THESIS

ACUTE AND CHRONIC COBALT TOXICITY IN *DUGESIA DOROTOCEPHALA*

Submitted by

Andrea Sternenberger

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2011

Master's Committee:

    Advisor: Howard Ramsdell

    Marie Legare
    Steven Strauss

ABSTRACT

ACUTE AND CHRONIC COBALT TOXICITY IN *DUGESIA DOROTOCEPHALA*

Limited data are available regarding cobalt toxicity for water quality criteria and risk assessment evaluation for freshwater organisms. These experiments were performed to establish median lethal concentrations from short term studies and to identify the most sensitive sublethal effect in a long term study in a flatworm species, *Dugesia dorotocephala*. Another study was conducted to elucidate the relationship between calcium, magnesium and cobalt toxicity in *D. dorotocephala*. During 4 and 7 day studies, median lethal concentrations of cobalt increased with total hardness indicating a protective effect. Calcium was shown to be more protective than magnesium in the presence of cobalt. A 60 day study showed fissioning rate, a process essential for maintenance of the *D. dorotocephala* population, to be the most sensitive endpoint. A lowest observable adverse effect concentration (LOAEC) of 1 mg Co/L (nominal) was incorporated into the calculation of an acute to chronic ratio (ACR), an important metric for risk analysis accounting for sublethal effects that may occur by mechanisms different from lethal ones. A NOAEC (no observable adverse effect concentration) was not determined because significant effects were observed at the lowest cobalt concentrations tested (<1 mg Co/L). Without an exact NOAEC, the ACR values established in this study represent the upper and lower bounds of the acute to chronic ratio for cobalt in *D. dorotocephala.*

ACKNOWLEDGMENTS

TABLE OF CONTENTS

I. INTRODUCTION

The toxicity of cobalt to freshwater organisms is relatively poorly known. Cobalt, the 33rd most abundant element in the Earth's crust (ATSDR 2004), has natural and anthropogenic sources of exposure to the environment. The ecological risk assessment and development of water-quality criteria for cobalt are currently hampered by insufficient knowledge about the toxicity of cobalt to freshwater organisms (DeSchamphelaere et al. 2008).

Dugesia dorotocephala, a freshwater, asexual flatworm, is an important species to aquatic ecology (Kenk 1980). They model vulnerable systems of higher animals and may have the potential to become a sentinel species for toxicity in the environment. This work was conducted to establish acute LC$_{50}$ cobalt values in *D. dorotocephala* at varying levels of hardness, to establish valid sublethal endpoints in chronic studies and to evaluate the role of calcium in cobalt toxicity.

l. Cobalt

Cobalt is a naturally occurring element that appears in the periodic table between iron and nickel. Pure cobalt is a silver gray, insoluble solid with one stable isotope, ^{59}Co (Greenwood and Earnshaw 1997). Cobalt commonly occurs in three valence states: elemental cobalt, +2 and +3 (Greenwood and Earnshaw 1997, Kim 2006). Cobalt (II) is stable in aqueous solutions and is the major form of cobalt found in simple salts (Greenwood and Earnshaw 1997). Cobalt (III) is a strong oxidizing agent and is unstable in aqueous solutions (Greenwood and Earnshaw 1997). Cobalt (III) has biochemical importance forming a coordination complex with four pyrrole rings to result in an essential mammalian vitamin, vitamin B$_{12}$ (Lehninger et al. 2005). See Table 2.1 for the properties of elemental cobalt.

Table 2.1. Properties of cobalt and a common cobalt compound

	Cobalt	Cobalt (II) Chloride Hexahydrate
CAS	7440–48–4	7791–13–1
Synonyms	Cobalt–59, cobalt metal	Cobalt dichloride, cobaltous chloride
Molecular Formula	Co	$CoCl_2 \cdot 6H_2O$
Atomic/Molecular Weight (g/mol)	58.93	237.93
Color, Physical State	Silvery gray, solid	Rose red, solid crystals
Melting Point (^{o}C)	1,495	87
Boiling Point (^{o}C)	2,870	1,049
Vapor Pressure	1 mm Hg at 1,910 ^{o}C	40 mm Hg at 770 ^{o}C
Density (g/cm^3)	8.9 at 20 ^{o}C	1.92 at 20 ^{o}C
Water Solubility	Insoluble	76.7 g/100 mL at 0 ^{o}C

Of the 26 known radioactive isotopes of cobalt, ^{57}Co and ^{60}Co are the two most important. The most commonly used source of gamma radiation is ^{60}Co (Mahara and Kudo 1981). Radioactive cobalt can be released to the environment as a result of nuclear research and development, nuclear accidents, operation of nuclear power plants, and radioactive waste dumping in the sea or in radioactive waste landfills (Mahara and Kudo 1981). On the Environmental Protection Agency's National Priority List, which identifies the most seriously contaminated hazardous waste sites in the nation, ^{60}Co is present at 13 of the 1,636 sites (ATSDR 2004).

Sources:

As the 33rd most abundant metal in the Earth's crust at a concentration of 25 mg/kg, cobalt is found naturally in the soil, seawater, surface and ground water (Smith and Carson 1981, p. 21). Cobalt occurs at an average concentration of 45–48 mg Co/kg in basaltic rocks, 4–

147 mg Co/kg in schists, 19–20 mg Co/kg in clays and 1.2 mg Co/kg in sandstone and limestone (Smith and Carson 1981, p. 21). Presumably, cobalt substitutes for Fe(II) and Mg(II) ions in many minerals which results in mining in an attempt to concentrate the cobalt content (Smith and Carson 1981, p. 22). Cobalt is associated with arsenic, antimony, lead, nickel, silver, iron, zinc and copper ores and as arsenides, sulfides and oxides in mineral form (Kim 2006, Smith and Carson 1981, p. 22). Natural atmospheric deposition from windblown soil, seawater spray, volcanic eruptions, and forest fires contribute to the atmospheric cobalt emissions in the environment at an estimated 6,100 tonnes per year (Nriagu 1989).

Elevated levels in soils and water are observed as a result of anthropogenic activities such as mining, applying phosphate fertilizers, disposing of cobalt-containing wastes and atmospheric deposition from activities such as the burning of fossil fuels and smelting and refining of metals (Smith and Carson 1981, p. 422). Anthropogenic sources of cobalt include fossil fuel and waste combustion, vehicular and aircraft exhausts, processing of cobalt and cobalt containing alloys, copper and nickel smelting and refining, and the manufacture and use of cobalt chemicals and fertilizers derived from phosphate rocks (Smith and Carson 1981, p. 407).

Use:

The United States is the world's largest cobalt consumer (USGS 2010). Cobalt's metallurgical application in materials such as superalloys is commonly used by the military (USGS 2010). Cobalt is easily magnetized and can retain its magnetism under a wide range of environmental conditions (Young 1948). Cobalt is therefore also useful in magnetic alloys which resist corrosion and wear (ATSDR 2004). It is used to increase strength for cutting tools, to add pigmentation to paints, glass and ceramics, as the main component of paint driers and to serve

4

as catalysts in chemical reactions such as desulfurization processes used by the oil/gas industry (ATSDR 2004). A growing use of cobalt is in Ni-Cd batteries and lithium ion cells (ATSDR 2004) which are used in cell phones, computers and hybrid vehicles. Gamma rays from ^{60}Co are used to treat cancer, to sterilize instruments and food-borne pathogens and to extend the shelf-life of food by delaying ripening (ATSDR 2004). The gamma rays can also destroy harmful organisms in sludge, water and wood (ATSDR 2004).

Exposure:

Exposure to cobalt occurs through four media: water, soil, food and air (Kim 2006). Atmospheric cobalt is associated with particulate matter at levels below one ng Co/m^3 at unpolluted sites and in open-ocean environments (Smith and Carson 1981, p. 32, Kim 2006). In source areas, cobalt concentrations may exceed 10 ng Co/m^3 but the highest recorded atmospheric level, 48 ng Co/m^3 was recorded near a nickel refinery in England (Smith and Carson 1981, p. 32).

Water

In ambient, uncontaminated environments, the cobalt concentrations are generally below 5 μg Co/L (Nagpal 2004). Surface and groundwater cobalt concentrations of <1 μg Co/L are found in pristine areas and can be up to 10 μg/L in urban areas (Smith and Carson 1981, p. 428). Concentrations may be higher in mining and agricultural areas. Levels in an Arizona creek near a copper mine and smelter were recorded at 4500 μg Co/L and 6500 μg Co/L was found in a Missouri river which receives mining and milling effluent (Smith and Carson 1981, p. 428). Mean seawater concentrations of 0.078 and 0.39 μg Co/L were observed in the Caribbean Sea and Indian Ocean respectively (Hamilton 1994). The National Community Water Supply Study found an average 2.2 μg

Co/L in drinking waters but up to 107 μg Co/L have been reported (Smith and Carson 1981, p. 425). Rainwater samples were found to have levels ranging from 0.045 μg Co/L to 2.9 μg Co/L (Smith and Carson 1981, p. 428).

Soil

Found in greater abundance than cadmium, molybdenum and lead, the average U.S. soil sample contains 7.2 mg Co/kg (Kim 2006). Soils containing 0.5–3 mg Co/kg are considered deficient for the required intake by ruminants (Kim 2006). Airports, ore smelting facilities, ore deposits, mines, highway traffic or other industrial sites may contain elevated soil concentrations up to 12,700 mg Co/kg (Abraham and Hunt 1995, Smith and Carson 1981, p. 26). Unpolluted freshwater sediments generally contain less than 20 mg Co/kg, similar to cobalt-sufficient soil, but polluted lake and river sediments can contain up to 133 mg Co/kg (Smith and Carson 1981, p. 28, 38).

Cobalt sorbs to particles and may settle into the sediment or may be sorbed directly by the sediment (ATSDR 2004). It may precipitate from overlying water as carbonates and hydroxides or with mineral oxides (ATSDR 2004). Cobalt may also sorb to humic acid substances in water (ATSDR 2004). Cobalt can also be transported in the dissolved form or as suspended sediment by rivers to lakes and the sea or by ocean currents (ATSDR 2004, Smith and Carson 1981, p. 34). Environmental factors such as pH, redox potential (Eh) and the presence of chelating agents will affect the fate and transport of cobalt: At a lower pH, more cobalt will be present in soluble forms and it will have greater mobility

(Smith and Carson 1981, p. 33, 532, 582, ATSDR 2004). Cobalt adsorbs strongly and rapidly to soil and sediment, in which it is retained by metal oxides, minerals and natural organic matter (ATSDR 2004).

Plants

As a result of the presence of cobalt in the soil and sediment, it has been detected in living plants as well at a mean level of 0.48 µg Co/g (Bowen 1966). Grasses normally contain 0.20–0.35 µg Co/g but in deficient soil, concentrations average 0.02–0.06 µg Co/g (Hamilton 1994). The copper flower plant (*Haumaniastrum robertii)*, can actually hyperaccumulate cobalt at a mean level of 4,304 mg Co/kg dry weight (Brooks 1977). Cobalt has been shown to affect the growth and metabolism of plants depending on several factors including complexation with other minerals and physical conditions such as pH, temperature and salinity (Palit et al. 1994). Toxic effects on plants include inhibited active transport of ions, photosynthetic inefficiency, change in morphology, impaired DNA replication and RNA synthesis, and alteration of the sex of plants (Palit et al. 1994). Stimulated algae growth has been seen at low concentrations of Co^{+2} suggesting that cobalt chloride may be used effectively as a trace metal (Ahluwalia and Kaur 1988).

Food

Food represents the largest potential source of cobalt exposure for humans (Kim 2006). The Centers for Disease Control and Prevention recommends a daily intake of 0.1 µg Co/day (ATSDR 2004). Grains and cereals have the highest cobalt content followed by potatoes, fish, sugar and coffee (Smith and Carson 1981, p. 60). The estimated intake from food is 5–40 µg Co/day, most of which is inorganic cobalt (Kim 2006). However,

studies reported a wide range of concentrations in these foods. Several studies have

identified cobalt in unfiltered coffee effusions at an average level of 0.93 mg Co/kg dry

coffee in one study (Smith and Carson 1981, p. 60). Cooking and canning may cause a

loss of cobalt from the fresh food (Smith and Carson 1981, p. 60). Up to 10% of cobalt

can be lost by roasting and grilling, 90% by stewing and boiling and 70% by canning

(Smith and Carson 1981, p. 60). The foam-stabilizing additives for beer production

contain cobalt at a maximum authorized concentration of 1 mg Co/L due to the fatal

cases of cardiomyopathies as a result of the synergistic action between alcohol and

cobalt at higher concentrations (Smith and Carson 1981, p. 61, 69).

Air

Cobalt, as particulate in the air, will return to land or surface water as wet or dry

deposition depending on size, density and meteorological conditions (ATSDR 2004).

Particles greater than 2 μm in diameter may deposit within 10 km from the point of

emission but finer particles may travel further (ATSDR 2004).

Concentration Factors:

Aquatic concentration factors describe the level of cobalt in an organism compared to

that in water. Marine zooplankton have concentration factors (CFs) ranging from 1,000 to

10,000 (Smith and Carson 1981, p. 58, 802). Rooted freshwater plants have CFs less than 10

which suggest the absorption occurs through the roots in the cobalt-enriched sediment (Smith

and Carson 1981, p. 59, 802). Freshwater algae, with CFs from 400 to nearly 2 million, reflect the

effect of their large surface–to–volume ratio on accumulation (Smith and Carson 1981, p. 58,

802). Insects and insect larvae show very high CFs, up to 100,000, possibly due to adsorption on

the exoskeleton of adult insects and the exposure of larvae living in sediment (Smith and Carson

1981, p. 58, 802). Freshwater fish and fish muscle CFs range from 10 to 1,000 (Smith and Carson 1981, p. 58, 802). Overall, the higher an organism is in the food chain, the lower the CF (Smith and Carson 1981, p. 803). Some variables that influence concentration factors include the high surface-to-volume ratio of plankton species which favors adsorption, absorption via ingestion of food particles by invertebrates and absorption via the gills in aquatic vertebrates (Smith and Carson 1981, p. 58).

Essential Nutrient:

Cobalt forms a unique carbon–metal bond in vitamin B_{12}, an essential nutritional molecule. This vitamin is not synthesized by plants or animals but rather by a few microorganisms. This vitamin is essential to convert methylmalonyl coenzyme A to succinyl coenzyme A, an intermediate of the citric acid cycle, and the formation of s-adenosylmethionine, an essential cofactor for methylation reactions. The most common congeners of vitamin B_{12} (cobalamin) have three portions to the molecular structure (Hillman 1996). The first two portions are the corrin ring system of coenzyme B_{12} which coordinates to cobalt and a dimethylbenzimidazole ribonucleotide. One of four groups complete the molecule and form the corresponding congener: cyanide, hydroxyl, methyl or 5-deoxyadenosyl (Hillman 1996). Cobalt $^{+1}$ complexes with 5'-deoxyadenosine through a weak covalent bond. This bond is easily broken with exposure to visible light and produces a radical and Co^{+2}. Cobalt $^{+3}$, when complexed with a hydroxyl group in the sixth coordination position instead of 5-deoxyadenosine, can be reduced by a reductase enzyme and NADH to cobalt $^{+2}$ and further to cobalt $^{+1}$ (Lehninger 2005, Stryer 1988). The two active forms of this coenzyme necessary are the

deoxyadenosine and methyl forms. However, the cyano and hydroxyl forms are the more commonly encountered forms which are converted to the active forms (Hillman 1996, Lehninger 2005, Stryer 1988).

Ruminants are completely dependent on the microflora within the rumen for vitamin B_{12} synthesis and require more cobalt from the diet than nonruminants. Ruminants use vitamin B_{12} to convert propionate, their primary energy source to glucose. The estimated cobalt requirement is 0.07–0.10 mg/kg diet. The estimated intake of cobalt for nonruminants such as pigs and chicks is only 0.01–0.02 mg/kg diet because nonruminants require preformed vitamin B_{12} to meet their metabolic needs (Swenson 1977, Van Soest 1994).

Cobalt deficiencies have been associated with several diseases. An autoimmune disease in humans, pernicious anemia, produces insufficient amounts of a factor required for vitamin B_{12} absorption (Lehninger 2005). Several wasting diseases, including bush sickness, salt sickness, Grand Traverse disease, Mairoa dopiness and Morton Mains disease, have been reported globally as a result of cobalt insufficiencies in cattle and sheep (Young 1948).

Cobalt deficient herbage occurs as a result of cobalt deficient soil which can be corrected by applying cobalt-infused fertilizer or concentrated salt licks (Smith and Carson 1981, p. 25, Young 1948). When iron is present, cobalt and iron mutually antagonize the absorption of each other due to the greater affinity cobalt has in the transport system of the intestine (Thomson et al. 1971).

Toxicology:

Cobalt toxicosis is not likely to occur in nonruminants unless environmental contamination of feed or water occurs (National Research Council 2005). Cobalt toxicosis in animals is very rare because concentrations of cobalt normally present in animal diets are much lower than those needed to cause toxicosis (National Research Council 2005). However, errors in mineral supplement formulation in ruminants, for example, could result in cobalt toxicosis (National Research Council 2005). The LD_{50} in rats for $CoCl_2·6H_2O$ is 42.4 mg Co/kg (ATSDR 2004). When fed an iron-sufficient diet, 50 mg Co/kg diet did not affect weight gain but 100 mg Co/kg diet did (Huck 1975). Myocardial damage in adult rats (Morvai et al. 1993), testicular degeneration in male rats and mice (Corrier et al. 1985), impaired fetal development in mice and rats (Szakmary et al. 2001) and increased mortality in pregnant rabbits have all been reported (Szakmary et al. 2001). One suggested mechanism involves oxidative damage to the purine and pyrimidine bases of DNA caused by cobalt (II) exposure (Kasprzak et al. 1994). Vitamin E and selenium offered protection against cobalt-induced cardiomyopathy (Van Vleet et al. 1977).

Cobalt toxicity has been noted in humans. Cardiac damage in beer drinkers progressed from dyspnea and hypotension to death in more than 40% of the cases. Irregular histologic findings were noted not only in the heart but also in the thyroid (Smith and Carson 1981, p. 69).

Cobalt toxicity was observed as a result of treatments for anemia in humans prior to 1970 which included a daily dose of cobalt up to 200 mg Co/day. This practice was suspended due to the onset of goiters and classical hypothyroidism signs that developed as early as six weeks after cobalt therapy began. Iodine uptake was suppressed by 20 to over 90%. Other

11

symptoms including nausea, neurogenic deafness, anorexia, dermatologic conditions and tinnitus were also documented in humans receiving cobalt anti-anemia therapy (Smith and Carson 1981, p. 69).

Hypoxia inducible factor (HIF), a transcription factor, leads to angiogenesis and stimulation of erythropoietin. Under normoxic conditions, hydroxylase enzymes add a hydroxyl group to the alpha subunit of HIF on a conserved proline residue. When hydroxylated, the von Hippel-Lindau (VHL) protein binds to the alpha subunit which initiates proteasomal degradation. When the alpha subunit is not hydroxylated, as in hypoxic conditions, dimerization of the other subunit, HIF beta, occurs and translocates to the nucleus where transcription of angiogenic, anaerobic glycolytic and erythropoietic proteins begins (Klaassen 2008, p.91). Cobalt has been shown to inhibit VHL by blocking the VHL binding site on the alpha subunit or by replacing iron in the hydroxylase enzyme thus inactivating the enzyme (Yuan et al. 2003, Epstein et al. 2001). In addition, the antioxidant ascorbate is essential to stabilizing the $iron^{+2}$ in hydroxylase. It has been demonstrated that when cells are exposed to cobalt, less ascorbate is absorbed. This deficiency likely favors iron oxidation leading to inactivation of the enzyme (Salnikow et al. 2004).

Cobalt has been shown to cause toxic effects in primary murine glial cells including mitochondrial damage. This lead to the release of apoptogenic factors leading to apoptosis of the cell. Cobalt, similar to hypoxia, also stimulated the expression of HIF-1α regulated genes (Nip3 and iNOS) and depleted ATP production. Thus, metabolic and neurotoxic effects have been documented at concentrations of 0.2–0.8 mM $cobalt^{+2}$ (Karovic et al. 2007). Neurotoxic effects, specifically memory deficits, were also observed in ex-factory workers who were diagnosed with hard metals disease as a result of cobalt exposure (Jordan et al. 1997).

