* did have a strong response in a dose dependent manner showing a decline in F1 individuals from 0 to 800 μg /L. This indicates that the aqueous nickel could be having an effect on the F1 generation, but this is only assumed without confirmation from the fecundity index.

The fecundity index for the distilled water showed that there was no difference between the F1 larva per P0 adult ratio for *P. pacificus*. There was a decrease in the amount of F1 larva per P0 adult ratio for both *C. elegans* and *C. briggsae* in a dosage dependent manner as seen in Figure 4-6. From both of the fecundity indexes, the assays indicate that for *C. elegans*, the number of F1 individuals being recovered is most likely due to the aqueous nickel. Unlike the spiked sediment assays, the aqueous nickel is having an effect on the number of progeny being produced although how it is affecting the F1 individuals it is hard to say. The aqueous nickel is assumed to be effecting the development of the larva either in the gonad or the environment by delaying the development, it could be reducing the number of eggs produced in the P0 individual by inducing apoptosis within the germ line, or it could be retarding laying of the eggs thereby reducing the number of larva produced in the given assay time. From our experiments, it can only be assumed and not directly supported.

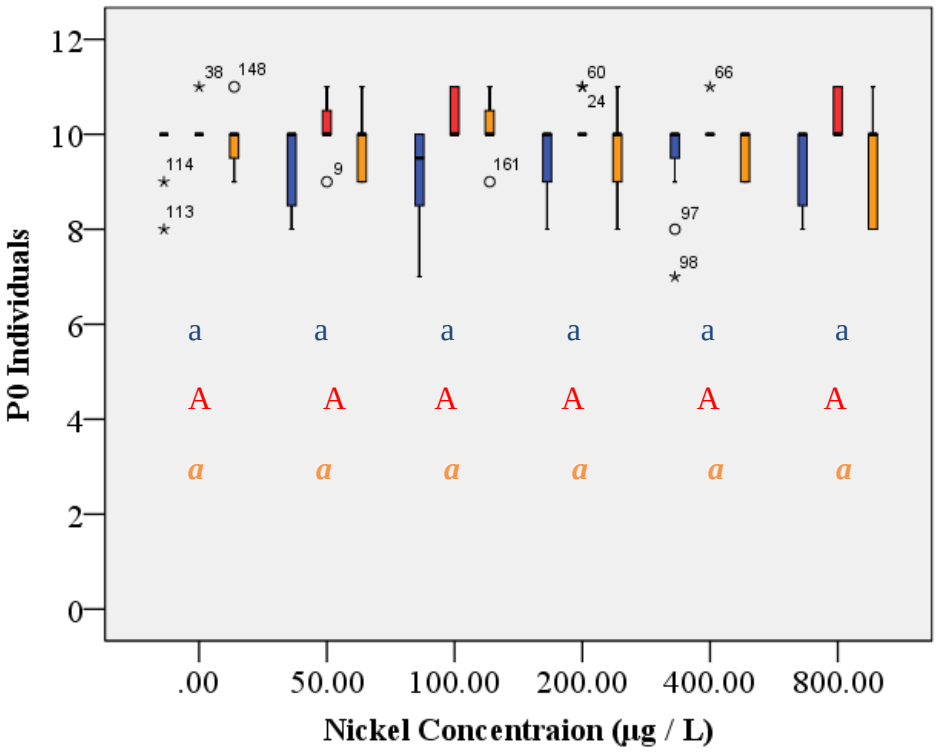

Figure 4-1. Recovery of adult P_0 animals from Ni(II) spiked hard water.
Blue – *C. elegans* Red – *P. pacificus* Orange – *C. briggsae*

