WORKSHOP

Heavy Metal: Investigating the Effects of Environmental Toxins on *C. elegans*

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Background Information

Environmental Toxicology and Bioassays

Environmental toxicology is the study of the effects of harmful chemicals on human health and the environment. This field rocketed into the public consciousness in 1962 with Rachel Carson's book "Silent Spring" which looked at the effects of dichlorodiphenyltrichloroethane (DTT) on humans and wildlife. Today, scientists from a range of backgrounds work to determine where a toxicant will end up in an ecosystem and how long it will remain. Using a series of bioassays – tests that measure the potency of a substance by observing its effects on molecules, cells, tissues or organisms – toxicologists also predict safe exposure limits that will keep the environment and us healthy.

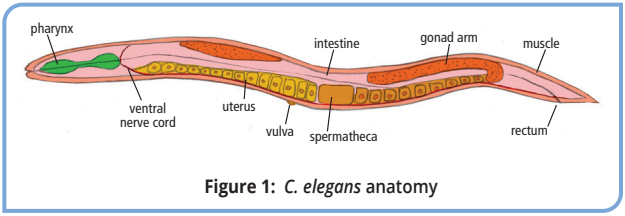


Figure 1: *C. elegans* anatomy

Predictive toxicology focuses on accurately forecasting the interaction between a toxicant and different biological systems and can be generally divided between in vitro and in vivo studies. In vitro, Latin for "in the glass", involves exposing molecules, cells, tissues and certain microorganism to both a standard neutral solution and to a concentration gradient of a toxicant. These targets are then monitored to see whether or when certain adverse effects — called endpoints — occur. For example, common endpoints in cell based in vitro studies are membrane damage, nuclear condensation, and apoptosis. In vitro tests are easy to carry out in large replicates, less expensive, and allow researchers to focus on specific biological processes.

Alternatively, the potency of a toxicant can be tested by observing how it affects the behavior, growth, reproduction, or lifespan of popular animal models. This is in vivo or "within the living" testing. These tests account for the fact that individuals have developed protective mechanisms to reduce the damaging effects of certain toxins, but that these mechanisms mainly manifest at the organism level. For example, when an animal is exposed to a high metal dose, several systems often work together to expel the absorbed molecules, decrease environmental uptake, and activate general stress responses. In addition, in vivo studies can highlight emergent health threats that may have been missed during studies that only focus on a certain cell type or a single organ. However, in vivo studies are also slow, costly, and raise both legal and moral issues related to animal rights.

Bioassays using Caenorhabditis elegans (see-no-rab-DITE-iss el-leh-GANS) offer many of the conveniences of in vitro testing with the fuller picture provided by in vivo testing. Because *C. elegans* is a popular model organism in other research fields like development, neurology, and genetics there is a wealth of scientific data that can guide and enhance basic toxicology tests. Today, these organisms are increasingly being used to test the safety of potential pollutants.

Why Study *C. elegans*

A model organism is any plant, animal or microorganism that allows us to study fundamental questions in biology that may be hard to study directly in complex organisms like humans. In the 1970s, Dr. Sydney Brenner established the nematode *C. elegans* as a model organism because they have a simple genome, a fast generation time, and are easy and inexpensive to

maintain. While characterizing the worm, Brenner, along with Drs. John Sulston and Robert Horvitz, discovered that the developmental fate of every cell in the worm is invariable between animals. They also discovered key genes involved in organ development and programmed cell death. For this work, Brenner, Sulston, and Horvitz were awarded the Nobel Prize in Physiology or Medicine in 2002.

C. elegans have become important to the study of embryogenesis, morphogenesis, development, nerve function, behavior and aging, and genetics. The *C. elegans* genome has been completely sequenced and several thousand genetic mutants are available for study. This allows scientists to correlate changes at the DNA level with changes in phenotype. Notably, by comparing DNA sequences, it was determined that over 35% of worm genes have human homologs. Many of these genes are important for human health and development.