Cobalt toxicity has been evaluated in aquatic organisms including microalgae, protozoa, invertebrates, fish and amphibians. The *Onchorynchus mykiss* (rainbow trout) LC_{50} was lower compared to other freshwater fish such as *Pimephales promelas* (fathead minnow). A flatworm species in the same genus as the species used in the studies for this thesis, *Dugesia tigrina*, had a 96-hour LC_{50} between the rainbow trout and fathead minnow (Table 2.2).

Table 2.2 Median lethal concentrations (LC_{50} values) for cobalt in other species

Common Name	Species	LC_{50} (mg Co/L)	Hardness (mg $CaCO_3$/L)	Reference
Fathead Minnow	*P.promelas*	21.8	130	Ewell et al. 1986
Worm	*D.tigrina*	11.3	130	Ewell et al. 1986
Rainbow Trout	*O.mykiss*	1.4	25	Marr et al. 1998

The LC_{50} values were based on a testing time of 96 hours. Hardness was determined in the dilution water. The contribution of cobalt to the total hardness was not accounted for when the exposure solutions were prepared.

Cobalt is either dissolved as cobalt carbonate or as cobalt (II) ions under most environmental conditions (Tipping et al. 1998). However, bioavailability appears to depend on the water chemistry and the concentration of calcium ions (Diamond et al. 1992). Therefore, when the hardness was varied, the 24-hour LC_{50} for *Ceriodaphnia dubia* increased from 2.4 to 5.3 mg Co/L as the hardness increased (Diamond et al. 1992). Using a gill-cobalt binding model, calcium competition and dissolved organic matter complexation were the most important factors preventing cobalt from binding at the gills in a series of natural water tests in *O. mykiss*. Calcium at 17 μM allowed cobalt to bind but inhibited binding at 90 μM (Richards and Playle 1998). The data based on binding affinities indicate that cobalt binds to gill sites more weakly (log K =5.1) than other metals such as cadmium (log K=8.6), lead (log K=6.0) and zinc (log K=5.3-5.5) (Niyogi and Wood 2004).
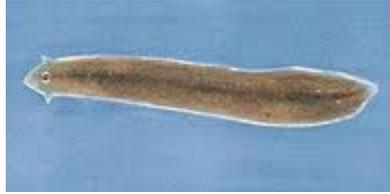
ll. Planaria

Figure 1. *Dugesia dorotocephala*.

Dugesia dorotocephala, freshwater planaria, are bilaterally symmetrical metazoans of

the phylum Platyhelminthes (Figure 1). These invertebrates are unsegmented, acoelomate, and

possess well-defined anteroposterior and dorsoventral axes (Adell et al. 2010). Along the AP

axis, the anterior cephalic region containing the brain and a pair of eyespots, a central region

with a pharynx and a ventral mouth opening, and a posterior tail region can be distinguished

(Adell et al. 2010) (Figure 1). They are best known for their ability to regenerate complete

animals from fragments of their own bodies in seven days, a generally short time frame (Adell et

al. 2010). The inclusion of planaria in a screening scheme would provide improved sensitivity in

detecting toxicity because planaria generally respond to lower levels of contamination than do

other species (Kapu and Schaeffer 1991). They can also be included in a higher tier because they

may exhibit complex responses including inhibition and stimulation of reproduction,

teratogenesis and tumorigenesis (Best and Morita 1982).

Freshwater planaria are important components of the aquatic ecology of relatively

unpolluted streams. They were incorporated in the biological assessments for water quality in

Europe. The sensitivity varies between species of planaria from very sensitive to mild sensitivity

to pollution in waters but generally are intolerant of heavy metal salts (Kenk 1980). They model

many of the toxicologically vulnerable systems of higher animals and may represent an

economical organism for toxicity screenings and bioassays (Best 1981, Best and Morita 1982).

This species occurs in nature as both sexual and asexual forms. The transformation of

asexual worms into sexual ones has been observed under conditions that simulate the drying up

of a pond (H.S. Ramsdell, unpublished observations). Morita and Best describe a distinct

circadian rhythm of fissioning under the influence of normal photoperiods in asexual

D. dorotocephala. This process, essential for maintaining and increasing their population, is

difficult to observe because fissioning occurs at night and is completed in three to four minutes.

Initially, a body constriction appears at the site where fissioning will occur; the rostral portion of

the body contracts and then pulls away from the caudal portion which adheres to the surface of

the container. The constricted region becomes narrower as it stretches. This process continues

until the stretched area ruptures. Finally, the rupture closes up when the circular body wall

muscles contract. After fissioning is completed, regeneration takes place to yield two complete,

but smaller, planaria (Morita and Best 1993). These observations suggest that fissioning

primarily involves neuromuscular events which are regulated by neurotransmitters or

neuromodulators within the central nervous system (Morita and Best 1993, Best et al. 1969).

Fissioning was a process previously believed to occur as a result of mucus or some other

substance being released into the water from the planarian. Instead, a system of population

feedback control has actually been identified. In the presence of other planaria, the brain exerts

an influence (probably neurohormonal) to suppress fissioning which decreases with axial

distance from the brain. The suppression is contingent on population density and thus is a part

of a feedback system for adjusting the rate of reproduction to population density. A reduced population density would lead to increased rates of fissioning whereas higher population densities suppress fissioning (Best et al. 1969).

lll. Studies Reported In This Thesis

The ecological risk assessment and development of water-quality criteria for cobalt are still currently hampered by insufficient knowledge about the toxicity of cobalt to freshwater organisms (DeSchamphelaere et al. 2008). Therefore, this work was conducted to establish acute LC$_{50}$ cobalt values in *D. dorotocephala* at varying levels of hardness, to establish valid sublethal endpoints in chronic studies and to evaluate the role of calcium in cobalt toxicity.

III. MATERIAL AND METHODS

I. Culture Maintenance

Asexual *Dugesia dorotocephala* were maintained in large white enameled dishpans that contained moderately hard reconstituted culture water. Eighteen liters of culture water were prepared at a time in a carboy using ultraviolet-treated water from Horsetooth Reservoir in Fort Collins, CO. To adjust hardness from the soft Horsetooth Reservoir water to moderate hardness, calcium sulfate (Mallinckrodt Analytical Reagent), magnesium sulfate heptahydrate (EM Science), potassium chloride (Fisher Scientific) and sodium bicarbonate (Fisher Scientific) were added. Aeration was applied for 12–24 hours to allow for complete dissolution of the salts, thorough oxygenation and equilibration of the solution with atmospheric carbon dioxide. After three uses of a given carboy or a six month period had passed, the carboy was disinfected by allowing a 5% bleach solution to sit in the carboy for thirty to forty minutes followed by extensive rinsing with Horsetooth Reservoir water followed by Type 1 water (ASTM 1999).

Planaria were collected from a tributary of the Cache La Poudre River in Bellvue, CO and acclimated over a two-week period by transitioning their water to the laboratory culture water. The planaria were transferred to culture pans and only handled with a small wet paintbrush to minimize stress and potential injury. All of the culture pans were covered with pieces of light-weight plastic fitted with holes to allow for air exchange and to prevent debris falling into the pans.

Once acclimated to the water, the planaria were divided by size in different culture pans to discourage cannibalism and promote growth. They were fed organic beef liver 1–2 times a week. The pan water was changed between four and 24 hours after feeding. The planaria were placed in new culture pans with the aid of a piece of plastic screen approximately every four to six weeks or earlier if algae had overgrown in the pans. The photoperiod, 16:8 hours light:dark, was maintained with an automatic timer.

II. Experimental Methods

All experiments were conducted in glass crystallizing dishes with a diameter of 105 mm containing 50 mL of the desired exposure solution. The replicates were randomly stacked with their respective concentrations after each water change to minimize the effect of variation in light intensity from the overhead light. An empty crystallizing dish was placed on top of each stack for protection and to limit dehydration.

Glassware used in a study was detergent-washed, rinsed five times with tap water, three times with ultra-pure water followed by an acid rinse of 10% hydrochloric acid. Three final rinses with Type 1 water prepared the glassware for the study (ASTM 1996).

Cobalt concentrations were prepared from a stock solution (either 625 or 400 mg Co/L) made with Type 1 water using $CoCl_2 \cdot 6H_2O$ (Mallinckrodt Analytical Reagent). The highest concentration for each study was prepared using the stock solution and a match water. The match water was formulated using calcium sulfate and magnesium sulfate to achieve the required hardness values while accounting for the hardness contribution of cobalt. Dilution water, prepared to match the desired overall hardness for each study, was used for the serial dilutions to the lower concentrations. Match and dilution waters were prepared separately for

18

every study. In an experiment where total hardness was maintained but cation concentrations varied, separate dilution and match waters for each test were prepared in individual containers. In this manner, test solutions were formulated such that hardness was held constant while cobalt concentrations varied. An initial seven-day mortality study was conducted without hardness matching.

Planaria, 10–15 mm in length, to be used for a study were segregated into a separate pan four or seven days prior to the start of the study in the appropriate dilution water in the hardness to be used during the study. Feeding occurred three days prior to the start of the study.

Observations were recorded prior to a water change if one was scheduled to occur that day, typically within three hours of the time of day when the study began. Observations took place under a magnifying light and a dissecting microscope using a paintbrush to manipulate the planaria if necessary. Morphology, behavior, and any abnormalities were recorded.

In studies when behavioral assessments were performed, tactile response, righting reflex and swimming speed were evaluated. Tactile response was measured by recording the time elapsed for a planarian to regain forward motility after being subjected to a touch stimulus. The planarian was observed for up to 60 seconds. If there was no movement by this time, the result was scored as "60 seconds." The righting reflex was evaluated by measurement of the time to regain normal forward motility after the body was inverted using a small paintbrush. As with the tactile response, failure of the planarian to achieve forward motility within one minute was scored as "60 seconds." Swimming speed was measured by counting the number of lines on a two centimeter by two centimeter grid crossed during a two-minute time interval. The same

planarian, one per replicate and randomly chosen by the observer, was used for the three assessments. Without a convenient method for identifying individual planaria, behavioral assessments at other time points may not have been done using the same planarian.

After observations, feedings would take place if required by the study design. A small piece of raw beef liver was placed in each dish. The remaining food was removed four to six hours later and the exposure solution renewed. Exposure solutions prepared within thirty minutes of use were added from a graduated cylinder as soon as the old water was removed. Vacuum filtration and a micropipette tip were used to remove the old water from each dish.

Water chemistry parameters were measured on the newly prepared dilutions and on a composite sample of old exposure solutions removed from dishes during exposure solution renewal, using meters generally calibrated on a daily basis. Temperature, conductivity, pH and dissolved oxygen were recorded for both new and composited old samples but ammonia was only analyzed in the composited old water samples. Subsamples of the new and/or old solutions were preserved at each water change with 1% trace metals grade nitric acid (Fisher Scientific). After every water change, the dishes were restacked randomly within each concentration and placed under the light source with a 16:8 light:dark photoperiod. The following table (3.1) presents the methods used to determine the specific parameter.

Table 3.1. Water Chemistry Methods

pH	SM 4500-H$^+$ B	(Standard Method, 1996)
Dissolved Oxygen	SM 4500-O G	(Standard Method, 1993)
Ammonia	ASTM D1426-98A, SM 4500-NH$_3$ D	(ASTM, 1998 and Standard Method, 1997a)
Conductivity	EPA 120.1	(EPA, 1982)
Alkalinity	SM 2320 B	(Standard Method, 1997b)
Hardness	SM 2340 C	(Standard Method, 1997c)
Temperature	ASTM E729-96	(ASTM, 1996)

III. Short Term Studies

Mortality studies were performed with 96 and 168 hours of exposure. A standard test period for many aquatic species is 96 hours (ASTM 1996). Previous toxicity testing using *D. dorotocephala* in this laboratory has demonstrated a pronounced increase in mortality in periods between 96 and 168 hours (H.S. Ramsdell, unpublished observations). Samples of the new exposure solution dilutions were collected and preserved once at the beginning of each study. Samples of the composited old waters were collected and preserved after each water change. When fissioning occurred during an acute study, it was recorded but no segregation of fragments was performed.

Prior to the renewal of exposure solutions, mortality and morphology were recorded. Exposure solutions were renewed on the third and fifth days of seven day studies. Exposure solutions were renewed daily in four day studies. Three behavioral assessments were conducted on the final day of each test.

IV. Long Term Study

A sixty day study was conducted to determine a nonlethal endpoint sensitive to cobalt exposure. Three endpoints were assessed: behavioral responses, fissioning and regeneration rates. Behavioral assessments were made every two weeks. Daily checks for fissioning events were performed and if an event had occurred, the head and the tail fragment were segregated in separate dishes. Segregated fragments were evaluated daily to see if regeneration was complete. If so, each fragment returned to the exposure dish from which the fragments had been removed. The daily checks were all made between 0700 and 1100.

Mortality and morphology were recorded twice weekly prior to the renewal of the exposure solutions for the planaria in the crystallizing dishes and those that were segregated as well. Feeding occurred once a week after observations followed by the test renewal four to six hours later. Samples of the new exposure solution dilutions were collected and preserved once a week and composited old exposure solutions samples were prepared twice a week for later ICP-AES analysis.

V. Cation Studies

An initial range finding study took place with just one replicate to determine the impact of calcium and magnesium concentrations on cobalt mortality. In a subsequent experiment, at a fixed concentration of 10 mg Co/L and an overall target hardness of 100 mg $CaCO_3$/L, the calcium and magnesium ratios were varied for a seven day mortality study. Observations took place daily after the initial 48 hours and behavioral assessments occurred at test termination. Samples of the new exposure solutions were preserved once at the beginning of the study.

Composited old samples were collected when test solutions were renewed which occurred on the third and fifth day of the seven day study.

VI. Analytical Chemistry

Samples preserved with 1% nitric acid were analyzed using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Table 3.2). Blank samples and two standard concentrations per element (calcium, cobalt and magnesium) were evaluated during every analysis.

Table 3.2.The settings used for the ICP-AES

ICP-AES	PerkinElmer Optima 7300 DV
Forward Power	1.3 kW
Plasma flow rate	15 L/min
Auxiliary flow rate	0.2 L/min
Nebulizer flow rate	0.8 L/min
Sample flow rate	0.75 mL/min
Nebulizer	Meinhard concentric
Spray chamber	Precision cyclonic with baffle
Internal Standard	10 µg/mL Yb at 0.25mL/min

VII. Statistics

The LC_{50} values from the short term studies were determined using the trimmed Spearman-Karber Method and Probit analysis using US EPA Toxicity Data Analysis Software Version 1.5 (1994). The Probit method is used if there are two or more non-control concentrations that have partial mortalities. The Spearman-Karber method is used when one partial mortality exists among non-control concentrations or when the Probit method is inappropriate due to the chi-square value calculated by the software.

Long term data including mortality, fissioning, behavioral and regeneration, were analyzed. Proportion data were transformed using the arcsine square root for statistical tests. One way and two way ANOVAs, paired T tests and Tukey's Method were calculated or performed using R Version 2.13.1 (R Development Core Team 2008).

The results of five experiments explored the various endpoints related to cobalt toxicity.

Three short term studies identified mortality-related values as the main effect. They were

conducted in two time frames: 7 days (Studies #1 and 2) and 4 days (Study #3). Study #1 had

varying levels of total hardness. Studies #2 and 3 had fixed total hardness levels. Data from

three behavioral assessments was evaluated also in Studies #2 and 3.

One long term study, #4, identified relevant sublethal endpoints including fissioning and

regeneration rates and behavioral assessments. Survival of intact worms, those that did not

fission, and of the fragments, head or tail, were also evaluated. Finally, Study #5 showed the

mortality that was observed when cobalt and total hardness were held at a fixed concentration

but the ions comprising total hardness, calcium and magnesium, varied.

4.1   Study #1

A 7 day mortality study using *D. dorotocephala* was performed. The initial 96 hours of

exposure resulted in no mortality but 80% mortality was observed in the highest cobalt

concentration by the seventh day. No mortality occurred in the dilution water, the

concentration that contained no cobalt, throughout the study. Mortality was significantly

increased at concentrations of 50 mg Co/L and above (Tukey, $p < 0.0009$) (Figure 4.1.a.). A 7 day

median lethal concentration (LC_{50}) of 50 mg Co/L was calculated for Study #1 (Trimmed

Spearman-Karber).

Based on the nominal concentrations and titration of the dilution water, the calculated total hardness range was 217- 323 mg $CaCO_3$/L. The $CoCl_2$ stock solution (made up in Type 1 water) was added to achieve the desired Co concentration. Due to the contribution of Co^{+2} to hardness, each level of Co exposure solution had a different total hardness (Table 4.1.a). As noted below, this could be expected to result in calculation of a higher LC_{50} value as a consequence of the protective effect of higher hardness.
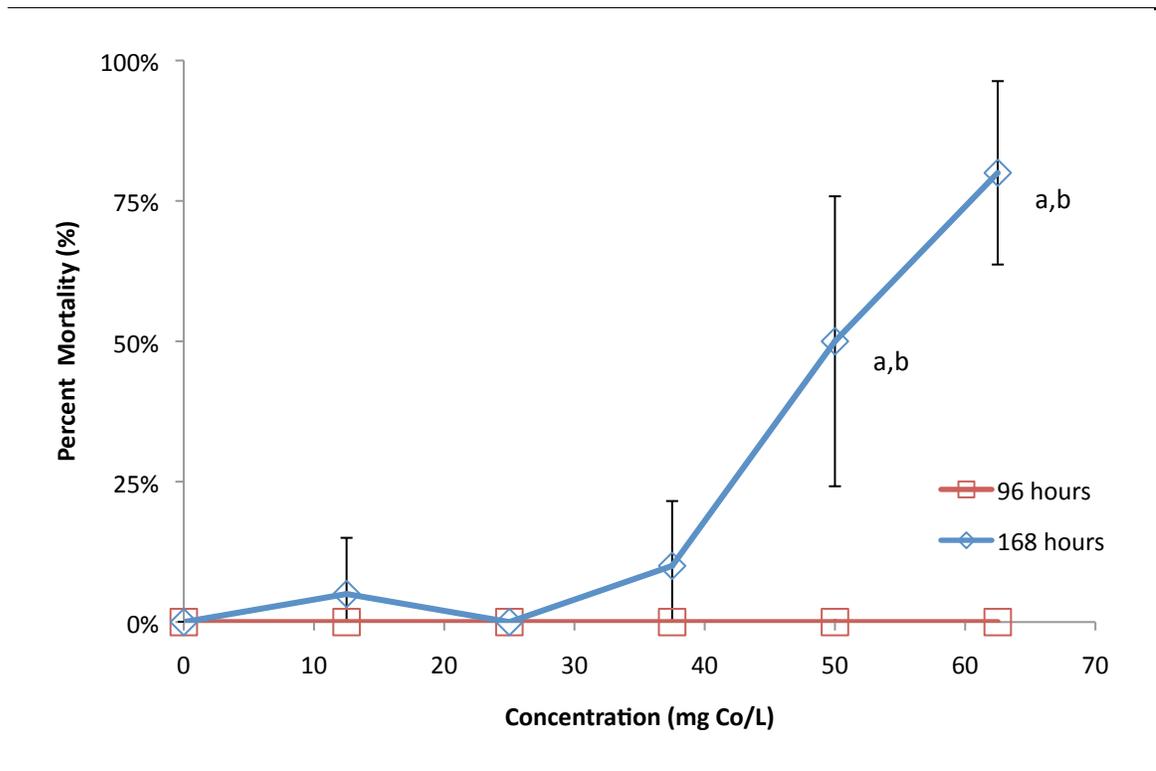
Figure 4.1.a. Mortality after the initial 96 hours and at 168 hours in Study #1

Symbols indicate the mean value of 4 replicates, each consisting of five planaria per dish. Exposure solutions were renewed on days 3 and 5. Observations were made daily after the first two days. Planaria were not fed during the study. An arcsine square root transformation was applied to the data for statistical analysis. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from control, p < 0.0009 (Tukey)
[b] Significantly different from 12, 25 and 37 mg Co/L, nominal, p <0.0009 (Tukey)

Table 4.1.a. Mortality and Calculated Total Hardness, Study #1

Co (mg/L)	Calculated Total Hardness (mg CaCO$_3$/L)	Percent Mortality at 96 hours	Percent Mortality at 168 hours (± S.D.)[a]
0	217	0%	0% (± 0)
12.5	238	0%	5% (± 0.1)
25	259	0%	0% (± 0)
37.5	281	0%	10% (± 0.1)
50	302	0%	50% (± 0.2) [b,c]
62.5	323	0%	80% (± 0.2) [b,c]
Calculated LC$_{50}$ (95% CI [d])=	50 mg Co/L (45, 56)	N/A	N/A

The dilution water was titrated for hardness determination once at test termination. The total hardness values for the cobalt concentrations were calculated based on the total hardness of the dilution water and the contribution from cobalt at each concentration. Percent mortality is based on a mean value of four replicates. An arcsine square root transformation was applied to the data for statistical analysis. The concentrations of cobalt are nominal values.
[a] One standard deviation
[b] Significantly different from the control, p<0.0009 (Tukey)
[c] Significantly different from 12, 25 and 37 mg Co/L, nominal, p <0.0009 (Tukey)
[d] Confidence Interval

The ranges reported in Table 4.1.b. were compiled from the data of both new and old exposure solutions. The reported hardness and alkalinity concentrations were determined from titrations performed with dilution water only. The highest ammonia value, 5.04 mg/L, was noted in the composited old exposure solution of the control on the third day. On that same day, the highest dissolved oxygen and pH values of all the composited samples were also recorded in the control as well.