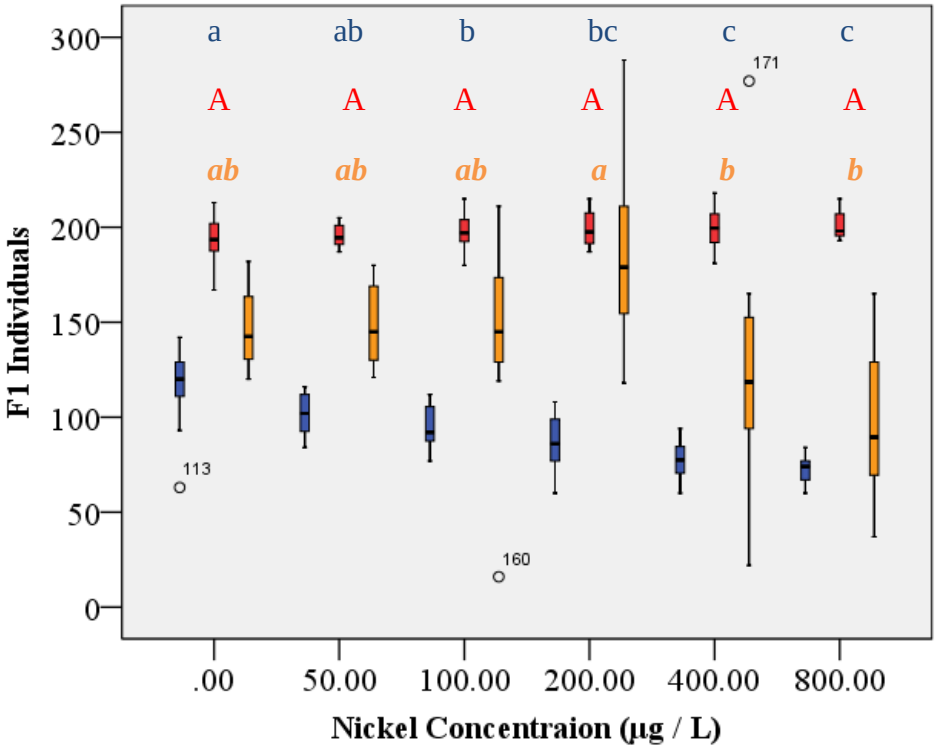

Figure 4-2. Recovery of L1/J2 F_1 larva from Ni(II) spiked hard water.
Blue – *C. elegans* Red – *P. pacificus* Orange – *C. briggsae*

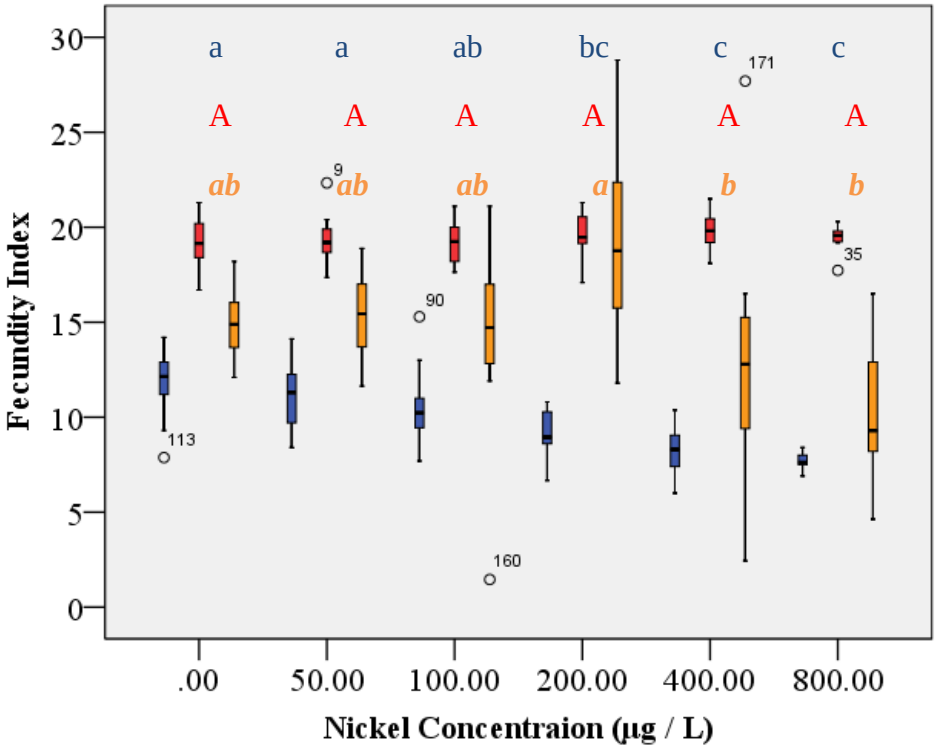

Figure 4-3. Fecundity index as a function of $NiCl_2$ dosage.
Blue – *C. elegans* Red – *P. pacificus* Orange – *C. briggsae*