C. elegans is a free-living, non-parasitic nematode that lives in temperate soil, where it feeds on microbes that are found in decaying organic matter. Adult worms measure approximately one millimeter (mm) in length. The outer cuticle of *C. elegans* is transparent, making it easy to visualize growth and development of internal structures like the pharynx, the intestine, the gonads and the muscles (Figure 1). The worm also has an extensive nervous system – in fact, the nervous system comprises almost 1/3 of the worm's 959 somatic cells! This makes *C. elegans* a valuable model system for neuroscientists (Figure 2).

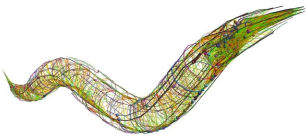


Figure 2: Neural anatomy of *C. elegans*. Source: OpenWorm project. Shared under an MIT License.

Growth and Development of *C. elegans*

There are two naturally occurring sexes in *C. elegans*. The vast majority of worms are self-fertile hermaphrodites, meaning that they produce both the sperm and the eggs used for reproduction. Free-living males represent <1% of the total nematode population. However, free-living males plus a hermaphrodite can produce over 1000 offspring in a generation; in contrast, self-fertilized hermaphrodite worms will produce about 300. Because their sperm will preferentially fertilize a hermaphrodite's eggs and produce more offspring, free-living males are often used to introduce specific genetic mutations into a worm population to be studied.

C. elegans develop from embryo to adult in four days, allowing for rapid studies in the laboratory (Figure 3). The worms are grown on agar plates or in liquid culture and they feed on *E. coli*. After being laid, the worm embryo will develop for approximately 14 hours before hatching. Juvenile worms progress through four larval stages (L1-L4) over the next two days, increasing in size with each stage. After the fourth larval molt (L4), the worms are reproductively mature, meaning that they can be used for further genetic studies. Adults will live for 2-3 weeks, over which time they gradually age and lose vigor.

Bioassays with Multiple *C. elegans* Strains

Another advantage of *C. elegans* is the availability of over 3000 strains whose behavior and genetic make-up has been researched and documented. In biology, a strain is a genetic variant or subtype that falls below the taxonomical level of species. By creating multiple strains, scientists can identify the function of different genes, observe how different genotypes respond to environmental stimuli, or modify a phenotype for a particular task. *C. elegans*' short generation

time, male fecundity, and susceptibility to multiple DNA alteration techniques have allowed scientists to create thousands of different mutational strains in this species. In environmental toxicology, different strains are used to better understand how an individual's genetics and its environment interact to determine the biological consequences of toxic exposure. In addition, mutant strains that are more susceptible to toxicants enable toxicologists to run faster and more sensitive bioassays. In this experiment you will be using a strain with a loss of function mutation in the adr-2 gene that makes individuals more vulnerable to environmental pollution.