Table 4.1.b. Water chemistry values, Study #1

	Range	Mean (± S.D. [a])
pH	6.7-7.8	7.4 (± 0.28)
Dissolved Oxygen (mg/L)	4.52-8.23	6.3 (± 1.1)
Temperature (^{o}C)	17-18	17 (± 0.44)
Ammonia (mg/L)	<1.0-5.04	2.4 (± 1.5)
Hardness (mg/L CaCO$_3$)	217	N/A
Alkalinity (mg/L CaCO$_3$)	228	N/A

Water chemistry values were measured in new and old exposure solutions. Mean values were computed on days 3, 5 and 7. Dissolved oxygen, pH, ammonia and temperature were recorded in the old solutions on days 3, 5 and 7. Only pH and dissolved oxygen were measured on the new solutions on day 3. Dissolved oxygen and temperature was measured on day 5. The dilution water was titrated once at test termination. The total hardness values for the cobalt concentrations were calculated based on the total hardness of the dilution water and the contribution from cobalt at each concentration.
[a] Standard deviation

4.2   Study #2

In order to account for the influence of hardness on Co toxicity to planaria, a 7 day mortality study using *D. dorotocephala* was performed at three different total hardness levels. Calculated total hardness, including the contribution of Co^{+2}, was held constant by preparing working Co^{+2} stock solutions in a hardness "match" water formulated with calcium and magnesium salts such that the total hardness would be at the desired level. Dilution of the hardness-matched working stock solutions with reconstituted water with the same hardness value thus resulted in each exposure concentration having the same total hardness. Analysis of exposure solutions for Ca, Mg and Co by ICP-AES was used to verify actual total hardness values. Mortality and median lethal concentrations (LC_{50}) were calculated. Three different behavioral responses were also recorded.

After the initial 96 hours, the highest cobalt concentration at each level of hardness resulted in approximately 50% mortality (Figure 4.2.a.). For planaria that underwent fissioning during the study, mortality was assessed based on survival of only the head fragments. Complete mortality occurred in the higher Co exposure groups by 168 hours at all three levels of hardness (Figure 4.2.b.). Hardness alone did not significantly contribute to the mortality either at 96 or 168 hours (Two-way ANOVA, $p > 0.05$). When the effect of the cobalt concentration was accounted for in the statistical analysis of each level of hardness, the interaction of these two factors significantly contributed to the observed mortality at 96 and 168 hours (Two-way ANOVA, $p < 0.0001$).
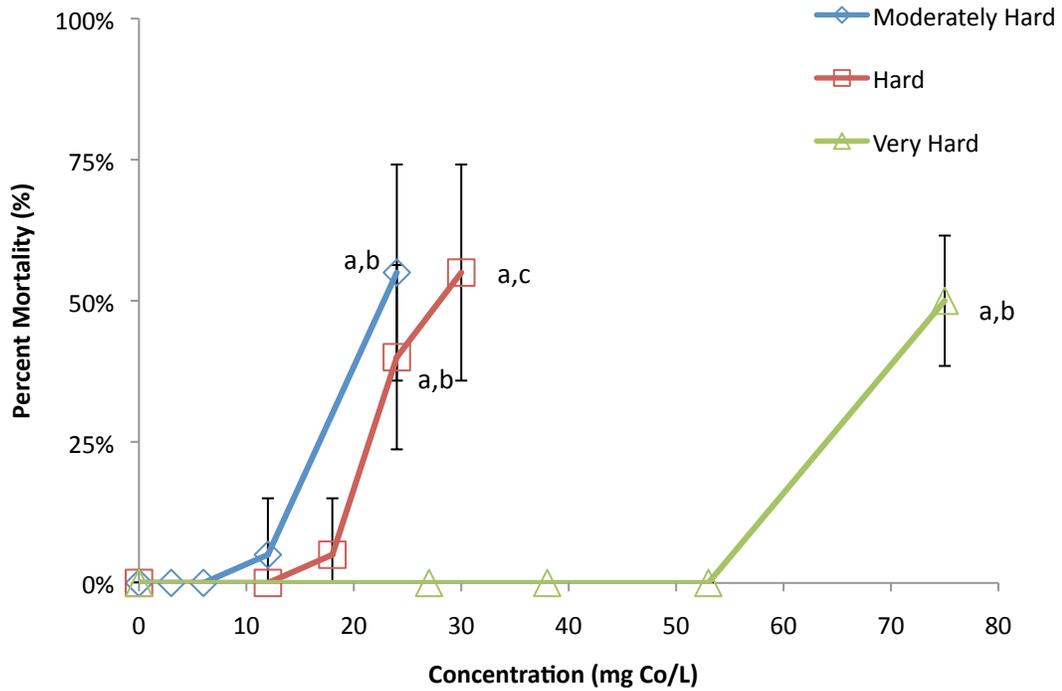
29
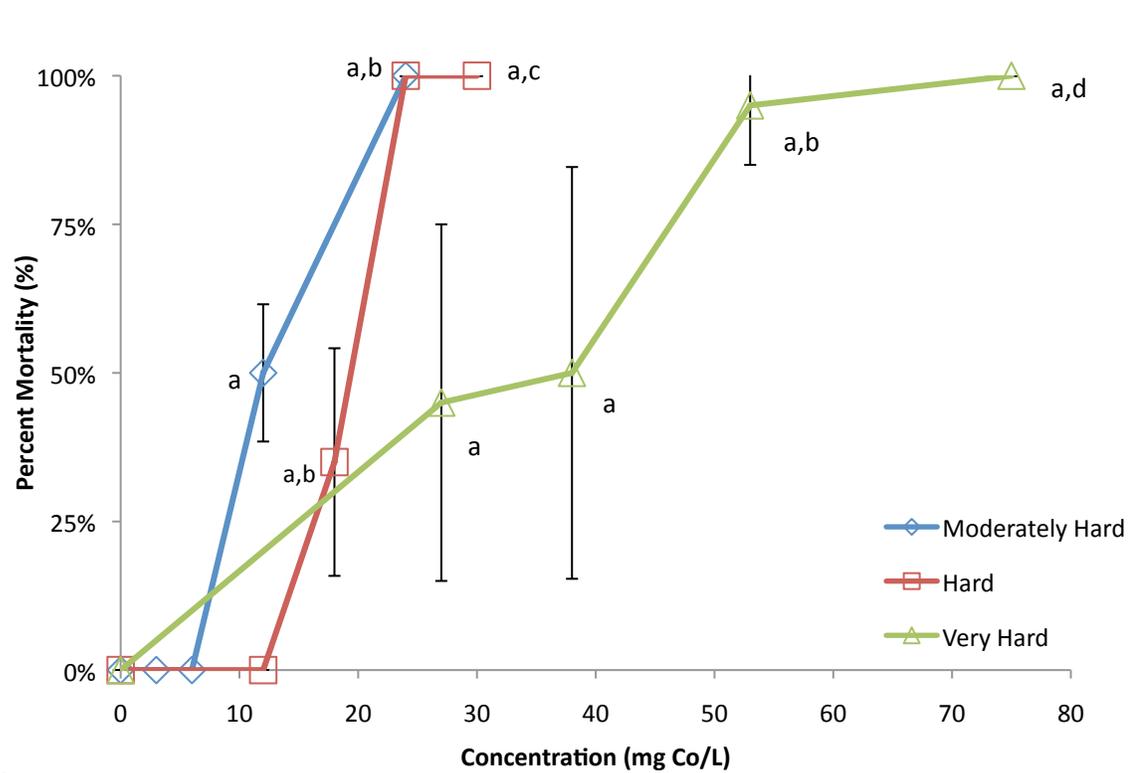
Figure 4.2.a. Mortality at 96 hours, Study #2

Observations were made daily after the initial 48 hours. Symbols represent a mean value of 4 replicates (five planaria per dish). Concentrations of calcium, magnesium and cobalt were analyzed by ICP-AES for total hardness calculations. Planaria were fed once prior to the start of the study while they were segregated in their appropriate dilution water. An arcsine square root transformation was applied to the data for statistical analysis. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.

[a] Significantly different from the control, p<0.0001 (Tukey)
[b] Significantly different from all lower concentrations, p <0.002 (Tukey)
[c] Significantly different from 12 and 18 mg Co/L, p<0.002 (Tukey)

Figure 4.2.b. Mortality at test termination (168 hours), Study #2

Observations were made daily after the initial 48 hours. Symbols represent a mean value of 4 replicates (five planaria per dish). Concentrations of calcium, magnesium and cobalt were analyzed by ICP-AES for total hardness calculations. Planaria were fed once prior to the start of the study while they were segregated in their appropriate dilution water. An arcsine square root transformation was applied to the data for statistical analysis. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.

[a] Significantly different from the control, p<0.001 (Tukey)
[b] Significantly different from the lower concentrations, p <0.01 (Tukey)
[c] Significantly different from 12 and 18 mg Co/L, p <0.0001 (Tukey)
[d] Significantly different from 27 and 38 mg Co/L, p <0.01 (Tukey)

The temporal effect at a total hardness of 180 mg $CaCO_3$/L is shown in Figure 4.2.c. By 120 hours (five days), all planaria were dead at the highest concentration, 30 mg Co/L. At 24 mg Co/L, complete mortality required 168 hours (seven days). Time was significant (Two-way ANOVA, p=0.008) but when cobalt concentrations were also taken into account, the interaction of time and cobalt levels had a higher level of significance with respect to mortality (Two-way ANOVA, p<0.0001).
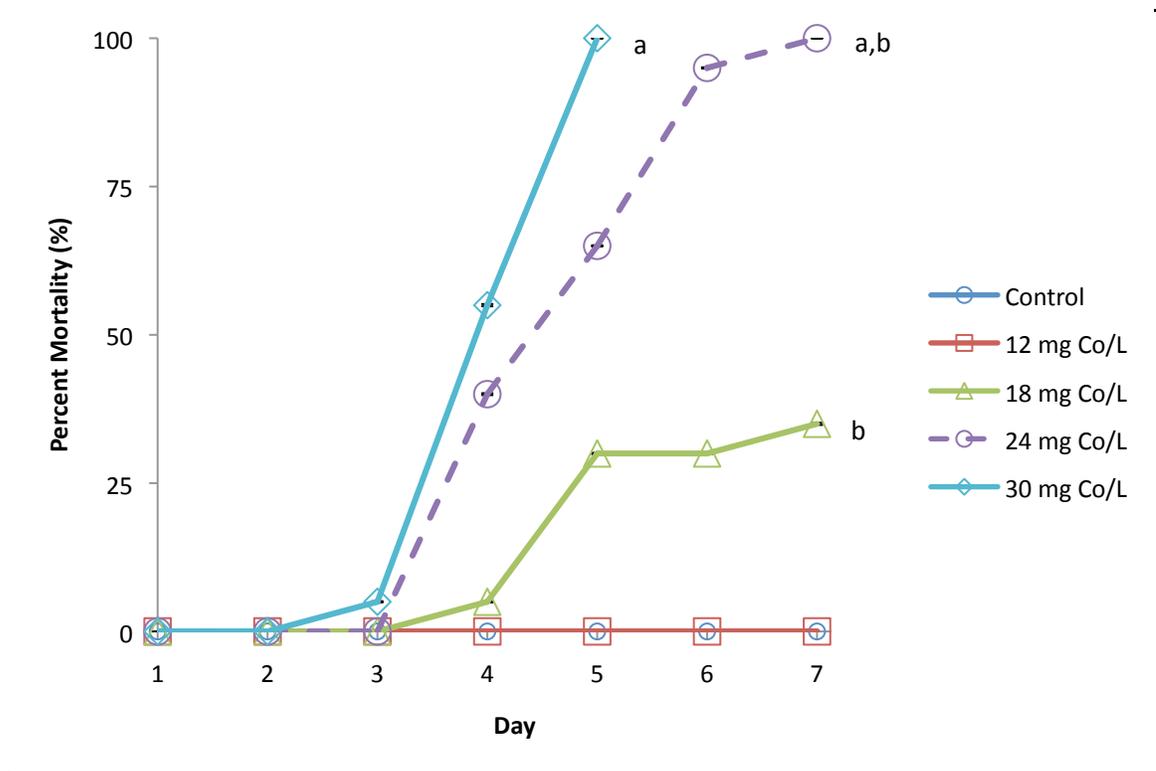
Figure 4.2.c. Mortality by day at varying cobalt concentrations for hard water exposure solutions, Study #2

Observations were made daily after the initial 48 hours. Symbols represent a mean value of 4 replicates (five planaria per dish). Concentrations of calcium, magnesium and cobalt were analyzed by ICP-AES for total hardness calculations. Planaria were fed once prior to the start of the study while they were segregated in their appropriate dilution water. An arcsine square root transformation was applied to the data for statistical analysis. Error bars that representing one standard deviation are smaller than the plot symbols. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, p<0.0001 (Tukey)
[b] Significantly different from the lower concentrations, p <0.01 (Tukey)

At the moderate level of hardness, the LC_{50} was 12 mg Co/L (Spearman-Karber Method) based on the original data (no transformation). As hardness increased, the LC_{50} values also increased to 18 and 32 mg Co/L in hard and very hard water respectively (Spearman-Karber and Probit, respectively) (Figure 4.2.d.). All of these concentrations were significantly different from each other because the Least Significant Difference intervals do not overlap (Table 4.2.a.)
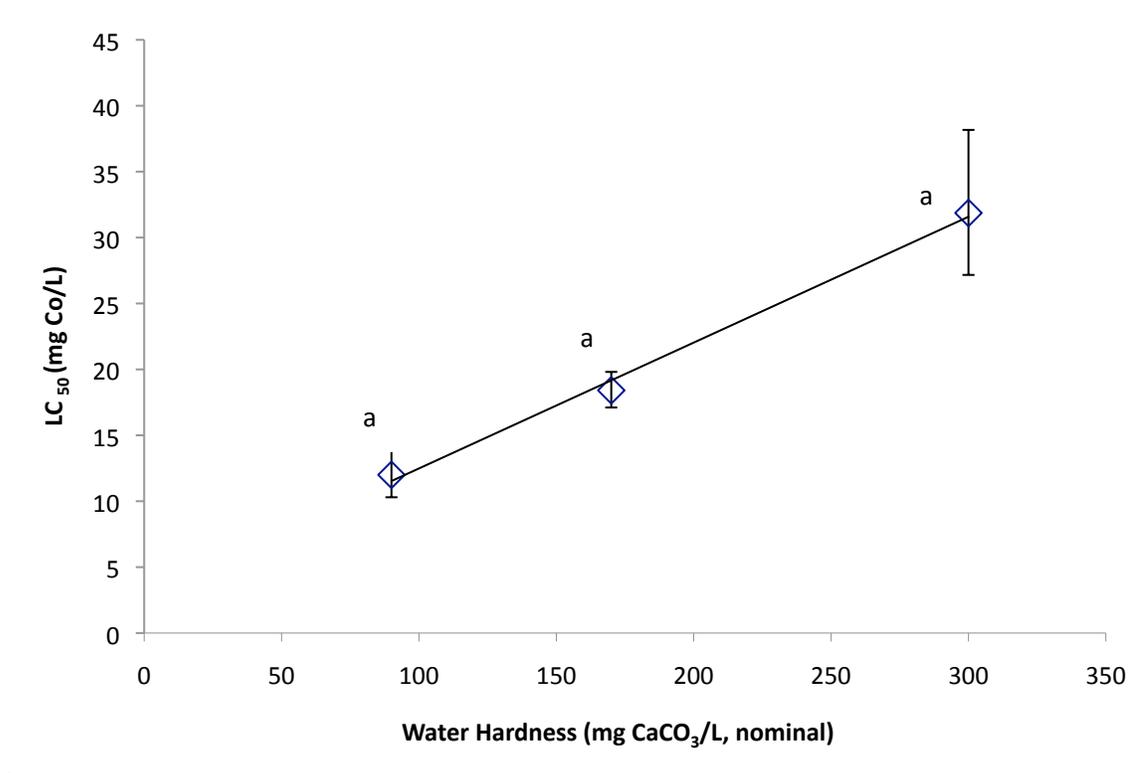
Figure 4.2.d. Cobalt toxicity (LC_{50}) to Planaria at Different Water Hardness Levels, Study #2

Median lethal concentrations were calculated using Spearman-Karber and Probit methods. Error bars that are displayed represent the 95% confidence intervals.
[a] Significantly different from the other treatment groups based on LSD Intervals.

Table 4.2.a. Median lethal concentrations for three nominal hardness levels, Study #2

	LC_{50} (mg Co/L)	95% Confidence Intervals	LSD[a] Intervals
Moderately Hard 100 mg $CaCO_3$/L	12	(10, 14)	(11, 13)
Hard 180 mg $CaCO_3$/L	18	(17, 20)	(17, 19)
Very Hard 320 mg $CaCO_3$/L	32	(26, 37)	(28, 36)

Each level of hardness had 4 replicates, each consisting of 5 planaria per dish. Exposure solutions were changed on days 3 and 5. The planaria were not fed during this study.
[a]Least Significant Difference

Three behavioral assessments, tactile response, righting reflex response and swimming speed, were measured at test termination (168 hours) at all levels of hardness tested. One planarian was randomly selected from each surviving replicate. As the concentration of cobalt increased and more adverse effects were observed (ruffled edges, lesions, mortality etc), fewer planaria were available for these assessments. Toxicity decreased when the swimming speed was higher but increased when the time for the tactile or righting reflex responses took longer.

Moderately Hard

The tactile response at 3 and 12 mg Co/L were significantly different compared to the control (Tukey, $p<0.05$) (Figure 4.2.e.). The righting reflex response was significantly delayed at 12 mg Co/L relative to the control (Tukey, $p<0.0001$). Both tactile and righting reflex responses were significantly longer at 12 mg Co/L compared to 3 and 6 mg Co/L (Tukey, $p<0.0001$). Swimming speed was significantly reduced in a concentration-related fashion in all of the exposure solutions with available subjects (3, 6 and 12 mg Co/L) (Tukey, $p<0.0001$).

Hard

The two timed behavioral responses were significantly affected at 12 and 18 mg Co/L relative to the control (Tukey, $p<0.0008$) (Figure 4.2.f.). Similar results were observed in the swimming speed as well at the same concentrations.