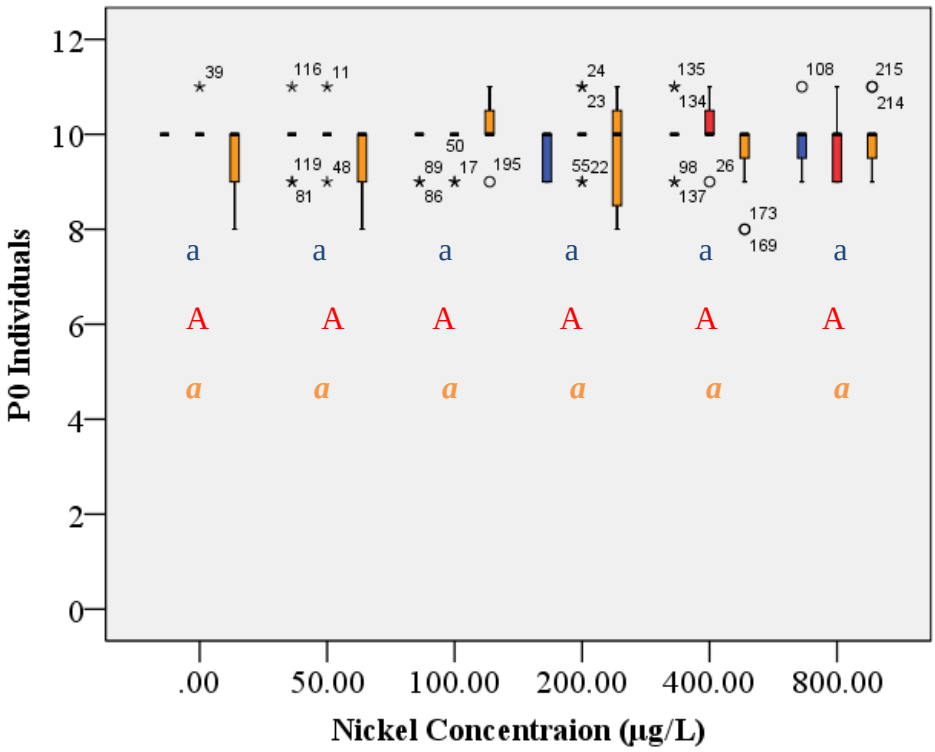

Figure 4-4. Recovery of adult P_0 animals from Ni(II) spiked di water.
Blue – *C. elegans* Red – *P. pacificus* Orange – *C. briggsae*

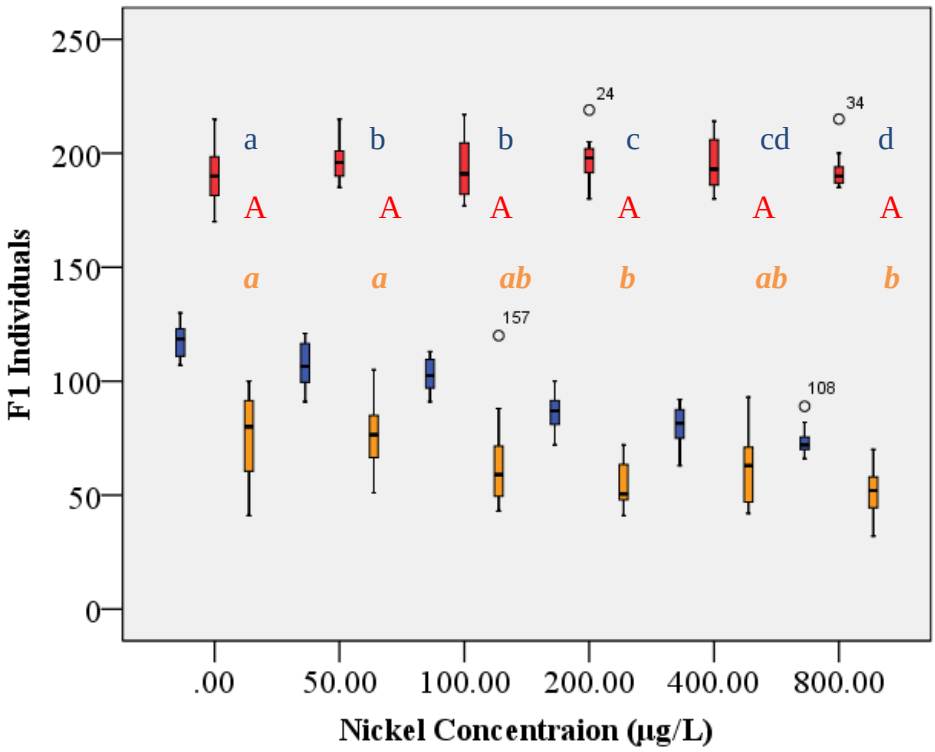

Figure 4-5. Recovery of L1/J2 F_1 larva from Ni(II) spiked di water.
Blue – *C. elegans* Red – *P. pacificus* Orange – *C. briggsae*