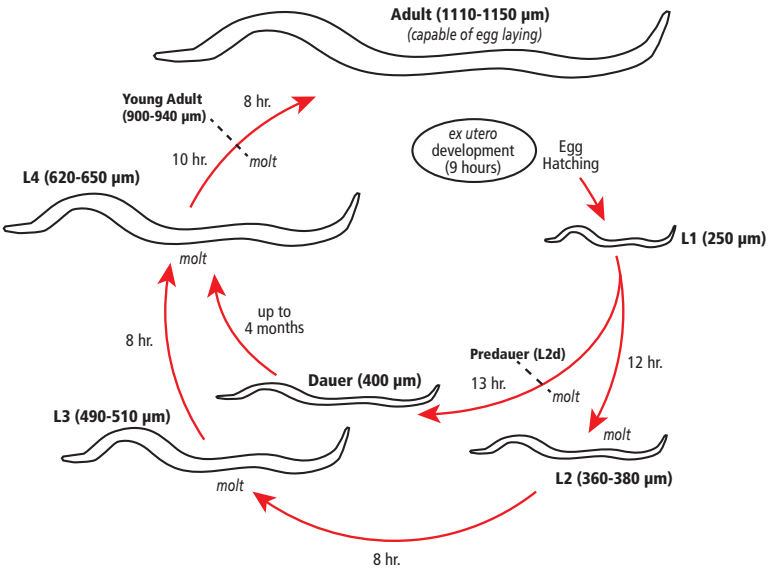


Figure 3: Life Cycle of *C. elegans*

Heavy Metal Pollution and Human Health

Heavy metals are a loosely defined group of high-density chemical elements (Figure 4). While naturally occurring, many of these elements have become concentrated in certain areas as a result of mining, industrial waste, agricultural runoff, and use in products like paint and treated wood. Heavy metals also tend to bioaccumulate in living organism because they are often stored in fat tissue rather than metabolized. Some heavy metals such as iron, zinc, copper, cobalt, and manganese play crucial biological roles in processes like oxygen delivery, free radical removal, and cell signaling. However, these

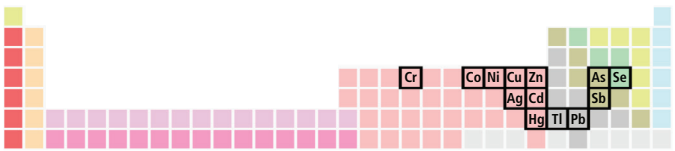


Figure 4: Examples of Toxic Heavy Metals.

trace nutrients can become toxic at higher doses. Other heavy metals such as mercury and lead have no known biological benefits but can cause long-lasting and irreversible biological damage.

Exposures to heavy metals are a common and dangerous environmental health issue. The fast metabolic rates and limited self-repair abilities of nerve cells, as well as the large number of chemical messengers used in interneuron communication, make the nervous system particularly vulnerable to these chemicals. This is especially true for the still-developing nervous systems of children and infants. Regardless of age, damage to the nervous system can affect everything from learning to motor coordination to the regulation of internal systems like circulation and digestion (Table 1). While some of these effects can be observed immediately after exposure, others take months or even years to manifest. Because it is often difficult to detect early symptoms of exposure and because damages to the nervous system can be permanent, health strategies related to heavy metal neurotoxicity focus on prevention through the early identification and removal.

C. elegans have a similar nervous system to humans and are susceptible to most heavy metals. Mercury, copper, lead, chromium, nickel, cadmium, aluminum, cobalt, zinc, and manganese have all been observed to have a negative effect on the wild-type strain. While toxicology studies have traditionally focused on lethal doses or lethal concentrations (LC), advances in chemical detection technologies have led to a growing interest in the effects of smaller, and often more environmentally relevant concentrations, as well as the synergistic effects of two or more chemicals.

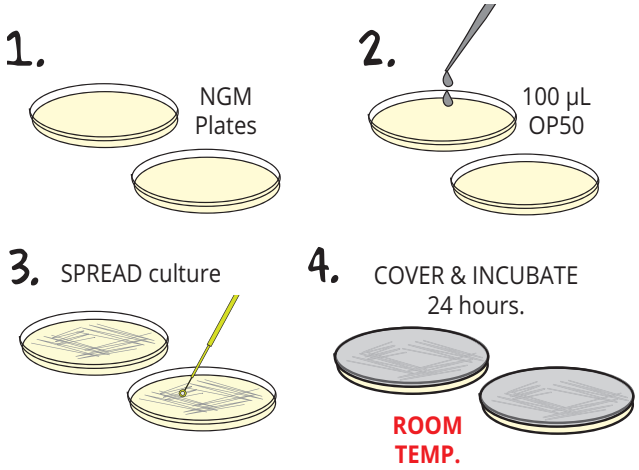
In this experiment, you will be investigating the effects of low-level heavy metal exposure on both wild-type and adr-2 mutant *C. elegans*. Each strain will be exposed to a potentially damaging chemical for four minutes. (Copper Sulfate, Cadmium Chloride, and Zinc Sulfate are provided but you or your teacher may bring in additional solutions to test.) You will observe and count the thrash rate of a single worm at the beginning, middle, and end of this time period. In addition, you will carry out controls where both strains are exposed to a non-harmful buffer solution for four minutes and again observe and calculate the start, middle, and end thrash rates. Results can be graphed and shared with other lab groups.