Very Hard

The tactile and righting reflex responses were significantly different from the control (Tukey, p<0.02) (Figure 4.2.g.). The swimming speed was significantly reduced relative to the control at 27, 38 and 53 mg Co/L (Tukey, p<0.0005) due to the immobility of the planaria.
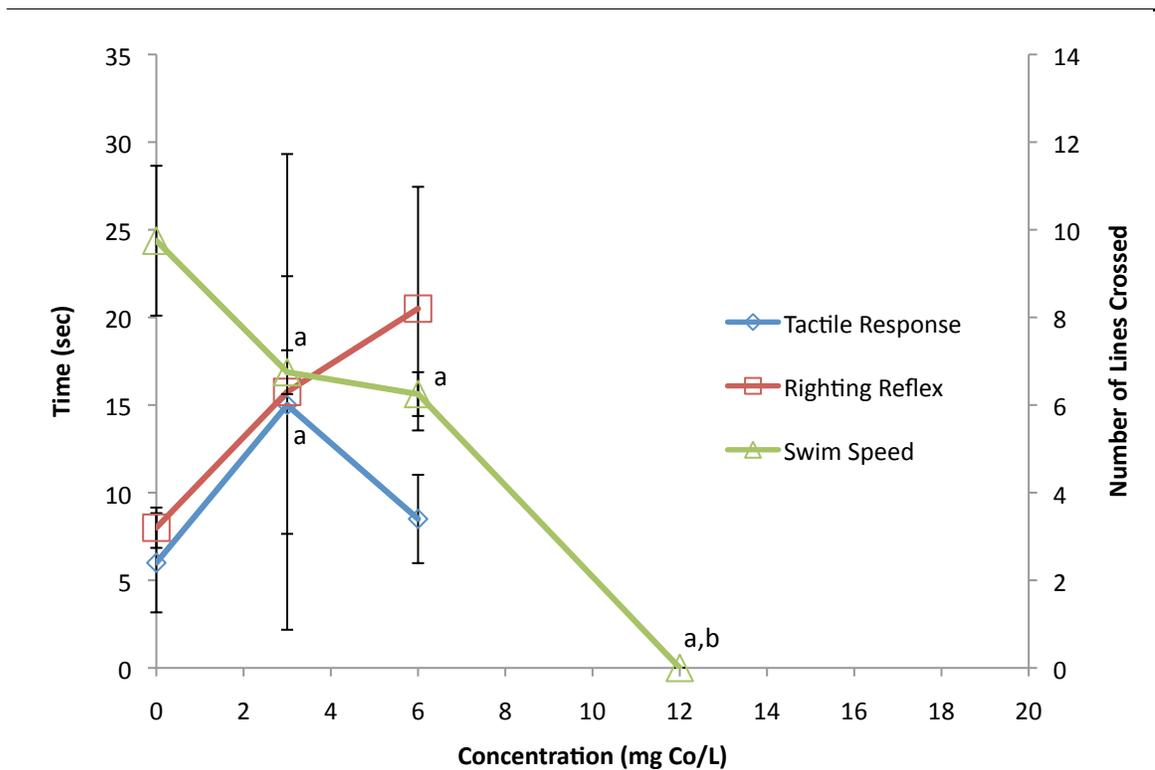

Figure 4.2.e. Three behavioral assessments performed in Study #2 at nominal total hardness of 100 mg CaCO$_3$/L (moderately hard)

Symbols represent a mean value based on four replicates for all three assessments. These assessments were performed at test termination. For either of the two timed responses, values are not shown if forward mobility was not regained within the one-minute observation time. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, p<0.05 (Tukey)
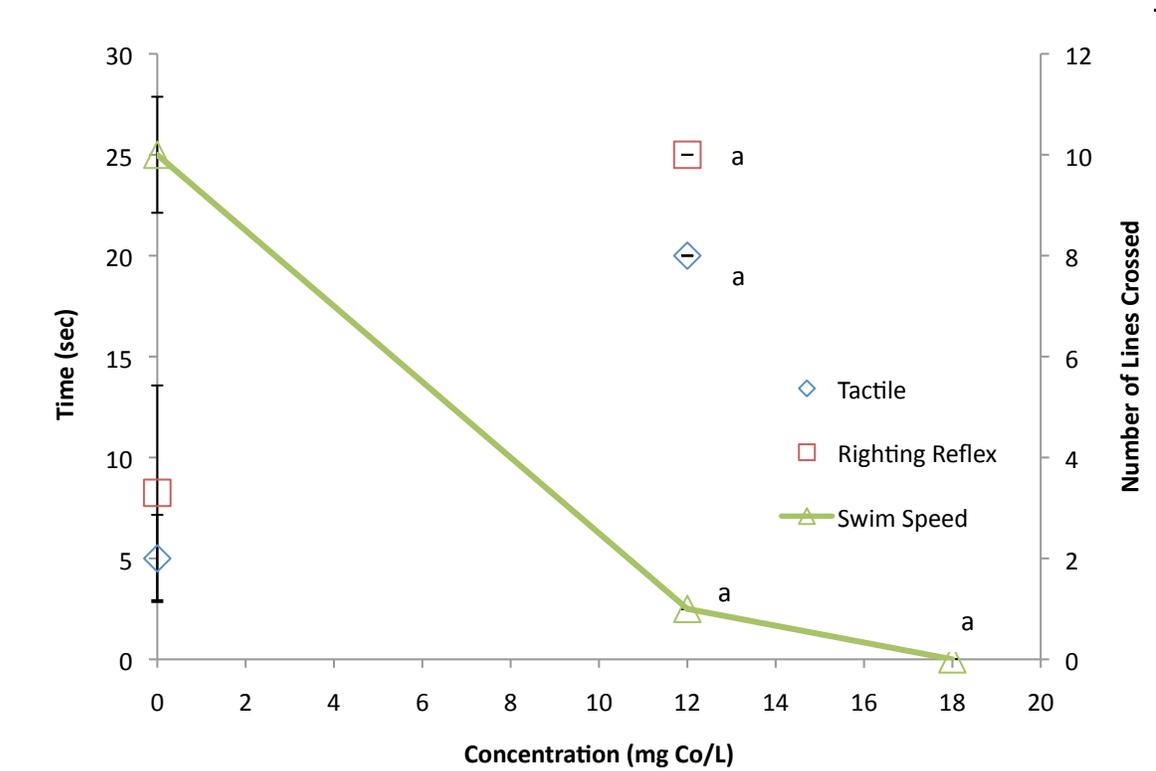[b] Significantly different from 3 and 6 mg Co/L, p <0.0001 (Tukey)

Figure 4.2.f. Three behavioral assessments performed in Study #2 at nominal total hardness of 180 mg CaCO$_3$/L (hard)

Symbols represent a mean value based on four replicates for the control for all three assessments, one replicate for 12 mg Co/L for all three assessments and four for 18 mg Co/L for swim speed. These assessments were performed at test termination. For either of the two timed responses, values are not shown if forward mobility was not regained within the one-minute observation time. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
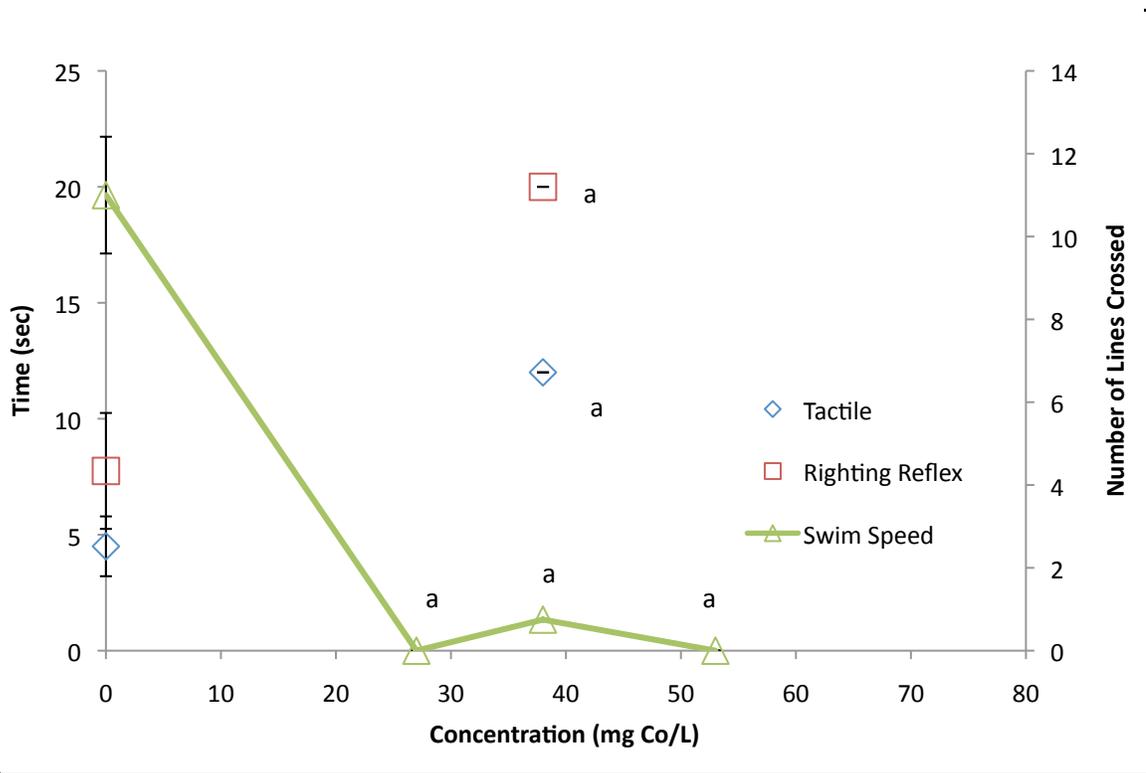[a] Significantly different from the control, $p < 0.002$ (Tukey)

Figure 4.2.g. Three behavioral assessments performed in Study #2 at nominal total hardness of 320 mg CaCO$_3$/L (very hard)

Symbols represent a mean value based on four replicates for all three assessments but only one replicate for 53.6 mg Co/L. These assessments were performed at test termination. For either of the two timed responses, values are not shown if forward mobility was not regained within the one-minute observation time. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, p<0.03 (Tukey)

Swimming Speed:

The swimming speed was analyzed for all three hardness levels because this assessment had the greatest response in all the concentrations. Hardness alone did not significantly reduce the swimming speed but Co concentration and hardness together explained the decreased response (Two-way ANOVA, p<0.001) (Figure 4.2.h.). Every concentration of cobalt experienced a significant reduction from the control and the planarian at 12 mg Co/L was significantly slower compared to 3 and 6 mg Co/L (Tukey, p<0.004).
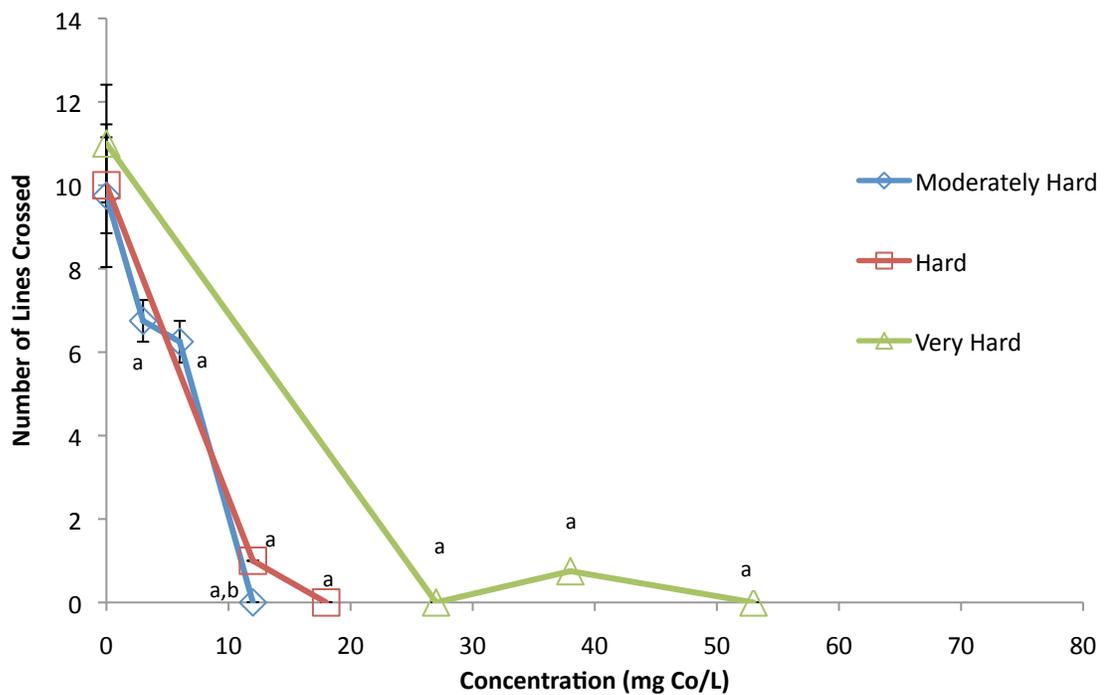
Figure 4.2.h. Swimming speed in all three levels of hardness, Study #2

Symbols represent a mean value based on four replicates but only one replicate for 53 mg Co/L. These assessments were performed at test termination. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, p<0.004 (Tukey)
[b] Significantly different from 3 and 6 mg Co/L, p <0.0001 (Tukey)

Water chemistry values for this study are listed in Table 4.2.b. Initial titrations of the dilution and match waters were within 10% of the nominal concentrations. The measured hardness range was within 11% of the mean value. The pH, conductivity, and alkalinity increased as hardness increased.

Table 4.2.b. Water chemistry values, Study #2

	Moderately Hard	Mean (± S.D.[a])	Hard	Mean (± S.D.[a])	Very Hard	Mean (± S.D.[a])
pH	7.9–8.1[b]	8.0 (± 0.05)	8.1–8.4	8.3 (± 0.08)	8.1–8.6	8.4 (± 0.1)
Dissolved Oxygen (mg/L)	6.9–7.4	7.2 (± 0.1)	7.0–7.3	7.2 (± 0.09)	7.0–7.4	7.2 (± 0.09)
Temperature (^{o}C)	18–19	18.2 (± 0.4)	18–19	18.1 (± 0.4)	18–20	18.8 (± 0.4)
Conductivity (µS)	331–360	342 (± 7.6)	561–591	574 (± 8.2)	811–995	910 (± 46)
Ammonia (mg/L)	<1.0–1.28	1.0 (± 0.09)	<1.0–1.10	1.0 (± 0.03)	<1.0–1.06	1.0 (± 0.02)
Hardness (mg/L $CaCO_3$)	99–108	102 (± 1.3)	170–187	175 (± 3.3)	218–276	245 (± 12)
Alkalinity (mg/L $CaCO_3$)	64	N/A	113	N/A	222	N/A

Water chemistry values were measured in new and old exposure solutions collected when the exposure solutions were changed on days 3, 5 and 7 (test termination). The dilution water was titrated once at test termination. Concentrations of calcium, magnesium and cobalt were analyzed by ICP-AES for total hardness calculations. Planaria were not fed during the study.
[a] Standard deviation
[b] Range of observed values.

The 7 day median lethal concentration of 50 mg Co/L calculated for Study #1 was significantly different from the LC_{50} values determined in Study #2 (Table 4.2.c.). The Least Significant Difference intervals did not overlap with any of those observed in Study #2.

Table 4.2.c. Median lethal concentrations from Study #1 and #2

	LC_{50} (mg Co/L)	95% Confidence Intervals	LSD[a] Intervals
Study #1	50	(44, 56)	(46, 54)
Study #2 100 mg CaCO$_3$/L	12	(10, 14)	(11, 13)
Study #2 180 mg CaCO$_3$/L	18	(17, 20)	(17, 19)
Study #2 320 mg CaCO$_3$/L	32	(26, 37)	(28, 36)

Median lethal concentrations were calculated using Spearman-Karber and Probit analysis. The LSD intervals were calculated based on the 95% confidence intervals.
[a]Least Significant Difference

4.3   Study #3

Another mortality study was performed at the standard acute aquatic testing duration of 96 hours (ASTM 2007) using higher cobalt concentration ranges to achieve a greater level of mortality at the earlier time point. The hardness contribution of cobalt was accounted for in this study as well. Mortality and median lethal concentrations (LC_{50}) were calculated and three behavioral responses were recorded as in Study #2.

Median lethal concentrations were calculated for each total hardness level. At a moderate level of hardness, the LC_{50} was 28 mg Co/L (Probit). As hardness increased, the 96 hour LC_{50} values also increased to 48 and 70 mg Co/L in hard and very hard water, respectively (Spearman-Karber and Probit, respectively) (Figure 4.3.a.). Two different statistical methods were used because there were two patterns of mortality. The Probit method is used if there are two or more non-control concentrations that have partial mortalities. The Spearman-Karber method is used when only one non-control concentration has a partial mortality or when the Probit method is inappropriate due to the calculated chi-square value. Based on the Least Significant Difference (LSD) intervals, these three LC_{50} values are all significantly different from each other (Table 4.3.a.). They are all also significantly different from their respective 168 hour LC_{50} values calculated in Study #2 (Figure 4.3.b.).
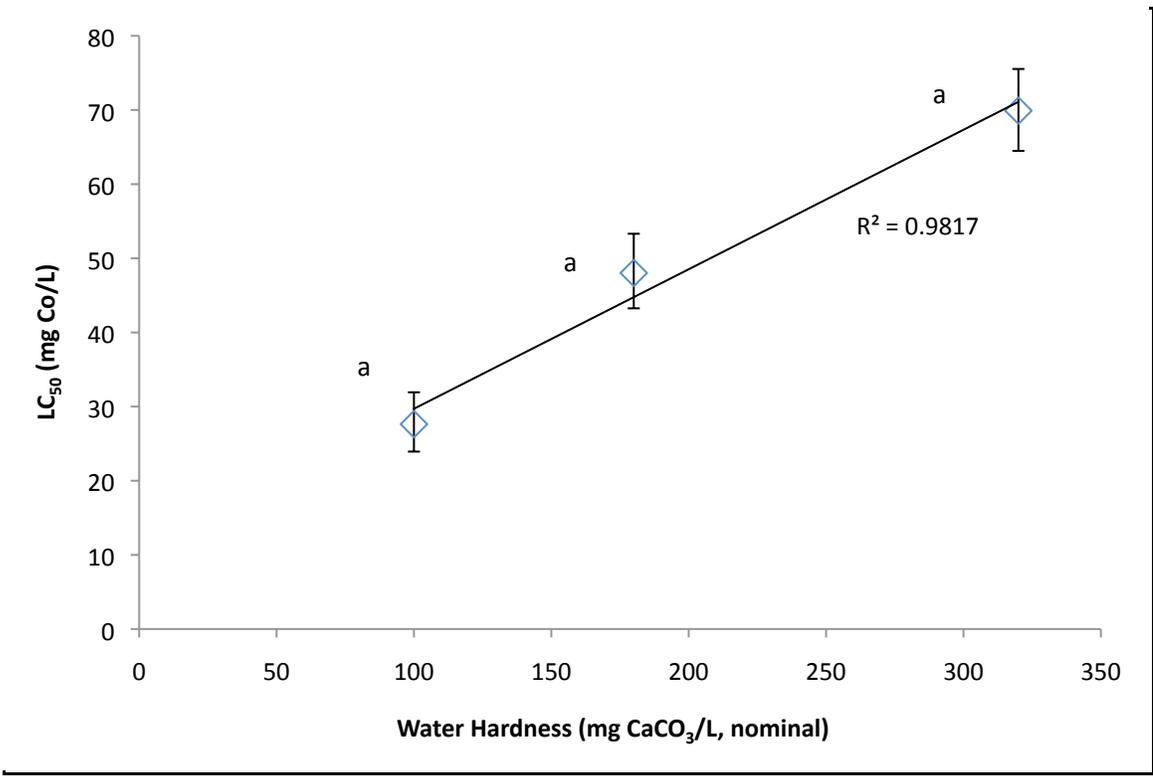
Figure 4.3.a. Median lethal cobalt concentrations to planaria at different water hardness levels, Study #3

Each level of hardness had 4 replicates, each consisting of 5 planaria per dish. Exposure solutions were changed daily after observations. The planaria were not fed during this study and were segregated in their corresponding dilution water 7 days prior to the start of the study. Spearman-Karber and Probit analysis yielded these median lethal concentrations. The error bars that are displayed represent the 95% confidence intervals.
[a] Significantly different from the other treatment groups based on LSD Intervals.
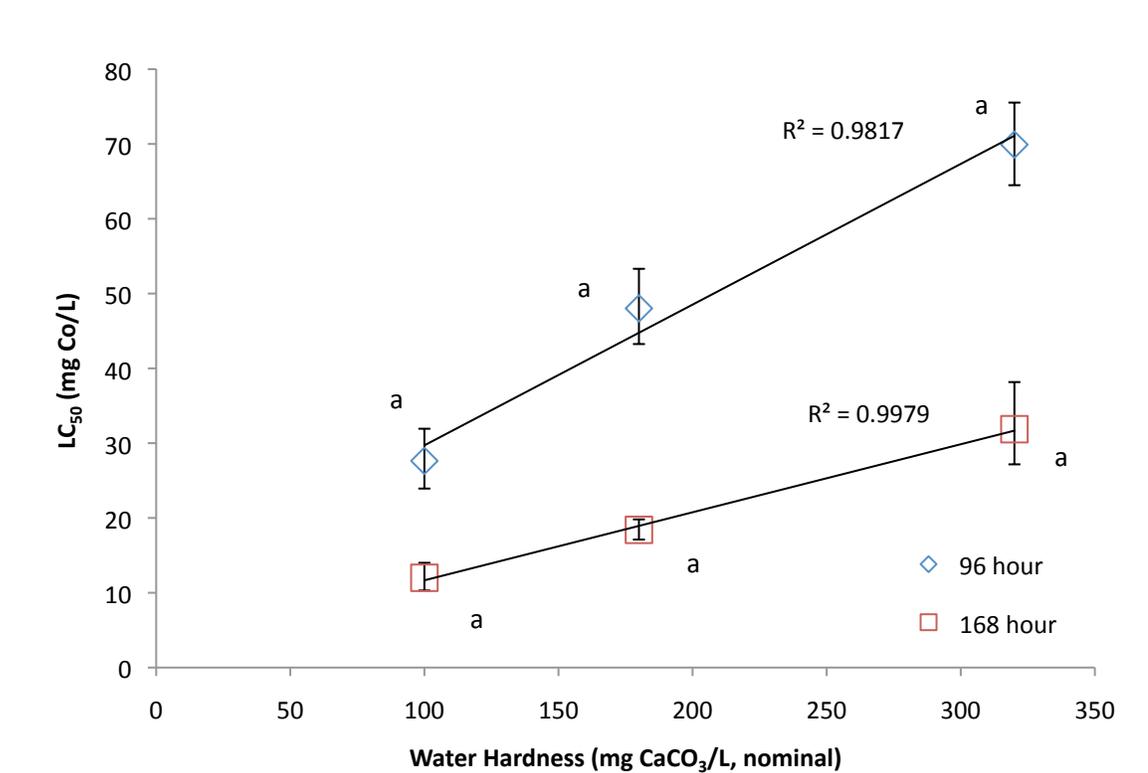
Figure 4.3.b. A comparison of LC_{50} values from Study #2 and Study #3

This comparison of median lethal concentrations is based on two studies, Study #2 and #3. Observations and water changes took place daily in the 96 hour study (Study #3) and on days 3 and 5 for the 168 hour study (Study #2). The error bars that are displayed represent the 95% confidence intervals.