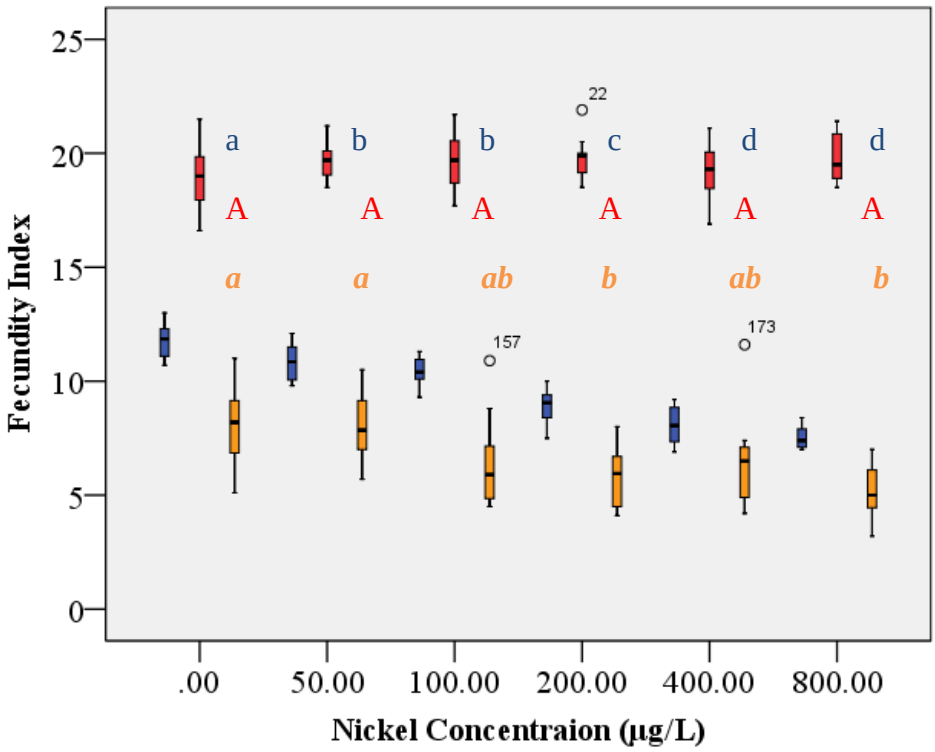

Figure 4-6. Fecundity index as a function of $NiCl_2$ dosage.
Blue – *C. elegans* Red – *P. pacificus* Orange – *C. briggsae*

CHAPTER 5: ANAYLSIS OF LONGEVITY OF *C. elegans* STRAIN *fog-2* IN NICKEL SPIKED SEDIMENTS SERIES OVER 22 DAYS

INTRODUCTION

With the evidence seen so far from the assays, there are many conclusions that have been formed. Nickel is clearly having a lethal effect when it is bound to a substrate within a sediment environment and having a detrimental effect to the next generation when in an aqueous state. All of the assay's were looking at the survival of nematodes that were newly hatched within an environment, and not at what would happen to an individual adult if it were to move into an environment with nickel present. This is important because if an adult can migrate into an area with nickel present, how long could it survive and could it produce offspring are the two most important questions needed to be asked. In regards to why is this important, nematodes, like other animal species, will migrate into areas in the search of food, mates, or to get away from a lethal environment. If a nematode was to migrate into that area, then what could happen? Would the adult survive? Could it produce offspring? Could it make it out of the area?

This assay was a 22 day assay to look at the longevity of the nematode *C. elegans* strain JK574: *Cel-fog-2 (q71) LGV* in different sediments with different levels of nickel and they were inserted as L4. The assay was setup in 22 wells in the same manner and requirements as the previously stated assays. Food would be added every 4^{th} day to ensure that the nematodes were not starving within the sediment. Nematodes were collected daily and evaluated on whether they were dead or alive. The 4 sediments chosen for this assay were WB-0, WB-2, WB-3, and WB-5. These sediments were chosen because of WB-0 having the greatest survival, WB-5 having the lowest survival rate, and WB-2 and WB-3 both having a decline in survivability before the estimated LD50 nickel concentration.