TABLE 1: Symptoms of Heavy Metal Neurotoxicity

- Headache
- Loss of Vision
- Imbalance
- Loss of Circulation
- Flu-like symptoms
- Tingling, Weakness or Paralysis of the Limbs
- Loss of memory and cognitive function
- Depression
- Uncontrollable obsessive and/or compulsive behavior
- Sexual dysfunction
- Chronic Fatigue
- Attention Deficit Disorder
- Predisposition to Alzheimer's Disease, Multiple Sclerosis, Parkinson's Disease

Module I: Preparation of *C. elegans* Food Source ("Seeding" the Plates) *Excerpts from Edvo-Kit #856*

In this module, you will seed two petri plates with a favorite *C. elegans'* food (*E. coli* OP50 strain bacteria).



1. **OBTAIN** two Nematode Growth Medium (NGM) plates, the OP50 culture, a small transfer pipet, and a sterile inoculating loop from your instructor. **LABEL** the bottom of both plates with your group number or names.

2. While maintaining sterile technique, **ADD** two drops (100 µL) of OP50 culture to each plate.

3. Using the loop, **SPREAD** the culture over the entire surface of the NGM plates. **COVER**.

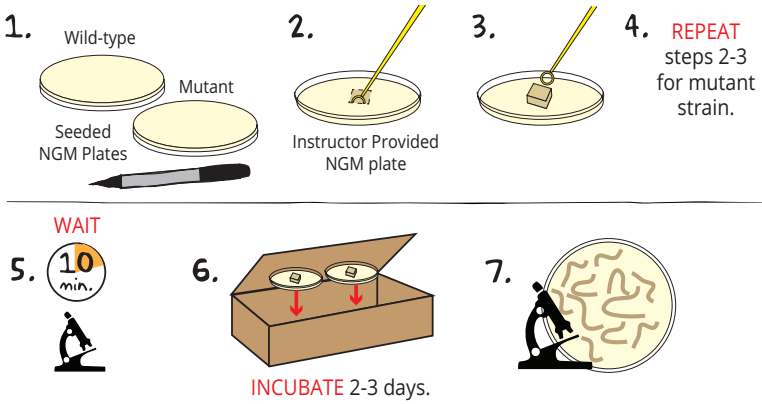
4. **INCUBATE** the plates inverted and at room temperature for 24 hours. ***NOTE: Seeded OP50 plates can also be prepared overnight (~12 hours) by incubating at 37°C.***

HINTS for Step 3:

- Avoid gouging or scratching the agar surface as this can affect visibility as well as worm movement.
- Widely spreading the bacteria creates a larger lawn for the worms but stop just before the plate's edge. This discourages the worms from crawling up the plate's sides and drying out.

Module II: Cultivation of *C. elegans* ("Chunking" the Plates) *Excerpts from Edvo-Kit #856*

In this module, you will be "chunking" your *C. elegans* strains. This means that you will transfer wild-type and ADR-2 mutant *C. elegans* to the plates you prepared in Module I. Over the next few days, both strains will establish new populations in their individual plates. Once these populations have expanded to at least 50 individuals you will continue to Module III.