[a] Significantly different from the other treatment groups based on LSD Intervals.

Table 4.3.a. Median lethal concentrations for three hardness levels in Study #3

	LC_{50} (mg Co/L)	95% Confidence Intervals	LSD Intervals[a]
100 mg $CaCO_3$/L	28	(24, 32)	(25, 31)
180 mg $CaCO_3$/L	48	(43, 53)	(44, 52)
320 mg $CaCO_3$/L	70	(64, 76)	(66, 74)

Spearman-Karber and Probit methods were used to calculate the median lethal concentrations. Based on the LSD Intervals, the LC_{50} values are significantly different from each other.

[a] Least Significant Difference

Three behavioral assessments: tactile response, righting reflex response and swimming speed were measured at test termination (96 hours) in each hardness level. One planarian was randomly selected from each surviving replicate. Similar to Study #2, as the cobalt concentration increased, fewer planaria were available for these assessments.

Moderately Hard

The tactile and righting reflex responses at 6 mg Co/L were not significantly different from the control (Tukey, $p > 0.5$) (Figure 4.3.c). Planaria in the other concentrations, 10.2, 17.3 and 29.5 mg Co/L were immobile during the one-minute observation time. (These data are not displayed on the graph.) The responses at these three concentrations of cobalt are significantly different from the control and 6 mg Co/L (Tukey, $p < 0.0001$). The swimming speed was significantly reduced compared to the control for all exposure solutions, 6, 10.2, 17.3 and 29.5 mg Co/L (Tukey, $p < 0.0002$). At 10.2, 17.3 and 29.5 mg Co/L, swimming speeds were significantly different from 6 mg Co/L (Tukey, $p < 0.0001$).

Hard

The tactile response was not significantly different in the two lowest exposure levels, 12 and 19 mg Co/L, compared to the control (Tukey, $p > 0.05$) (Figure 4.3.d.). At the remaining concentrations, 30.7 and 49.2 mg Co/L, tactile response times exceeded the one-minute observation time due to immobility and were significantly increased compared to the control (Tukey, $p < 0.0001$). Planaria responses to the righting reflex assessment were significantly different from the control at 12 and 19 mg Co/L (Tukey, $p < 0.0001$). Planaria in the two higher concentrations, 30.7 and 49.2 mg Co/L, also exceeded the one-minute observation time due to immobility and were significantly

44

different from the control, 12 and 19 mg Co/L (Tukey, p<0.0001). The swimming speed was not significantly decreased at 12 mg Co/L compared to the control (Tukey, p=0.96) but swimming speed in the remaining concentrations were significantly different from the control (Tukey, p<0.0001). At 19, 30.7 and 49.2 mg Co/L, swimming speeds were significantly different from 12 mg Co/L as well due to immobility (Tukey, p<0.02).

Very Hard

The tactile response was significantly impaired in all of the exposure levels with surviving planaria (50, 63.5 and 80.7 mg Co/L) compared to the control (Tukey, p<0.03) (Figure 4.3.e.). The righting reflex responses were different (Tukey, p<0.0001) from the control in all of the concentrations except the lowest, 50 mg Co/L (Tukey,p>0.06). At 63.5 and 80.7 mg Co/L, the planaria did not move during the one-minute observation time allotted for the tactile and righting reflex assessments and were thus significantly different from the responses at 50 mg Co/L (Tukey, p<0.0001). The swimming speed was reduced significantly in the three cobalt concentrations compared to the control (Tukey, p<0.0001).
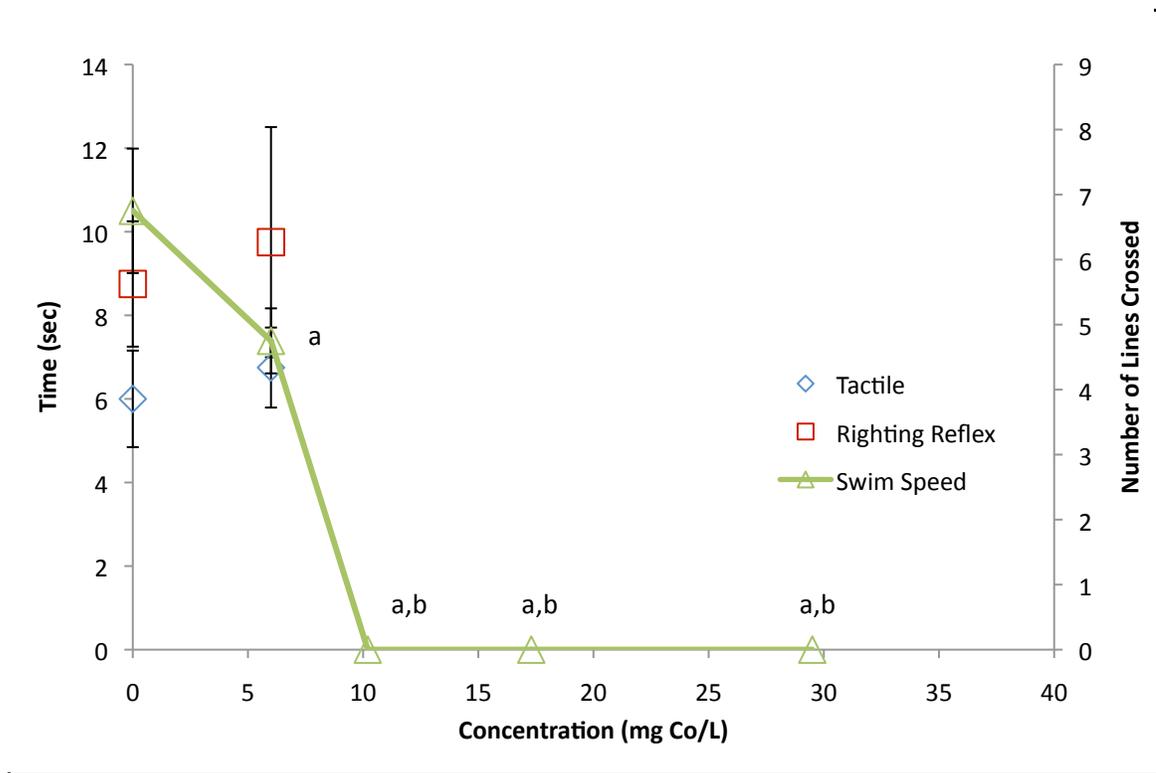
Figure 4.3.c. Planarian behavioral responses in moderately hard water (nominal, 100 mg $CaCO_3$/L) in Study #3

Symbols represent a mean value based on four replicates for all three assessments. These assessments were performed at test termination. For either of the timed responses, values are not shown if forward mobility was not regained within the one-minute observation time. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, p<0.0002 (Tukey)
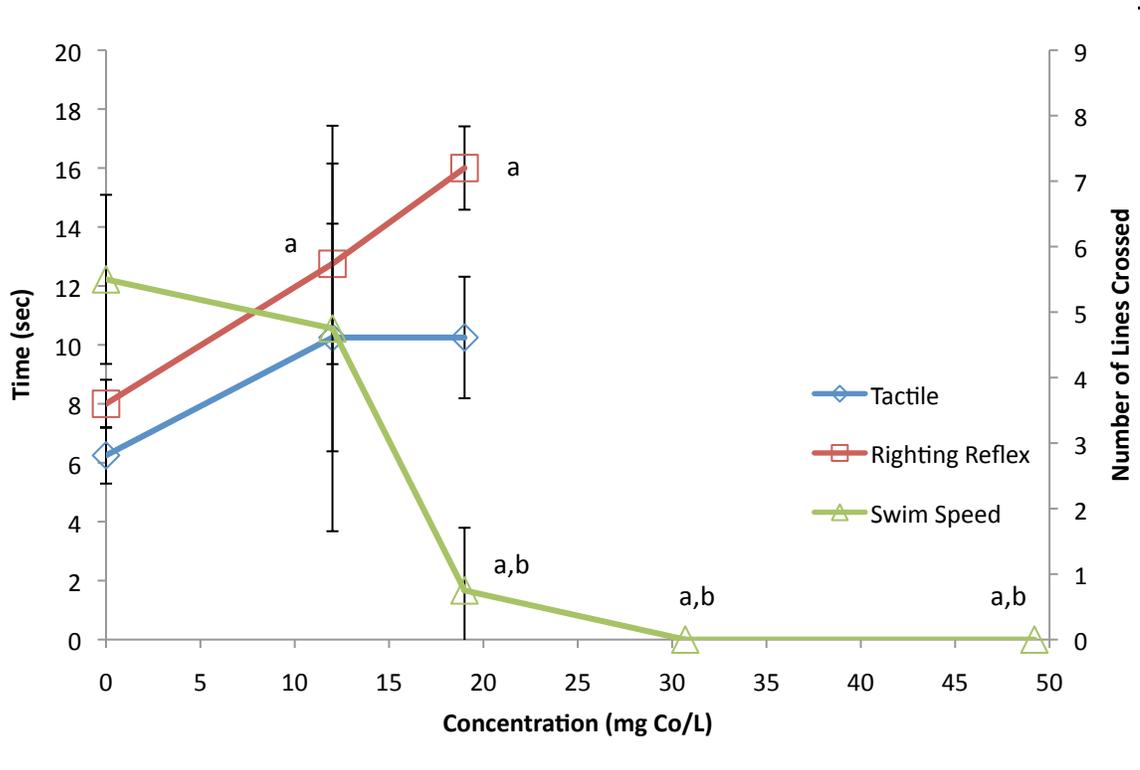[b] Significantly different from 6 mg Co/L, p <0.0001 (Tukey)

Figure 4.3.d. Planarian behavioral responses in hard water (nominal, 180 mg $CaCO_3$/L) in Study #3

Symbols represent a mean value based on four replicates for all three assessments. These assessments were performed at test termination. For either of the timed responses, values are not shown if forward mobility was not regained within the one-minute observation time. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, p<0.0001 (Tukey)
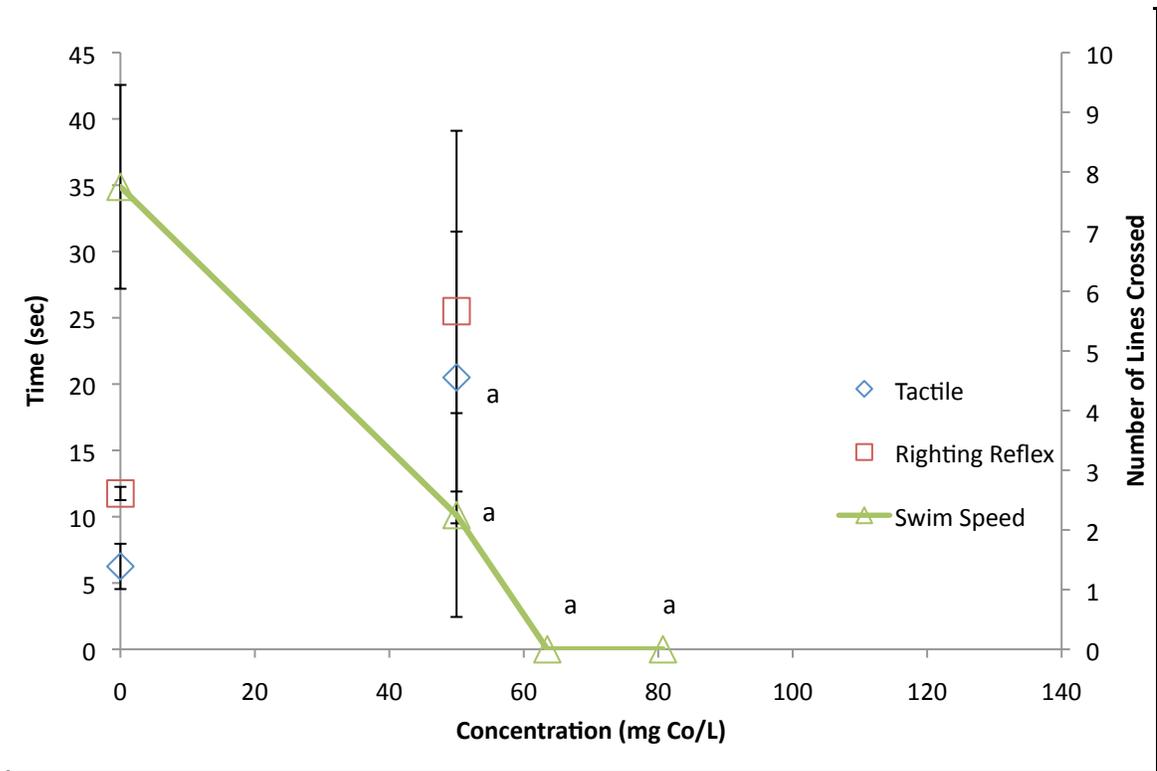[b] Significantly different from 12 mg Co/L, p <0.02 (Tukey)

Figure 4.3.e. Planarian behavioral responses in very hard water (nominal, 320 mg CaCO$_3$/L) in Study #3

Symbols represent a mean value based on four replicates for all three assessments. These assessments were performed at test termination. For either of the timed responses, values are not shown if forward mobility was not regained within in the one-minute observation time. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.

[a] Significantly different from the control, p<0.03 (Tukey)

The measured cobalt concentrations were compared to the nominal concentrations in Study #3 (Table 4.3.b.). Initial exposure solution samples were preserved once at the beginning of the test and old water samples were preserved daily for ICP-AES analysis. The reported mean values include both the initial and old exposure solutions. No significant variation in the concentrations was found in any level of hardness (Paired t-test, p>0.07). Variation in measured concentrations (except as noted below) was less than 13 % and mean values were within 13% of the nominal ones. One outlying value at 63.5 mg Co/L (very hard water) cannot be explained by any chemical nor biological reason. One possibility would be due to an error committed by the technician during the preservation process. The statistical conclusion is the same whether or not this value is included.

Table 4.3.b. Nominal concentrations of cobalt compared to the measured concentrations, Study #3

	Nominal (mg Co/L)	Mean (±S.D. [a])	Range
Moderately Hard	0	0.1 (± 0.0)	0.0–0.10
	6	6.0 (± 0.3)	5.6–6.3
	10.2	10.8 (± 0.1)	10.5–10.9
	17.3	18.5 (± 1.1)	16.7–19.5
	29.5	30.8 (± 0.9)	29.7–32.0
	50.1	50.3 (± 0.3)	50.1–50.6
Hard	0	0.1 (± 0.2)	0.0–0.4
	12	12.9 (± 0.2)	12.6–13.1
	19	20.7 (± 0.3)	20.2–20.9
	30.7	32.2 (± 0.7)	31.2–32.8
	49.2	49.9 (± 0.9)	49.1–51.1
	78.6	77.8 (± 2.3)	76.2–79.4
Very Hard	0	0.1 (± 0.0)	0.0–0.1
	50	46.7 (± 2.5)	43.5–50.4
	63.5	70.2 (± 21.9)	57.4–109.1[b]
	80.7	77.4 (± 11.6)	66.2–95.2
	102	89.0 (± 11.3)	80.1–101.8
	129	119.7 (± 12.9)	110.5–128.8

The actual values of cobalt are based on mean values of four replicates. Initial samples were preserved once at the beginning of the test and old water samples were preserved daily. Analysis was done by ICP-AES and the reported mean values include both the initial and old exposure solutions. No significant differences were observed in the analysis ($p > 0.07$) (Paired t-test).

[a] Standard deviation

[b] One outlying value in the 63.5 mg Co/L nominal exposure level was collected on the third day of the study when no unusual death or change in water chemistry values occurred.

The water chemistry values (Table 4.3.c), measured in initial and old exposure solutions, collected daily at each water change, show trends similar to those in Table 4.2.b. in Study #2. The pH values and conductivities generally increased as the hardness level increased. The ammonia concentrations remained low and the dissolved oxygen concentrations were at least 75% of saturation. The measured hardness was within 9% of the mean value for moderately hard water, 24% for hard water and 15% for very hard water.

Table 4.3.c. Water chemistry values for three hardness levels, Study #3

	Moderately Hard	Mean (± S.D. [a])	Hard	Mean (± S.D. [a])	Very Hard	Mean (± S.D. [a])
pH	7.9–8.2 [b]	8.0 (± 0.09)	8.1–8.6	8.3 (± 0.09)	8.2–8.6	8.3 (± 0.12)
Dissolved Oxygen (mg/L)	7.4–7.7	7.6 (± 0.09)	7.4–7.7	7.6 (± 0.09)	7.4–7.7	7.5 (± 0.09)
Temperature (^{o}C)	17	17 (± 0)	17–18	17.1 (± 0.3)	17–18	17.4 (± 0.5)
Conductivity (µS)	345–362	352 (± 4.8)	559– 612	584 (± 11)	848–1032	944 (± 50)
Ammonia (mg/L)	< 1.0	<1.0 (± 0)	<1.0	<1.0 (± 0)	< 1.0	<1.0 (± 0)
Hardness (mg/L $CaCO_3$)	98–114	108 (± 2.2)	173–200	153 (± 6)	261–332	305 (± 19)
Alkalinity (mg/L $CaCO_3$)	63	---	109	---	222	---

Values represent measurements of initial and old exposure solutions. Hardness and alkalinity were measured at the beginning of the study by titration of the control and match waters. Calculated hardness values reported in this table were based on analysis by ICP-AES in preserved samples collected daily throughout the study.
[a] Standard deviation
[b] Range of observed values.

4.4   Study #4

Based on the results from studies #1-3, a 60 day chronic study was conducted at the

most sensitive hardness level, moderate hardness, to assess sublethal effect of cobalt on

planaria. Total hardness, including the contribution of cobalt, was held constant by preparing a

working stock of cobalt solution in a hardness "match" water prepared based on the desired

total hardness. Observations and water changes were performed twice weekly. Once a week,

the planaria were fed organic beef liver for four to six hours. Observations on these days were

made prior to the feeding times and water changes occurred after the feeding time ended. A

daily check was performed to segregate any fragments if a fissioning event had occurred. Mean

mortality over the entire study was calculated based on the number of intact planaria. Mortality

of the segregated fragments was also assessed. The number of fissioning events and the time it

took for a head or tail fragment to regenerate was analyzed. A head fragment is defined as a

planarian that needed to regenerate a new tail following fissioning and a tail fragment is a

planarian that must regenerate a new head. Behavioral assessments were performed as well.