MATERIALS AND METHODS

This assay was conducted using the above described sediments from Tables 1-4 and the nematode specie *C. elegans* strain JK574: *Cel-fog-2 (q71) LGV*. Sediment, weighed out to 1 mL, was placed into 22 wells for the sediments WB-0, WB-2, WB-3, and WB-5. Two trials were setup with Trial 1 consisting of sediments WB-0 and WB-5 and Trial 2 consisting of WB-2 and WB-3. For Trial 1, around 55 L4 *fog-2* females were added to each well and for Trial 2 around 75 L4 *fog-2* females were added. For comparison of these two trials, the results were normalized so that the number of individuals recovered were divided by the average number of individuals added. Antibiotically killed *E. coli* strain OP50 was added to each well at 0.5 mL. The test was allowed to run for 22 days. Recovery was done by adding 3 mLs of a silica solution (2 parts water to 1 part silica suspension) to the wells and mixing the sediment up. The sediment solution mixture was removed and placed into 15 mL conical tubes upon which 5 mLs of additional silica solution was added. The conical tube was spun down at 800 gs to allow the nematodes to be removed from the sediment and into the overlying silica solution. This sediment was washed two more times with 8 mLs of silica solution to ensure all nematodes were removed.

RESULTS AND CONCLUSION

From the assay, Figure 5-1 was created. The nematode assays were not all performed at the same time so different amounts of nematodes were added. This led to the decision to normalize the graph. The nematodes survived in the WB-0, WB-2, and WB-3 sediments for the full 22 day period. The nematodes in the WB-5 sediment were not recovered alive after day 11. Using Table 1-5, the estimated day of reaching a 50% recovery (.5) was day 14.052 for WB-0, 18.172 for WB-2, 7.751 for WB-3, and 2.991 for WB-5. If adult nematodes are introduced into an environment and substrate bound nickel is present, then the nematodes may or may not have a chance at survival. From the data, nematodes that were introduced into the WB-0 and WB-2 sediments lived out their lives as they normally would. This indicates that the next generation would be produced; they would have an equal chance of surviving and producing the next generation. The nematodes introduced into the WB-3 sediment were recovered throughout the 22 day test cycle except that they reached an LD50 twice as fast as sediments WB-0 and WB-2. This has a range of consequences indicating that a full life cycle could be reached, they could produce the next generation, but the nickel is proving to be lethal and reducing survival. This also is important because this means as the generations go on, the number of larva surviving each generation would be at a level much reduced compared to a population within sediment where trace amounts of nickel would be present. WB-5 gave the greatest result reaching a 50% recovery 7 to 9 times faster than the WB-0 and WB-2 sediments. The day needed to reach a 50% recovery was 2.991 which indicated that not even one full generation has passed. This means if an adult were to migrate into this area, they would be able to lay the next generation, but that generation as well as the adult would not survive. This is also import because if a L1 were to do

the same thing, then the larva would not make it to adulthood, not lay the next generation, and essentially die out.