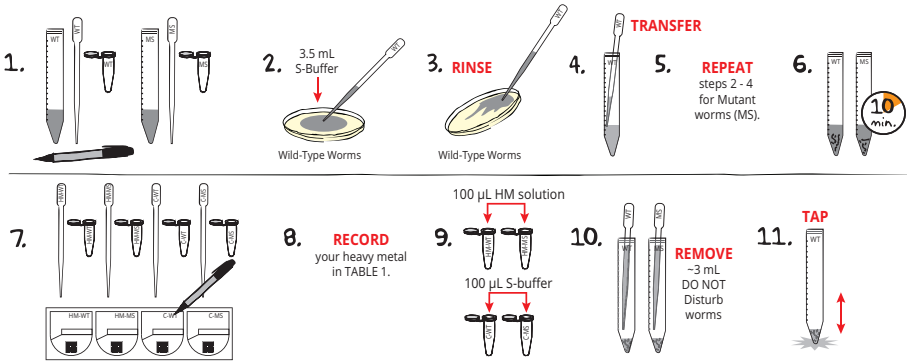
1. **LABEL** the bottom of the seeded NGM plates (from Module I) with "Wild-type" or "Mutant".
2. Your instructor will have NGM plates containing wild-type or mutant *C. elegans*. Using a sterile loop, **CUT OUT** a small portion of the NGM plate containing the wild-type strain (~1 cm square). Make sure to completely cut the agar by pushing the loop all the way to the bottom plate.
3. **REMOVE** the "chunk" from the plate. **PLACE** the chunk, worm side down, in the center of the Module I Wild-type plate.
4. With a new sterile loop, **REPEAT** step 2 and 3 for the mutant strain.
5. After 5-10 minutes, use a microscope to **CONFIRM** the presence of *C. elegans* on the "chunked" plates.
   NOTE: Occasionally, a "chunk" is transferred nematode side up. Worms in this position will eventually migrate to the plate. If the "chunk" is incorrectly positioned, keep the plate but wait 1 hour before confirming the presence of *C. elegans* and continuing on to Step 6.
6. **COVER** and **PLACE** the plates into a cardboard box. **INCUBATE** at room temperature for 2-3 days.

OPTIONAL STOPPING POINT:
Plates may be stored for up to a week but need extra OP50 to avoid drying and to feed the growing population. See Appendix A.

7. **CHECK** growth of *C. elegans* under a microscope. If the plate contains 50 or more worms, proceed with Module III. If the plate contains fewer than 50 worms, continue incubating at room temperature. ***HINT for Step 7: To quickly confirm worm numbers, divide the plate into quarters. If you see 12 or more in the first quarter, the plate is ready.***