Complete mortality occurred at the highest concentration, 16 mg Co/L, by the eleventh

day of the study (Figure 4.4.a.). Partial mortality was observed at 4 and 8 mg Co/L and no

mortality occurred at the two lowest concentrations (1 and 2 mg Co/L). Based on four replicates,

only the mortality that was observed at 8 and 16 mg Co/L was significantly different from the

control and lower concentrations (Tukey, $p<0.0001$). Therefore, 4 mg Co/L was the highest

concentration that caused no effect on mortality and 8 mg Co/L was the lowest concentration

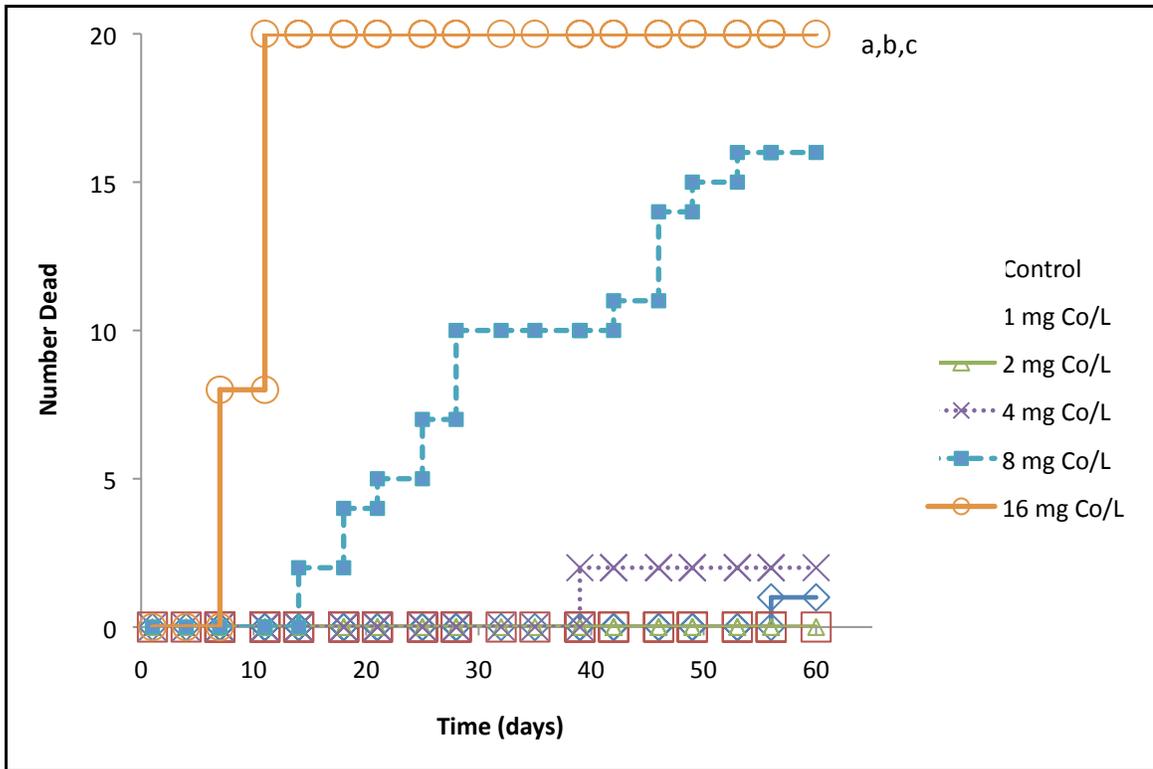that was observed to have an effect on mortality.

Figure 4.4.a. Planaria mortality by day, Study #4

Mortality was recorded based on the number of intact worms in each dish twice a week. Planaria were fed once a week and water changes occurred twice a week. Symbols represent the total number of planaria that died in four replicates. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, p<0.0001 (Tukey)
[b] Significantly different from 1, 2 and 4 mg Co/L, p <0.0001 (Tukey)
[c] Significantly different from 8 mg Co/L, p <0.0001 (Tukey)

Reproduction is critical for species sustainability and *D. dorotocephala* must fission to maintain and increase their population density. Fissioning events were significantly reduced within the first 28 days of the study and also during the entire length of the study (0-60 days) (Table 4.4.a.). During the initial 28 days, the mean number of fissioning events, based on four replicates, at 1, 2 and 4 mg Co/L was significantly different from the control (Tukey, p<0.04).

During the entire length of the study, the number of fissioning events at 1, 2, 4 and 8 mg Co/L were all significantly different from the control (Tukey, p<0.0001). Thus, the lowest observable adverse effect concentration (LOAEC) in this study was 1 mg Co/L.

Table 4.4.a. Mean fissioning events

	28 days	60 days
0	3.5 ± 1.9 [a]	8 ± 1.7 [a]
1 mg Co/L	0.75 ± 0.96 [a,b]	2.8 ± 0.5 [a,c]
2 mg Co/L	1 ± 0.82 [a,b]	2 ± 0.82 [a,c]
4 mg Co/L	0.5 ± 0.58 [a,b]	0.5 ± 0.58 [a,c,d]
8 mg Co/L	1.2 ± 1.2 [a]	1.2 ± 1.2 [a,c]

Observations to check for fissioning events in this 60 day study occurred daily. Mean fissioning rates are based on four replicates during the first 28 days. Thereafter, mortality in the highest concentration (8 mg Co/L) lowered the number, eventually to one. The planaria were fed once a week and the exposure solutions were changed twice a week. The concentrations of cobalt are nominal values.
[a] Mean ± one standard deviation
[b] Significantly different from control, p<0.04 (Tukey)
[c] Significantly different from control, p<0.0001 (Tukey)
[d] Significantly different from 1 mg Co/L, p<0.04 (Tukey)

Based upon results with exposure to the lowest concentration of added cobalt, a No Observed Adverse Effect Concentration (NOAEC) could not be determined. Because the mean cobalt concentration measured in the control exposure water was 0.0086 mg Co/L, this could be considered the lower bound of the NOAEC.

Table 4.4.b. Nominal concentrations of cobalt compared to the measured concentrations, Study #4

	Nominal (mg Co/L)	Mean (±S.D. [a])	Range
Moderately Hard	0	0.0086 (± 0.0098)	0.0018–0.041
	1	1.06 (± 0.0547)	0.999–1.22
	2	2.15 (± 0.0795)	1.98–2.34
	4	4.31 (± 0.152)	4.02–4.59
	8	8.30 (± 0.3970)	6.88–9.09
	16	16.9 (± 0.467)	16.3–17.4

The measured values of cobalt are based on mean values of four replicates. Initial samples were preserved once a week and old composited water samples were preserved twice a week. Analysis was done by ICP-AES and the reported mean values include both the initial and old exposure solutions. No significant differences between measured and nominal concentrations were observed in the analysis ($p>0.58$) (Paired t-test)
[a] Standard Deviation

Head fragment mortality in the cobalt exposure solutions was not significantly different compared to the fragment mortality in the control but there was a difference in mortality of the tail fragments (linear regression, $p=0.12$ and <0.01 respectively) (Figure 4.4.b.). Significant increase in tail fragment mortality was observed at 4 and 8 mg Co/L compared to the control.
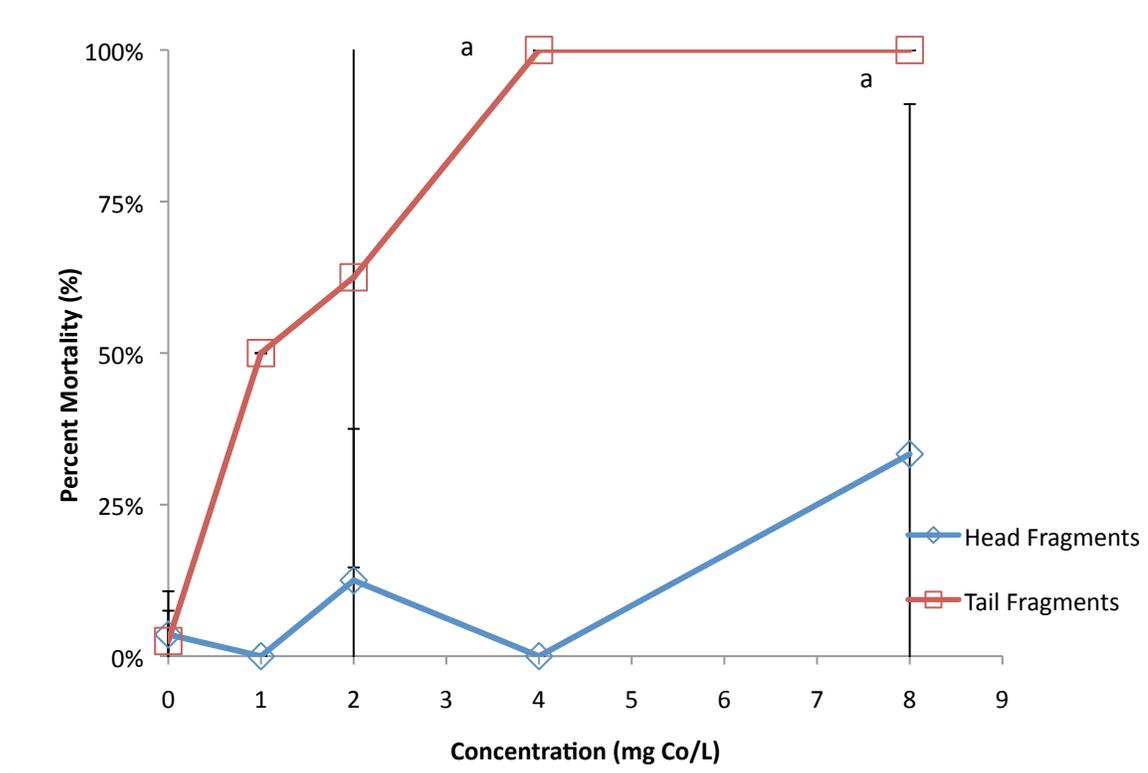
Figure 4.4.b. Percent mortality of head and tail fragments, Study #4

Symbols represent a mean value based on 32 planaria for the control, 10 for 1 mg Co/L, seven for 2 mg Co/L, 2 for 4 mg Co/L and 5 for 8 mg Co/L. Mortality of fragments were recorded on a daily basis. The head fragments were fed while segregated once a week after observations occurred. An arcsine square root transformation was applied to the data for statistical analysis. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from control, p<0.02 (Tukey)

Regeneration of the head fragments occurred more frequently than of the tail fragments. Fewer tail fragments actually survived. The time for a head regeneration at 1 and 2 mg Co/L was not significantly different from the control (One-way ANOVA, Tukey, p>0.16) (Figure 4.4.c.). However, the time for tail regeneration of a head fragment was significantly different (One-way ANOVA, p<0.0002) (Figure 4.4.d.). At 8 mg Co/L, the tail regeneration rate was significantly different from the control and all of the lower concentrations (Tukey, p<0.003).
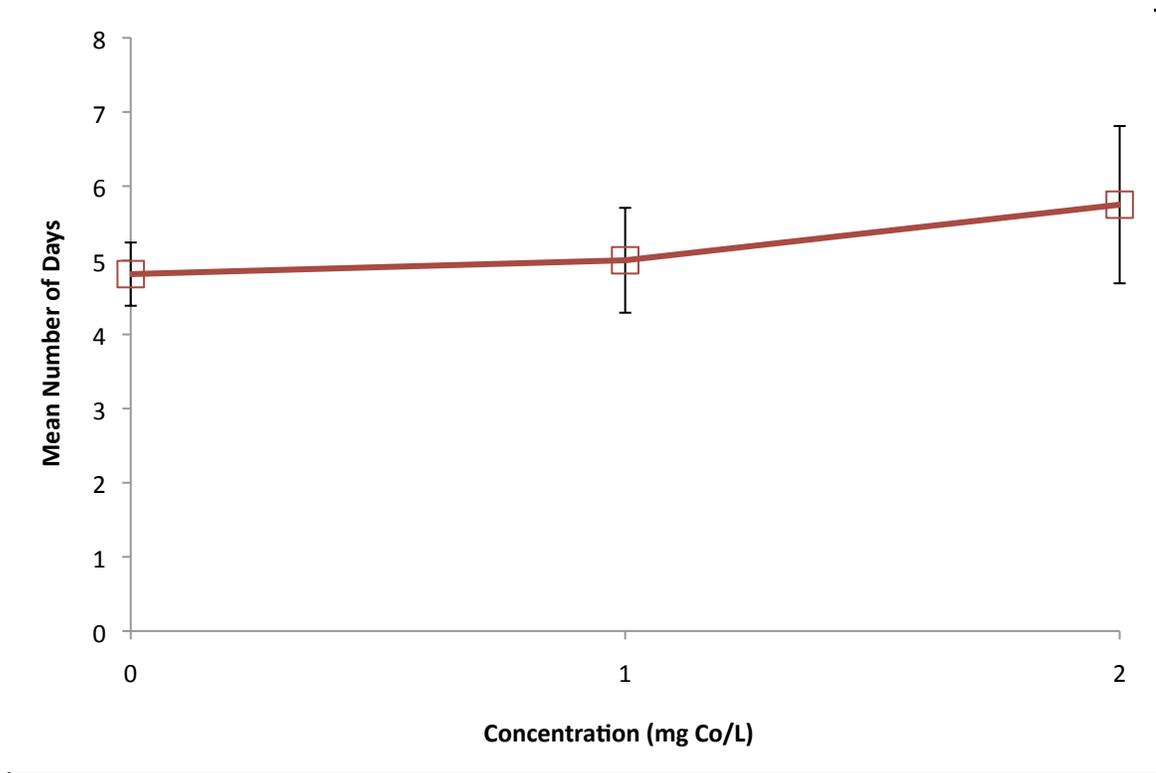
Figure 4.4.c. Regeneration of a head in tail fragments in Study #4

Symbols represent a mean value based on 32 planaria for the control, 10 for 1 mg Co/L, seven for 2 mg Co/L, 2 for 4 mg Co/L and 5 for 8 mg Co/L. Mortality of fragments was recorded on a daily basis. There were no significant differences in the number of days a tail fragment needed to regenerate a head (Tukey, $p > 0.3$). The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
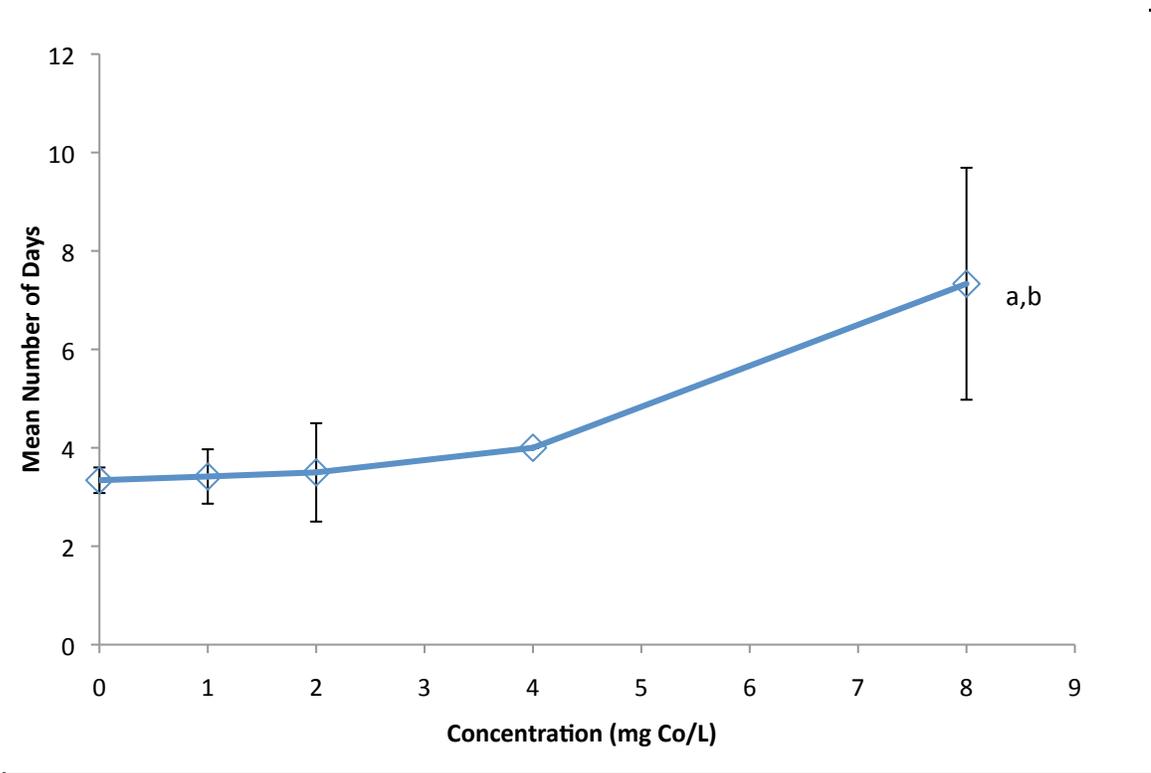
Figure 4.4.d. Regeneration of a tail in head fragments in Study #4

Symbols represent a mean value based on 32 planaria for the control, 10 for 1 mg Co/L, seven for 2 mg Co/L, 2 for 4 mg Co/L and 5 for 8 mg Co/L. Mortality of fragments was recorded on a daily basis. The tail fragments were not fed while segregated. The error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.
[a] Significantly different from control, $p<0.003$ (Tukey)
[b] Significantly different from 1 and 2 mg Co/L, $p<0.03$ (Tukey)

Three behavioral assessments, tactile response, righting reflex response and swimming speed, were measured every two weeks and at test termination (days 7, 21, 35, 49 and 60). One planarian was randomly selected from each surviving replicate. As the concentration of cobalt increased and more adverse effects were observed (ruffled edges, lesions, mortality etc), fewer planaria were available for these assessments.

In all three assessments, the number of days that passed during the study did not influence the results. The exposure to cobalt significantly affected the responses (Two-way ANOVA, $p<0.001$). There were no statistical differences in the tactile (Figure 4.4.e.) or the righting reflex (Figure 4.4.f.) responses at the three lowest concentrations (1, 2 and 4 mg Co/L) (Tukey, $p>0.05$). At 8 mg Co/L, the planarian exceeded the one-minute observation time for the tactile and the righting reflex responses on days 49 and 60. At 4, 8 and 16 mg Co/L, the swimming speeds were significantly different from the control (Tukey, $p<0.0003$). At 4 mg Co/L, the swimming speed was also significantly different from planaria exposed to 1, 2, 8 and 16 mg Co/L (Tukey, $p<0.0001$). At 8 and 16 mg Co/L, the swimming speed was lower than at 1, 2 and 4 mg Co/L (Tukey, $p<0.0001$) (Figure 4.4.g.). The lowest concentration where no effect on swimming speed was observed was at 2 mg Co/L but the lowest effective concentration was 4 mg Co/L for tactile and righting reflex responses (Figure 4.4.h.).
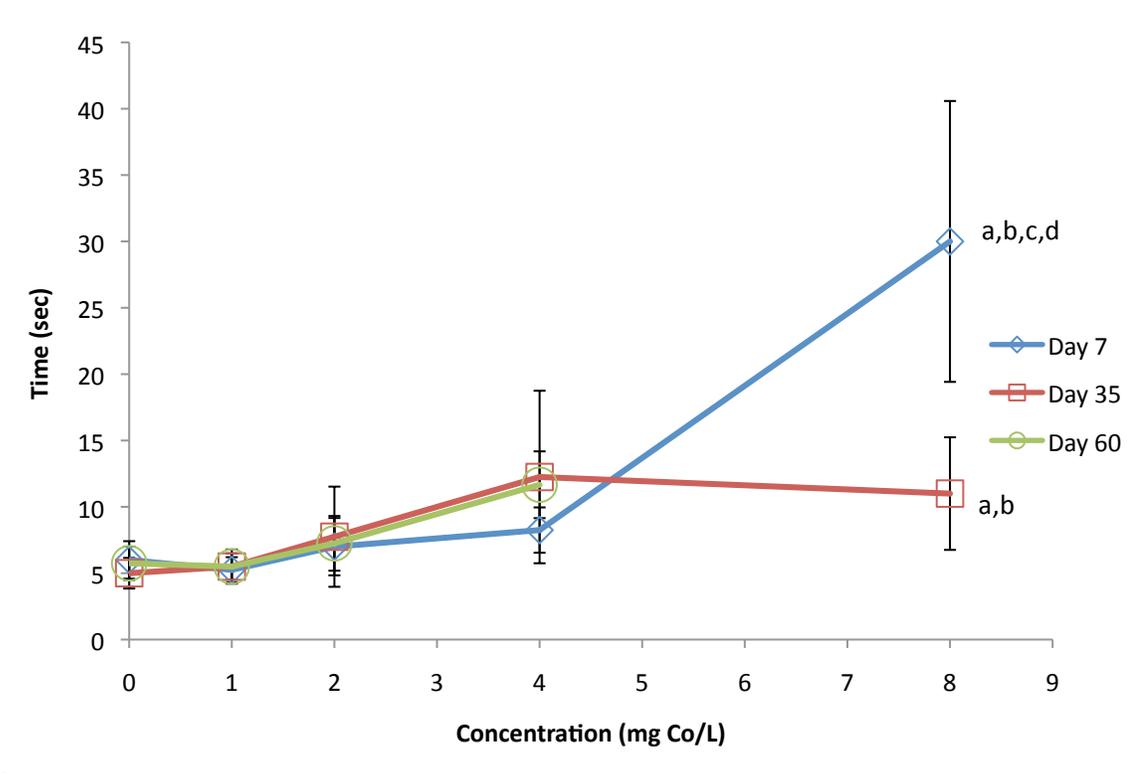
Figure 4.4.e. Planarian behavioral assessment in Study #4: Tactile Response