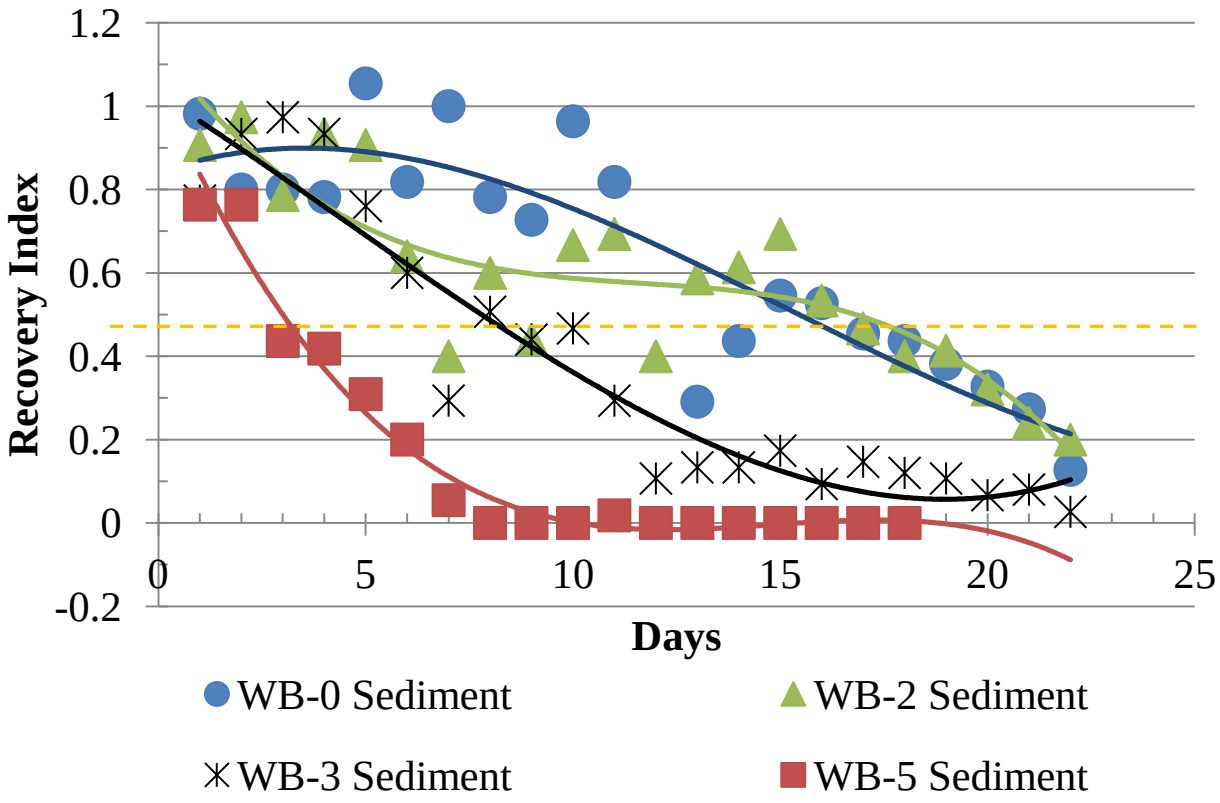

Figure 5-1. Recovery index for adult longevity in nickel spiked sediment

CHAPTER 6: CONCLUSION

From the assays performed a number of conclusions can be made. First is that the nematodes *C. elegans* and *P. pacificus* are sensitive to nickel when bound in a sediment and they can be used as bioindicator organisms. *C. elegans* appear to be best suited for sediments with large amounts of dissolved organic carbon while *P. pacificus* could be used as a broad species to look at a variety of sediments. *C. briggsae* needs more testing to determine what sediment requirements are needed in order for this species to be thought of as a bio indicator species. Second, Nickel (II) when bound in a substrate is more lethal than when it is in an aqueous state to the nematodes directly. This means that direct survival of the organism is effected by the presence of substrate bound nickel. From our assays, it also appeared that the sediment bound nickel was not directly affecting the progeny of the nematodes. Third, aqueous nickel does have an effect on the nematodes but it appears to be species dependent as well as effecting only the F1 generation. *C. elegans* did show a sensitivity to the aqueous nickel in reducing the number of F1 progeny in a dosage dependent manner while *P. pacificus* showed no ill effects on its F1 progeny in the aqueous nickel concentrations tested. The effects seen in *C. elegans* F1 individuals could be due to retardation in the embryo development, genomic damage within the gonad and developing oocytes, or it could have an effect on overall laying rate of the adult. More research is needed to determine where in the animal and life cycle aqueous nickel is having an effect as well as to determine what effects of aqueous nickel have on the *C. briggsae* progeny as well. The last major conclusion was that substrate bound nickel has an effect on the longevity of the animal. As the amount of nickel bound in the sediment increased, there was a decrease in the number of recovered individuals to a point where, at the highest levels of nickel bound in the

sediment, the time needed to reach a 50% recovery was 7-8 times faster than that of the control sediment.

Overall nickel is an extremely hazardous element and better care is needed to limit its effect on the environment. It should be noted that bound nickel is a definite environmental toxin (i.e. lethal in high concentrations) and it appears to have effects as an aqueous element although more studies are needed to determine the detrimental effects on the environment. Nematodes provide scientist and field researchers with a great field bio indicator organism. Due to its fast life cycle, ease of use in sediment and water trials, and because of their overall successful reproduction of results, it should be noted that these animals should be used when possible for quick and accurate assessment of environmental toxins and the issues these toxins can pose to plants, animals, and the overall health of the environment.

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APPENDIX

Recovery of *C. briggsae* from 8 control sediments over 3 trials.