Module III: Collection of *C. elegans* and Toxicity Bioassay

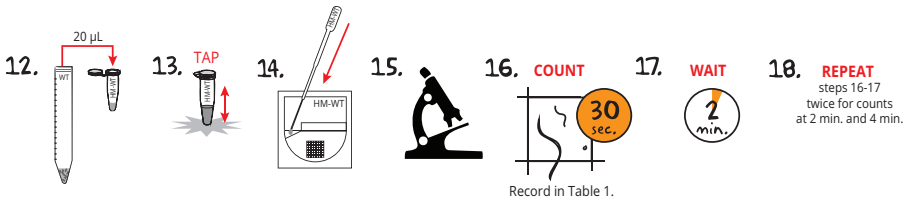
Excerpts from Edvo-Kit #856



1. **LABEL** one 15 mL tube of S-buffer, large transfer pipet, and snap top tube with "WT" (Wild-Type) and the other 15ml tube of S-buffer, large transfer pipet, and snap top tube with "MS" (Mutant Strain).
2. Using the WT large transfer pipet, **TRANSFER** 3.5 mL of S-buffer from the WT conical tube to the Petri dish containing the wild-type *C. elegans*.
3. **DISLODGE** worms by rinsing the dish several times. **RINSE** the dish by either (a) swirling the plate or (b) holding the plate at a slight angle and allowing the buffer to collect near the bottom. Next suck up the buffer using the WT transfer pipet and then expel the buffer near the top so that the buffer runs down the plate.
4. Once most worms are suspended in the buffer, **TRANSFER** the worms and buffer back to the WT 15 mL conical tube using the WT transfer pipets.
5. **REPEAT** steps 2-4 for mutant worms using the MS labeled items.
6. Keep the conical tubes still and upright to ALLOW the worms to settle to the bottom of the tubes (~10 minutes). While you wait, REVIEW Appendix B and complete Steps 7 through 9.
7. **LABEL** four 1.5 mL snap-top test tubes, counting chambers, and small transfer pipets with "HM-WT", "HM-MS", "C-WT", and "C-MS".
8. **RECORD** which heavy metal you plan to test in your lab book or in Table 1 (page 9).
9. **ADD** 100 µL of your heavy metal solution to both "HM" tubes and 100 µL of the S-buffer to both "C" tubes.
10. Using the large transfer pipets, slowly **REMOVE** ~3 mL of the cleared S-buffer supernatant without disturbing the worms that have settled to the bottom of the tubes. Between 300 and 500 µL of buffer with worms should remain at the bottom of both tube.
11. **MIX** the WT tube by tapping the bottom 3-4 times or until worms are uniformly resuspended throughout.

Module III: Collection of *C. elegans* and Toxicity Bioassay

Excerpts from Edvo-Kit #856



12. Immediately, **ADD** 20 µL of the wild-type worms to the "HM-WT" tube.
13. **MIX** by tapping this tube 3-4 times.
14. Using the HM-WT small pipet, **TRANSFER** 1 drop of the mixture from the "HM-WT" tube to the opening of the corresponding chamber.

    NOTE: If correctly placed, the solution will rapidly move into the chamber by capillary action. If the solution does not move into the chamber, check that the chamber is orientated so that the triangular opening is facing upwards.

15. Quickly **PLACE** the chamber under a microscope and **IDENTIFY** an adult worm (900-1200 µm long) for observation.
16. **COUNT** the number of thrashes that occur over a 30-second time period. **RECORD** your count in Table 1.
17. **WAIT** 2 minutes.
18. **REPEAT** steps 16 and 17 twice so that you have additional counts at 2 and 4 minutes.
19. After the final count, **RECORD** any additional notes about the final appearance of the worms including movement, body shape, body curvature, and any clumping. Also record any general observations about the effect of each heavy metal on the worms and how these changed over time.
20. To collect data for the effects of your chosen heavy metal on the mutant strain, **REPEAT** steps 11 through 19 using the mutant strain and your HM-MS labeled items. **RECORD** your results in Table 1.

TABLE 1: Heavy Metal_____

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

Module III: Collection of *C. elegans* and Toxicity Bioassay

Excerpts from Edvo-Kit #856

21. To collect data for the effects of the control solution on the wild-type strain, **REPEAT** steps 11 through 19 using the wild-type strain and your C-WT labeled items. **RECORD** your results in Table 2.
22. To collect data for the effects of the control solution on the mutant strain **REPEAT** steps 11 through 19 using the mutant strain and your C-MS labeled items. **RECORD** your results in Table 2.

TABLE 2: Control Solution

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

23. (Optional) If class time permits, **TEST** the other two provided heavy metals. Four test tubes, counting chambers, and small transfer pipets are provided. **LABEL** theses items, **RECORD** the selected heavy metals, and **REPEAT** steps 11 through 19 with these two new solutions on both strains. **RECORD** the results in Tables 3 and 4. Note that the control does not need to be repeated.

TABLE 3: Heavy Metal_____

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

TABLE 4: Heavy Metal_____

	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.		
2 min.		
4 min.		

24. (Optional) **GRAPH** the change in thrash rate over time for each condition you tested (solution and strain combination) with time on the x-axis and thrash rate on the y-axis.
25. (Optional) **SHARE** your group's findings with another lab group or with the class.