Symbols represent a mean value based on four replicates in all exposure solutions for Day 7. A mean value based on four replicates at 8 mg Co/L is displayed on Day 35 but only one replicate at 8 mg Co/L is displayed on Day 60. These assessments were performed every two weeks but only data from the beginning, middle and end of the study are shown. The error bars that are displayed represent one standard deviation. Values are not shown for 16 mg Co/L at Day 7, prior to complete mortality in this concentration, or for 8 mg Co/L at Day 60 because forward mobility was not regained in the one-minute observation time. The concentrations of cobalt are nominal values.
[a] Significantly different from the control, $p < 0.04$ (Tukey)
[b] Significantly different from 1 mg Co/L, $p < 0.04$ (Tukey)
[c] Significantly different from 2 mg Co/L, $p < 0.02$ (Tukey)
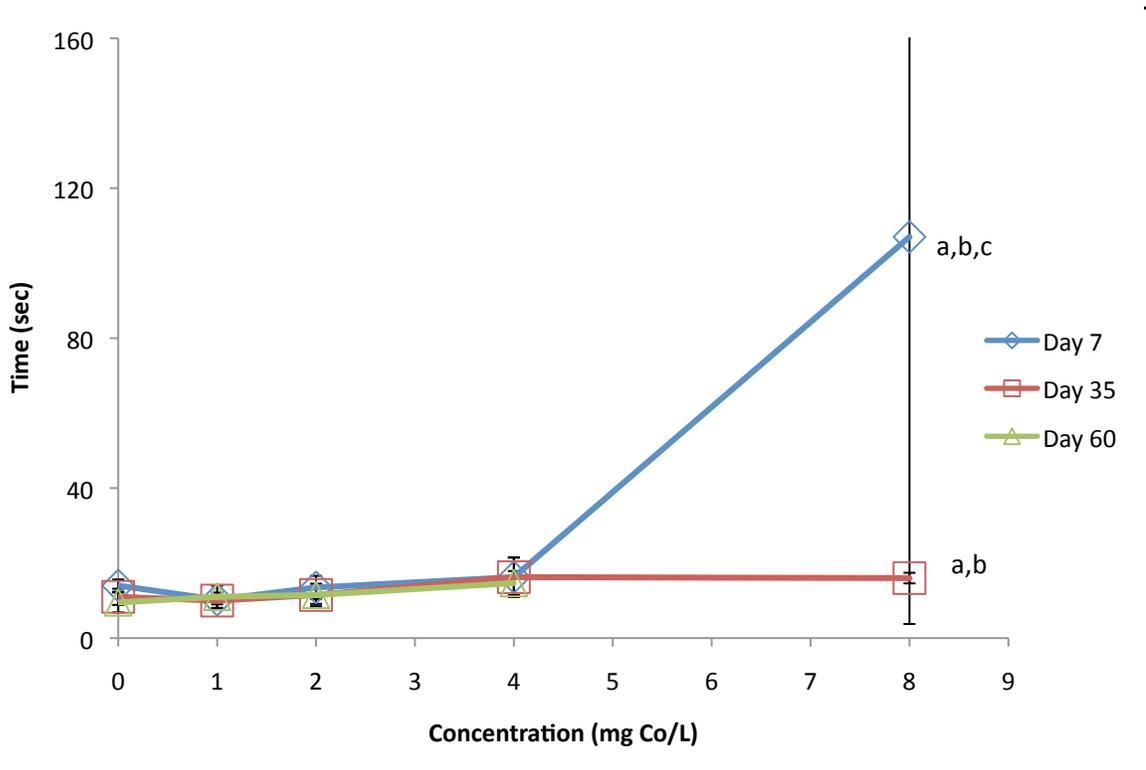[d] Significantly different from 4 mg Co/L, $p < 0.003$ (Tukey)

Figure 4.4.f. Planarian behavioral assessment in Study #4: Righting Reflex Response

Symbols represent a mean value based on four replicates in all exposure solutions for Day 7. A mean value based on four replicates at 8 mg Co/L is displayed on Day 35 but only one replicate at 8 mg Co/L is displayed on Day 60. These assessments were performed every two weeks but only data from the beginning, middle and end of the study are shown. Values are not shown for 16 mg Co/L at Day 7 or for 8 mg Co/L at Day 60 because forward mobility was not regained in the one-minute observation time. Two replicates at 8 mg Co/L exceeded the observation time as well on Days 7 and 35. The concentrations of cobalt are nominal values. The error bars that are displayed represent one standard deviation.

[a] Significantly different from the control, p<0.04 (Tukey)
[b] Significantly different from 1 and 2 mg Co/L, p <0.05 (Tukey)
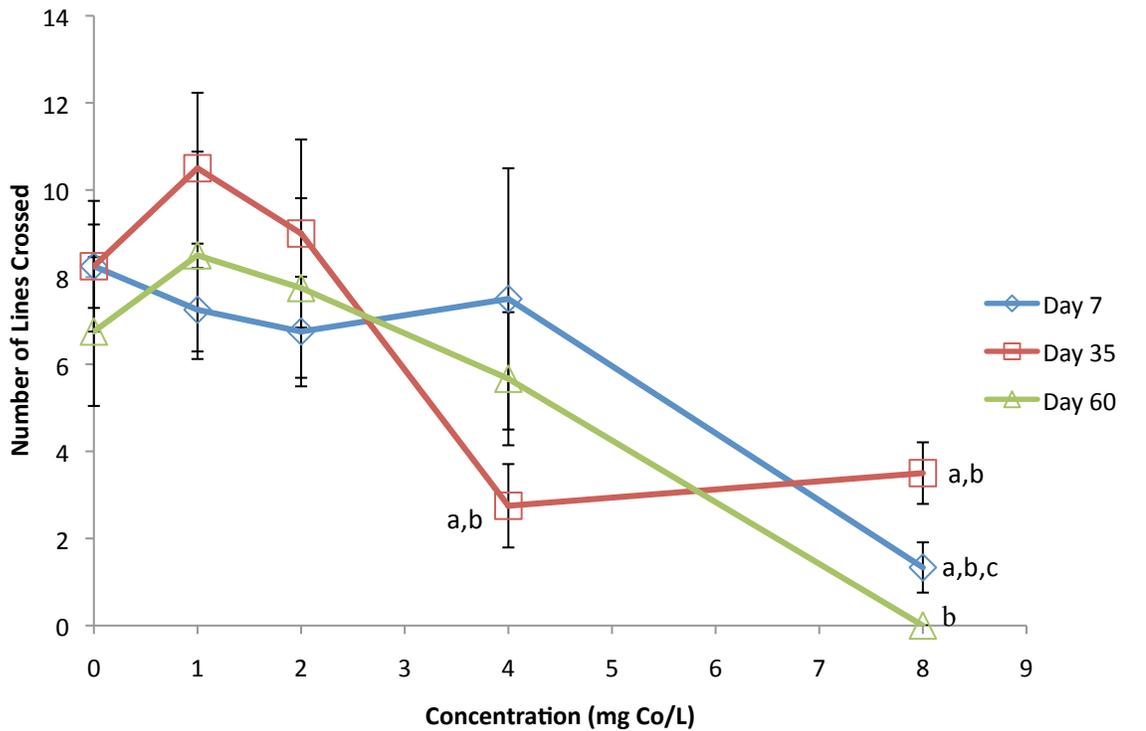[c] Significantly different from 4 mg Co/L, p <0.0001 (Tukey)

Figure 4.4.g. Planarian behavioral assessment in Study #4: Swimming Speed

Symbols represent a mean value based on four replicates in all exposure solutions for Day 7. A mean value based on four replicates at 8 mg Co/L is displayed on Day 35 but only one replicate at 8 mg Co/L is displayed on Day 60. These assessments were performed every two weeks but only data from the beginning, middle and end of the study are shown. Data for 16 mg Co/L are not shown because no planarian in any replicate crossed any lines during the two-minute observation time on Day 7 and, due to complete mortality, no data were available on the subsequent days. Error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.

[a] Significantly different from the control, $p < 0.004$ (Tukey)

[b] Significantly different from 1 and 2 mg Co/L, $p < 0.04$ (Tukey)

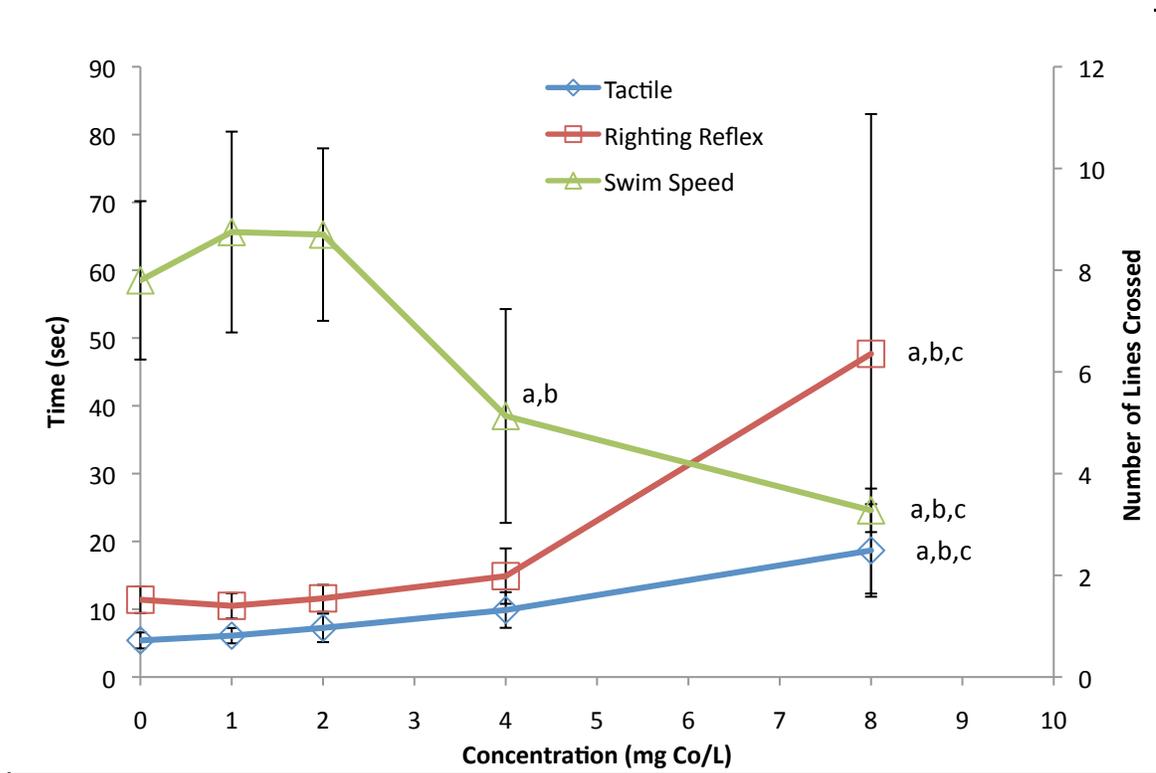[c] Significantly different from 4 mg Co/L, $p < 0.0002$ (Tukey)

Figure 4.4.h. Mean responses of three planarian behavioral assessments on days 7, 21, 35, 49 and 60, Study #4

These assessments were performed every two weeks. Symbols represent a mean value based on four replicates for the control, 1, 2 and 4 mg Co/L. The mean value of 8 mg Co/L began at four replicates but changed throughout the study and eventually was reduced to one replicate at the end of the study. Data for 16 mg Co/L are not shown because no planarian in any replicate regained forward mobility or crossed any lines during the observation periods on Day 7 and, due to complete mortality, no data were available on the subsequent days. Error bars that are displayed represent one standard deviation. The concentrations of cobalt are nominal values.

[a] Significantly different from the control, p<0.004 (Tukey)
[b] Significantly different from 1 and 2 mg Co/L, p <0.04 (Tukey)
[c] Significantly different from 4 mg Co/L, p <0.0002 (Tukey)

The water chemistry values in Table 4.4.c. vary slightly more in the pH, dissolved oxygen, conductivity and hardness compared to the moderate level of hardness in studies #2 and #3. The initial titrations of the dilution and match waters were within 10% of the nominal concentrations. The measured hardness was within 17% of the mean value.

Table 4.4.c. Water chemistry values for Study #4

	Range	Mean (± S.D.[a])
pH	7.1–8.8	8.0 (± 0.2)
Dissolved Oxygen (mg/L)	6.5–8.5	7.5 (± 0.4)
Temperature (^{o}C)	16–18	17.1 (± 0.7)
Conductivity (µS)	273–485	362 (± 38)
Ammonia (mg/L)	<1.0–2.6	1.4 (± 0.4)
Hardness (mg/L $CaCO_3$)	105–130	108 (± 9)
Alkalinity (mg/L $CaCO_3$)	65–70	68 (± 1.5)

Water chemistry values were measured twice a week during this 60 day study based on initial and old exposure solutions. Hardness and alkalinity were measured weekly by titration of the control and match waters. Hardness values reported in this table were based on ICP-AES analysis from preserved samples that were collected twice a week throughout the study.
[a] Standard deviation

4.5   Study #5

To better understand the role of hardness in cobalt mortality, a 7 day mortality study was conducted with varying calcium and magnesium concentrations, with total hardness maintained at a constant level (100 mg CaCO$_3$/L) and a single cobalt concentration of 10 mg Co/L. To verify the total hardness effect, an extra exposure group at 180 mg CaCO$_3$/L and 10 mg Co/L was included at the standard cation concentrations: 0.576 mmol Mg/L and 0.399 mmol Ca/L. To ensure the calcium and magnesium levels were not too high or too low to sustain planaria, concurrent controls were performed at a moderate hardness level without cobalt (Test A Control and Test B Control).

Complete mortality did not occur in this study but Test A (low Ca, high Mg) showed the greatest amount of toxicity. This exposure had significantly reduced survival compared to every other control or exposure solution (Tukey, p<0.04) (Table 4.5.a.). Mortality occurred first in this exposure group (low Ca, high Mg) as well (Figure 4.5.a.). Mortality was observed in the dilution control water (5%) but was not significantly different compared to the concurrent controls (Test A Control and Test B Control). The exposure solution that contained the standard concentrations of calcium and magnesium taking into account 10 mg Co/L (Cobalt/Match) had significantly different mortality from the dilution control water (Tukey, p<0.04). Test B (high Ca, low Mg) achieved mortality that was significantly different compared to Test B Control (Tukey, p<0.04). A mean of 10.0 mg Co/L and standard deviation 0.87 was measured in the preserved exposure samples by ICP-AES.

Table 4.5.a. Percent mortality of planaria at varying cation concentrations, Study #5

	Calcium (mg/L)	Magnesium (mg/L)	Total Hardness (mg CaCO$_3$/L)	Cobalt (mg/L)	Mean Percent Mortality	Standard Deviation
Control	16	14	100	0	5%	0
Cobalt/Match	14	12	100	10	35%[a]	10%
Test A	2	19	100	10	95%[b]	10%
Test B	30	2	100	10	30%[c]	26%
Test C	27	24	180	10	5%	10%
Test A Control	2	23	100	0	0	0
Test B Control	37	2	100	0	0	0

Mortality is based on a mean value of four replicates. Exposure solutions were changed on days 3 and 5. Observations were made daily after the initial 48 hours. Planaria were segregated seven days prior in their respective exposure solutions, without cobalt, and fed once during this time. They were not fed during the study. An arcsine square root transformation was applied to the data for statistical analysis. Cation and total hardness concentrations are nominal values.
[a] Significantly different from control, p<0.04 (Tukey)
[b] Significantly different from every control or exposure group, p<0.04 (Tukey)
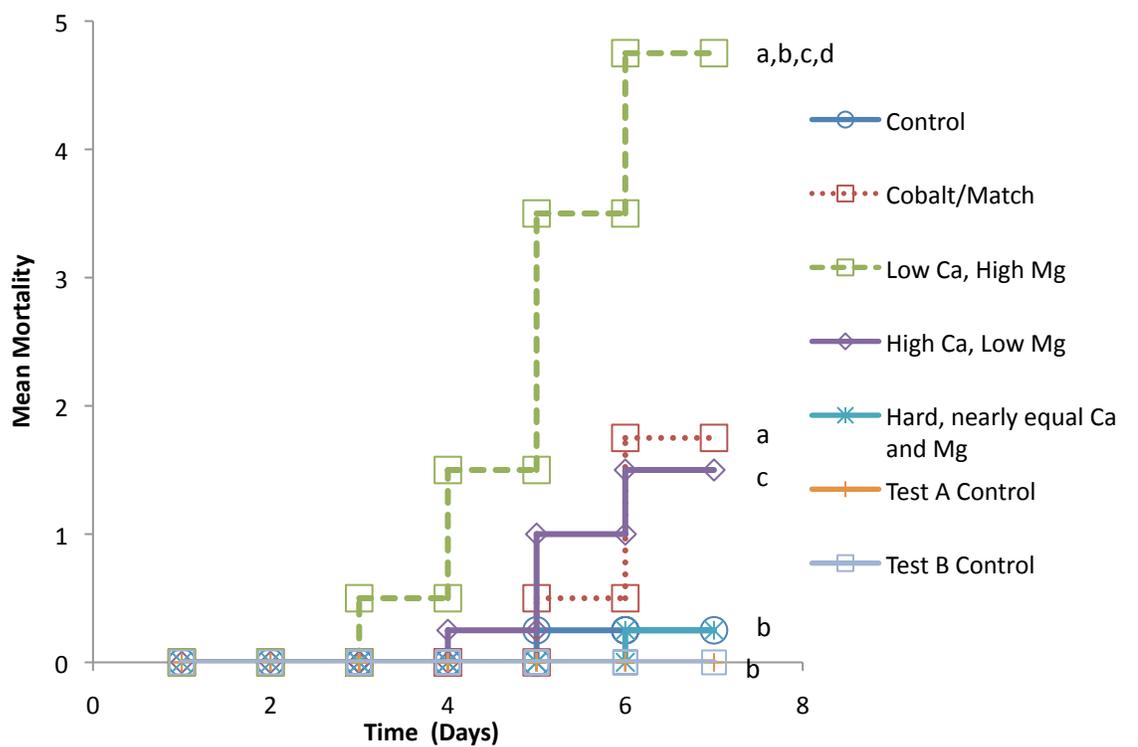[c] Significantly different from Test B control, p<0.04 (Tukey)

Figure 4.5.a. Mean mortality of planaria at varying cation concentrations, Study #5

Total hardness was maintained at a constant level, 100 mg $CaCO_3$/L, and cobalt concentration was maintained at 10 mg Co/L. The ratio of calcium and magnesium concentrations varied for the exposure groups low calcium/high magnesium and high calcium/low magnesium. To verify the total hardness effect, an extra exposure group at 180 mg $CaCO_3$/L and 10 mg Co/L was included. Mortality is based on a mean value of four replicates. Exposure solutions were changed on days 3 and 5. Observations were made daily after the initial 48 hours. Planaria were segregated seven days prior in their respective exposure solutions, without cobalt, and fed once during this time. They were not fed during the study. An arcsine square root transformation was applied to the data for statistical analysis.

[a] Significantly different from Control, $p < 0.04$ (Tukey)
[b] Significantly different from Cobalt/Match, $p < 0.04$ (Tukey)
[c] Significantly different from Test A and Test B Control, $p < 0.04$ (Tukey)
[d] Significantly different from High Ca/Low Mg (Test B) and Hard (Test C), $p < 0.0001$ (Tukey)

Mean percent mortality decreased as calcium concentrations increased (Figure 4.5.b.).