TRIAL 1				TRIAL 2				TRIAL 3			
SED.	WELL	P0	F1	SED.	WELL	P0	F1	SED.	WELL	P0	F1
SR-0	1	1	2	SR-0	1	0	0	SR-0	1	1	4
SR-0	2	1	2	SR-0	2	0	0	SR-0	2	0	0
SR-0	3	2	0	SR-0	3	0	0	SR-0	3	0	0
SR-0	4	0	0	SR-0	4	2	0	SR-0	4	0	0
SR-0	5	1	2	SR-0	5	0	0	SR-0	5	0	0
SR-0	6	1	3	SR-0	6	1	0	SR-0	6	2	4
SJ-0	1	6	6	SJ-0	1	3	0	SJ-0	1	7	0
SJ-0	2	6	7	SJ-0	2	2	0	SJ-0	2	7	0
SJ-0	3	4	8	SJ-0	3	3	0	SJ-0	3	9	0
SJ-0	4	2	2	SJ-0	4	2	3	SJ-0	4	9	0
SJ-0	5	4	10	SJ-0	5	4	5	SJ-0	5	10	0
SJ-0	6	3	8	SJ-0	6	6	10	SJ-0	6	7	0
P30-0	1	6	2	P30-0	1	0	0	P30-0	1	4	2
P30-0	2	5	4	P30-0	2	1	0	P30-0	2	3	2
P30-0	3	4	0	P30-0	3	0	0	P30-0	3	4	2
P30-0	4	4	3	P30-0	4	0	0	P30-0	4	6	4
P30-0	5	1	0	P30-0	5	0	0	P30-0	5	5	2
P30-0	6	1	2	P30-0	6	1	0	P30-0	6	6	3
DOW-0	1	1	2	DOW-0	1	6	7	DOW-0	1	3	1
DOW-0	2	2	2	DOW-0	2	5	5	DOW-0	2	3	2
DOW-0	3	2	2	DOW-0	3	4	6	DOW-0	3	2	1
DOW-0	4	2	3	DOW-0	4	4	5	DOW-0	4	-	-
DOW-0	5	5	2	DOW-0	5	5	4	DOW-0	5	-	-
DOW-0	6	1	0	DOW-0	6	6	5	DOW-0	6	-	-
RR2-0	1	8	0	RR2-0	1	3	2	RR2-0	1	10	2
RR2-0	2	8	0	RR2-0	2	4	0	RR2-0	2	9	2
RR2-0	3	7	0	RR2-0	3	5	1	RR2-0	3	9	2
RR2-0	4	9	0	RR2-0	4	5	6	RR2-0	4	9	3
RR2-0	5	7	0	RR2-0	5	6	6	RR2-0	5	8	4
RR2-0	6	1	0	RR2-0	6	4	4	RR2-0	6	7	1
STM-0	1	4	1	STM-0	1	2	0	STM-0	1	1	7
STM-0	2	4	1	STM-0	2	3	5	STM-0	2	1	5
STM-0	3	6	0	STM-0	3	7	6	STM-0	3	1	6
STM-0	4	4	0	STM-0	4	4	3	STM-0	4	1	6
STM-0	5	2	3	STM-0	5	2	0	STM-0	5	1	7
STM-0	6	3	3	STM-0	6	4	0	STM-0	6	1	9

	TRIAL 1				TRIAL 2				TRIAL 3		
SED.	WELL	P0	F1	SED.	WELL	P0	F1	SED.	WELL	P0	F1
RR3-0	1	2	2	RR3-0	1	3	16	RR3-0	1	5	2
RR3-0	2	1	2	RR3-0	2	2	7	RR3-0	2	6	1
RR3-0	3	2	3	RR3-0	3	2	11	RR3-0	3	7	1
RR3-0	4	3	2	RR3-0	4	8	20	RR3-0	4	8	1
RR3-0	5	2	1	RR3-0	5	5	15	RR3-0	5	8	0
RR3-0	6	5	4	RR3-0	6	4	14	WB-0	6	10	2
WB-0	1	8	30	WB-0	1	4	29	WB-0	1	0	0
WB-0	2	6	45	WB-0	2	6	22	WB-0	2	0	0
WB-0	3	8	15	WB-0	3	6	21	WB-0	3	0	0
WB-0	4	7	22	WB-0	4	5	17	WB-0	4	0	0
WB-0	5	8	28	WB-0	5	6	14	WB-0	5	0	0
WB-0	6	8	41	WB-0	6	5	21	WB-0	6	0	0