Experimental Results and Analysis

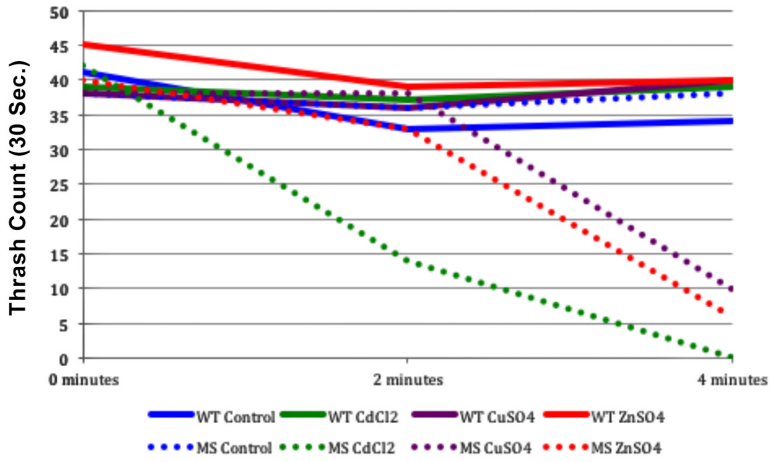
Copper, zinc, and cadmium should have a minimal effect on wild-type worms. In contrast, thrash rates in the mutant strain should decrease in the presence of these heavy metals. Zinc and copper can cause significantly slowing in the mutant strain worms while cadmium can cause complete mobility loss within the time frame of this experiments. Below are representative results from in house testing. Exact values will vary.

Heavy Metal - COPPER		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	39	38
2 min.	40	38
4 min.	38	10

Control Solution		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	45	38
2 min.	43	36
4 min.	41	38

Heavy Metal - ZINC		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	44	40
2 min.	39	33
4 min.	37	6

Heavy Metal - CADMIUM		
	Wild Type Thrash Count (30 sec.)	Mutant Strain Thrash Count (30 sec.)
0 min.	39	42
2 min.	37	14
4 min.	39	0



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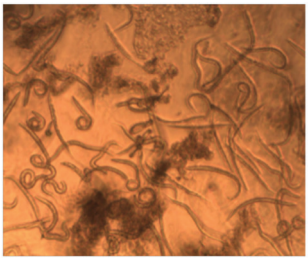
Cat. 856

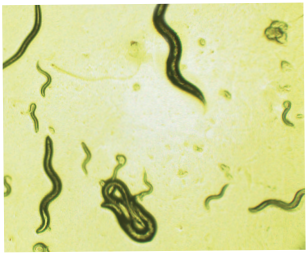
Ennvironmental Toxicity Response in *C. elegans*



For 10 Groups. *Caenorhabditis elegans* is a soil nematode with great potential for educational research, partly because of its rapid (3-day) life cycle, small size (1.0-mm-long adult), and ease of laboratory growth cultivation. In this experiment, students will observe and compare the effects of heavy metals found in the environment on normal and mutant strains of *Caenorhabditis elegans (C. elegans)*.

Cat. 851

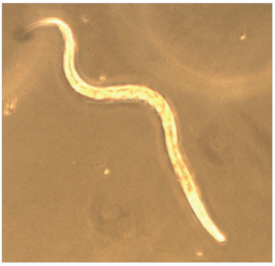
Effects of Alcohol on *C. elegans*



For 10 Groups. You will not believe how similar we are to worms! The genome of the tiny worm, *C. elegans*, was sequenced and found to be 40% similar to the human genome. It is now used as a model system by researchers to address fundamental questions in developmental biology, neurobiology and behavioral biology. The objective of this experiment is to observe and record the effects of alcohol on normal and alcohol mutant strains of *C. elegans*.

Cat. 852

Chemotaxis: The Science of Attraction in *C. elegans*



For 10 Groups. All organisms are affected by "scent" molecules in the environment, including a multicellular organism called *Caenorhabditis elegans.* These worms are composed of 959 somatic cells, of which 300 are neurons comprising organs for taste, smell, temperature and touch. In this experiment, students will observe and record the phenomenon by which normal and mutant strains of *C. elegans* can direct their movement in response to certain chemicals in the environment.