When calcium was low (2 mg Ca/L), significant mortality occurred compared to the mortality

when calcium concentrations increased to 14 and 30 mg Ca/L (Tukey, $p < 0.04$). There was no

significant difference in mortality between the higher calcium concentrations (14 and 30 mg

Ca/L) (Tukey, p>0.97).
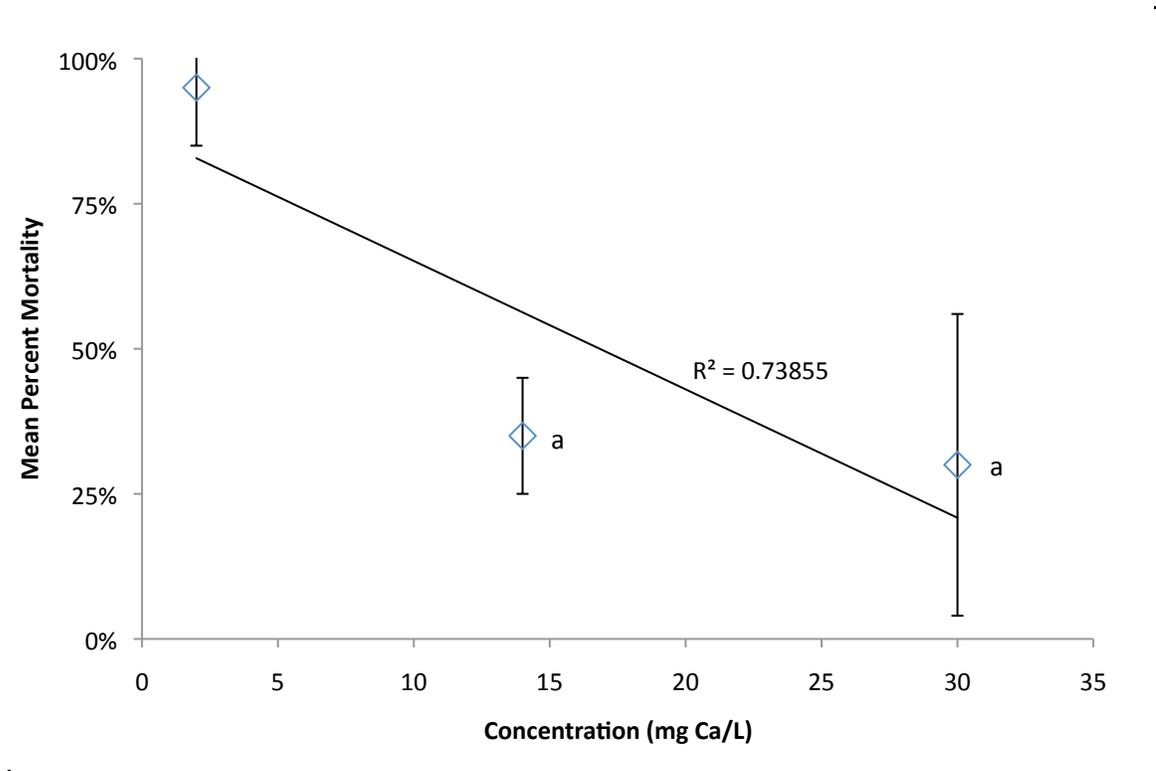
Figure 4.5.b. Mean percent mortality and calcium concentrations, Study #5

Mortality is based on a mean value of four replicates. Exposure solutions were changed on days 3 and 5. Observations were made daily after the initial 48 hours. Planaria were segregated seven days prior in their respective exposure solutions, without cobalt, and fed once during this time. They were not fed during the study. An arcsine square root transformation was applied to the data for statistical analysis. The error bars that are displayed represent one standard deviation. Calcium concentrations are nominal.
[a] Significantly different from the value at 2 mg Ca/L, p<0.04 (Tukey)

Water chemistry values for Study #5 are presented in Table 4.5.b. Except for ammonia, temperature and dissolved oxygen, all of the chemistry values increased as hardness increased. The measured hardness range was within 6% of the mean value.

Table 4.5.b. Water chemistry values for Study #5

	Moderately Hard	Mean (± S.D. [a])	Hard	Mean (± S.D. [a])
pH	8-8.3 [b]	8.2 (± 0.1)	8.3-8.4	8.4 (± 0.04)
Dissolved Oxygen (mg/L)	7.5-7.9	7.8 (± 0.09)	7.7-7.8	7.8 (± 0.05)
Temperature (^{o}C)	17-18	17.1 (± 0.31)	17-18	17.3 (± 0.52)
Conductivity (µS)	331-431	351 (± 17)	556-577	565 (± 8.5)
Ammonia (mg/L)	< 1.0	< 1.0 (± 0)	< 1.0	< 1.0 (± 0)
Hardness (mg/L CaCO$_3$)	102-109	108 (± 16)	183-188	166 (± 40)
Alkalinity (mg/L CaCO$_3$)	63-69	65 (± 2.6)	112	---

Total hardness was maintained at a constant level nominal, 100 mg CaCO$_3$/L, except for the hard exposure group (180 mg CaCO$_3$/L). Exposure solutions were changed on days 3 and 5. These values were obtained from old and new exposure solution values. The reported hardness values were calculated from ICP-AES analysis. Alkalinity was determined from titrations performed on each of the seven waters at test initiation. Planaria were segregated seven days prior in their respective exposure solutions, without cobalt, and fed once during this time. They were not fed during the study.
[a] Standard deviation
[b] Range of observed values.

The results of these experiments have identified lethal and relevant sublethal endpoints related to cobalt toxicity in the planarian, *D. dorotocephala*. Median lethal concentrations increased with total hardness indicating a protective effect regardless of the length of the study. Very hard water exhibited the lowest amount of toxicity in the presence of cobalt. Because total hardness is directly correlated to calcium concentrations, similar trends in mortality were seen in the acute and cation studies. As total hardness or calcium concentrations increased in the presence of cobalt, less mortality was observed. Calcium was shown in the cation study to be more essential of the two ions primarily responsible for hardness in uncontaminated waters (calcium and magnesium) in the presence of cobalt consistent with the biotic ligand model of the interaction with metal ion toxicity in aquatic organisms. (Di Toro 2001, McDonald 1989). The presence of more calcium ions may result in competition for the same binding site as cobalt ions.

I. Water Quality Parameters

Water chemistry values were consistent among the different hardness levels. Temperature fluctuated no more than the recommended 3°C differential (ASTM 1996) within each study. The range for all of the studies, 16-20°C, may have been too wide and could have altered the outcome by affecting the sensitivity to the effects of cobalt. However, in a sludge

worm, *T.tubifex*, temperature had little influence on acute cobalt toxicity (Rathore and Khangarot 2002). Future cobalt studies should be conducted with *D. dorotocephala* to evaluate their response to temperature variations.

II. Fissioning

Suppression in planarian reproduction was the most sensitive endpoint of toxicity when exposed to cobalt in a chronic time frame. This could ultimately influence the survival of *D. dorotocephala*. Despite concentrations of cobalt in environmental waters generally being less than 5 µg Co/L, cobalt has been found at levels up to 27 mg Co/L in industrial and mining areas (Nagpal 2004).

III. Behavioral Assessments

Considering all of the sublethal endpoints that were evaluated in these studies, planarian behavioral responses were the less sensitive to cobalt exposure. Limited data were collected, restricting the power of the tests. This could be studied in the future with more replicates and concentration levels to better evaluate the behavioral effects associated with cobalt toxicity. Variation between observers in the interpretation of the responses persisted despite limiting the number of observers involved in conducting the tests. Also, it was not possible to select the same planarian each time behavioral assessments were performed. That particular planarian could have been stronger or weaker compared to the rest of the population in the dish or could have perished. There is no way to determine if the selected planarian for each assessment was representative of effects at that exposure concentration. Stressors such as a declining population in a dish could have influenced the results. Finally, some planaria could

perform in the tactile and righting reflex tests but would not perform during the swimming assessment. This resulted in unequal sample sizes between the behavioral evaluations and weakened the interpretation of the results.

Future work may include segregating individual planaria for behavioral assessments for a given day and attempting to identify a potential time of the day when less stress (for example, stress from reproduction) would be occurring. Morita and Best (1984) describe a distinct influence of a photoperiod on fissioning. Based on their observations, fissioning rates increased with less than one hour of light. Thus, behavioral assessments may be altered if performed at night. Development of a way to follow the same worm throughout the exposure study would be ideal as well. Finally, increasing the number of replicates may allow for more data collection and a better detection of behavioral effects.

IV. Acute to Chronic Ratio

Establishing an acute to chronic ratio (ACR) for *D. dorotocephala* would be useful to estimate the maximum acceptable toxicant concentration (MATC) for other species of which only acute toxicity data are available (Kenaga 1982, EPA 1999). The practicality of estimating chronic toxicity from acute data is essential for agencies performing ecological risk assessments.

Chronic testing at moderate hardness for 60 days yielded a lowest observable adverse effect concentration (LOAEC) of 1 mg Co/L and a NOAEC (no observable adverse effect concentration) less than 1 mg Co/L based on the most sensitive endpoint, fissioning rate. This produced the lower bound of the ACR of 28 (Table 5.1.). Applying the concentration of cobalt as measured in the dilution water by ICP-AES, 0.0086 mg Co/L, the upper bound of the ACR was calculated to be 301. This background concentration of cobalt can be used because one would

not expect to have a much lower concentration of cobalt than that in an ambient water. Also,

this value is more indicative of cobalt concentrations that have been observed in the

environment (Nagpal 2004).

Table 5.1. Calculation of an acute to chronic ratio for cobalt toxicity in *D. dorotocephala*

	LC_{50} (mg Co/L)	NOAEC[b] (mg Co/L)	LOAEC[c] (mg Co/L)	Geometric Mean (mg Co/L)	ACR[a]
96 hour Study (Study #3)	28	<1	1	<1	>28
96 hour Study (Study #3)	28	0.0086	1	0.093	<301

The ACR is calculated by dividing the median lethal concentration from the short term study
(Study #3) by the geometric mean of the lowest and no observed adverse effect concentrations
(LOAEC and NOAEC, respectively). The geometric mean is established from the fissioning events
that occurred in the long term study. The NOAEC could not be quantified for this study. The
lower bound of the ACR is assuming the geometric mean is 1 mg Co/L. Based on the background
level of cobalt in the dilution water determined by ICP-AES, an upper bound could be calculated.
[a] ACR = acute to chronic ratio = LC_{50}/geometric mean of NOAEC and LOAEC=LC_{50}/MATC
[b] No Observable Adverse Effect Concentration
[c] Lowest Observable Adverse Effect Concentration

       As seen with the ACRs from other cobalt toxicity studies, the length of the short term

test greatly influences an ACR. For example, an ACR for cobalt toxicity in *Dapnia magna* was

162, using acute data from a 48 hour study and 2.1 using 3-week LC_{50} data (Kenaga 1981).

Therefore, an ACR should be used cautiously with respect to species and time. A standard

testing length should be followed for that particular species or one in a similar genus. An ACR in

D.magna for iron was 2 (Kenaga 1981). For nickel, the element next to cobalt on the periodic

table of elements, an ACR in *D.magna* was 15 but 48 for *P.promelas* (Kenaga 1981). The final

ACR for cobalt toxicity in *Dugesia dorotocephala*, using the standard testing length of 96 hours

for *D.tigrina* (ASTM 1996), was 28. If, however, the 168 hour LC_{50} value (12 mg Co/L) is used, the

ACR value would be 12.

V. Mechanisms and Implications For Higher Organisms

In Niyogi and Wood's (2004) review of the development of the biotic ligand model, they describe the effect on respiration (in gills), when fish are exposed acutely to cationic metals, as being much less compared to the inhibitory effect on ion transporters. Copper and silver blocked sodium uptake while cadmium, zinc and cobalt blocked calcium uptake. A similar mechanism is proposed in *D. dorotocephala* exposed to cobalt. A disruption in the osmotic gradient of the planaria through inhibition of the absorption of essential cations may explain the observed acute toxicity. According to the mortality data in this thesis, as calcium increased, mortality decreased. Thus, a calcium transporter may be a likely target that cobalt inhibits.

The pathways to produce energy for a cell are disrupted by cobalt (Smith and Carson 1981, p. 66). Several identified pathways in the regeneration of planaria (Adell et al. 2010) could be disrupted by cobalt. Also, because cobalt induces a hypoxic–like state in humans, a similar condition may occur when planaria are exposed to cobalt. A hypoxic environment could trigger pathways yet to be identified involved with survival.

Toxicity testing in higher mammals would not be beneficial due to the rarity of cobalt toxicosis. The diseases that have developed over time due to cobalt insufficiencies would warrant a better understanding of the mechanism of cobalt toxicity in mammals. Similarly, a more precise mechanistic view of the biotic ligand model for aquatic vertebrates and invertebrates would be useful. Investigation of the biochemical mechanisms observed in higher organisms would be of interest to understand the processes involved in cobalt toxicity in planaria.

VI. Limitations

Some potential problems were noted during these studies. The ICP-AES data showed a mean background cobalt concentration in the control of 8.6 µg Co/L in the 60 day chronic study. This background exposure could have influenced the results as the planaria would have been pre-exposed to cobalt at this low level.

Another potential source of cobalt exposure to planaria, presumably in the form of vitamin B_{12}, was from the beef liver they were fed. This dietary contact may have affected their tolerance or sensitivity to the exposure solutions during the course of a study. A future study may consider an alternative food source investigating the dietary accumulation of cobalt in the planaria.

Another limitation was the individual variation in observations. A lab technician could have recorded behavioral or regenerative characteristics somewhat differently compared to another technician working on the project. This was minimized by identifying one person to conduct all the observations for a particular endpoint in a study, if possible.

The method to determine hardness differed by approximately ten percent depending on the two different methods used: calculation from ICP-AES data and standard colorimetric indicator titration. Future work could consider hardness analysis by ICP-AES, due to the heightened sensitivity, throughout the course of a study rather than after it has been terminated to evaluate ion concentrations. However, for the convenience of identifying the range of hardness during the study, titration values provide a good estimate to the actual total hardness concentrations.

VII. Future Work

In the future, different salts could be used to assess the effects of different anions on the planaria. This pertains to the salts used for making the reconstituted water and the salt used to make the cobalt stock solution. Further, the calcium and magnesium studies should be repeated with a broader range of concentrations and replicates to permit more thorough modeling of the role of cations in cobalt toxicity.

Coexposure to cobalt and other elements or compounds has been shown to reduce or increase absorption. Amino acids containing sulfur such as cysteine have been suggested to form stable complexes with cobalt reducing its availability for absorption (Southern and Baker 1981). Coexposure to cobalt during iron deficiencies competitively inhibits iron absorption but increases cobalt absorption (Thomson et al. 1971). Cobalt exposure during zinc deficiencies increases zinc availability (Chung et al. 1988). Cobalt decreases ascorbate resulting in hypoxic stress (Salnikow et al. 2004).

Experimental methods could be changed to assess certain endpoints more effectively. To ascertain regeneration data more accurately, post-pharyngeal sectioning experiments with cobalt solution exposure could be done. The timing of regeneration could be controlled more stringently and no confusion would be made between a dysmorphic head or tail region versus a fissioning event.

Using natural waters instead of synthetic or reconstituted water may be useful to understand the role of dissolved organic matter in planarian cobalt toxicity. In a study that analyzed cobalt and gill binding in *O.mykiss*, cobalt did not accumulate on or in the trout gills in a natural water and cobalt-spiked water due to calcium competition and DOM complexation (Richards and Playle 1998).

VI. REFERENCES

Abraham, J, and A. Hunt. "Environmental Contamination by Cobalt in the Vicinity of a Cemented Tungsten Carbide Tool Grinding Plant." *Environmental Research* 69 (1995): 67–74.

Adell, T, F Cebria, and E Salo. "Gradients in Planarian Regeneration and Homeostasis." *Cold Spring Harbor Perspectives in Biology* 2 (2010): 1–13.

Ahluwalia S and M Kaur. Effect of some heavy metal compounds on growth and differentiation in blue-green and green algae. *Microbios* 53 (1988): 37-46.

Agency for Toxic Substances and Disease Registry. April 2004. Toxicological profile for cobalt. Atlanta: Agency for Toxic Substances and Disease Registry, 2004.

ASTM Standard D1193-99, 1999, "Standard Specification for Reagent Water," ASTM International, West Conshohocken, PA, 1999, DOI: 10.1520/D1193-99, www.astm.org.

ASTM Standard D1426-98A, 1998, "Standard Test Methods for Ammonia Nitrogen In Water," ASTM International, West Conshohocken, PA, 1998, DOI: 10.1520/D1426-98, www.astm.org.

ASTM Standard E729-96, 1996, "Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians," ASTM International, West Conshohocken, PA, 1996, DOI: 10.1520/E0729-96, www.astm.org.

Best, JB, AB Goodman, and A Pigon. "Fissioning in Planarians: Control by the Brain." *Science* 164.3879 (1969): 565–566.

Best, JB, and B Abbotts. "Acute Toxic Responses of the Freshwater Planarian, Dugesia Dorotocephala, to Chlordane." *Bulletin of Environmental Contamination and Toxicology* 26 (1981): 502–507.

Best, JB, and M Morita. "Planarians as a Model System for In Vitro Teratogenesis Studies." *Teratogenesis Carcinogenesis and Mutagenesis* 2 (1982): 277–292.

Bowen, HJM. *Trace Elements in Biochemistry*. New York City: Academic Press, 1966.

Brooks, RR. "Copper and Cobalt Uptake by Haumaniastrum Species." *Plant and Soil* 48 (1977): 541–544.

Chung, AS, ML Sunde, RH Frummer, and WG Hoekstra. "Sparing Effect of Cobalt or Nickel on Zinc Nutrition and the Difference in Zinc Absorption Between Ancona and New Hampshire X Leghorn Cross Chicks." *Asian–Australasian Journal of Animal Sciences* 1.2 (1988): 73–79.

"Mineral Tolerance of Animals, Committee on Minerals and Toxic Substances in Diet and Water for Animals." *Cobalt*. Washington DC: National Research Council of the National Academies, 2005. 124–133.

Corrier, DE, HH Mollenhauer, DE Clark, MF Hare, and MH Elissalde. "Testicular Degeneration and Necrosis induced by Dietary Cobalt." *Veterinary Pathology* 22 (1985): 610–616.

DeSchamphelaere, KAC, JM Koene, DG Heijerick, and CR Janssen. "Reduction of Growth and Haemolymph Ca Levels in the Freshwater Snail Lymnaea Stagnalis Chronically Exposed to Cobalt ." *Ecotoxicology and Environmental Safety* 71 (2008): 65–70.

Di Toro, Dominic M, Herbert E Allen, Harold L Bergman, Joseph S Meyer, Paul R Paquin, and Robert C Santore. "Biotic Ligand Model of the Acute Toxicity of Metals.1. Technical Basis." *Environmental Toxicology and Chemistry* 20.10 (2001): 2383–2396.

Diamond, JM, EL Winchester, DG Mackler, WJ Rasnake, JK Fanelli, and D Gruber. "Toxicity of Cobalt to Freshwater Indicator Species as a Function of Water Hardness." *Aquatic Toxicology* 22 (1992): 163–180.

Environmental Protection Agency, United States. "User Guide Acute to Chronic Estimation." *US EPA Office of R & D EPA/600* R–98 (1999): 152.

EPA Standard 120.1, 1982, "Conductance (Specific Conductance, umhos at 25°C)," US

Environmental Protection Agency, Washington, DC, 1982.

Epstein, AC, JM Gleadle, LA McNeill, KS Hewitson, J O'Rourke, DR Mole, , M Mukherji, E Metzen, MI Wilson, A Dhanda, YM Tian, N Masson, DL Hamilton, P Jaakkola, R Barstead, J Hodgkin, PH Maxwell, CW Pugh, CJ Schofield, and PJ Ratcliffe. *C. elegans* EGL-9 and Mammalian Homologs Define a Family of Dioxygenases that Regulate HIF by Prolyl Hydroxylation. *Cell* 107 (2001): 43–54.

Ewell, WS. JW Gorsuch, RO Kringle, KA Robillard, and RC Spiegel. "Simultaneous Evaluation of the Acute Effects of Chemicals on Seven Aquatic Species." *Environmental Toxicology and Chemistry* 5 (1986): 831–840.

Greenwood, NN, and A Earnshaw. *Chemistry of the Elements*. Oxford: Butterworth–Heinemann, 1997.

Hamilton, El. "Science of the Total Environment." *The Geobiochemistry of Cobalt*. Unk: Unk, 1994. 150: 7–39.

Henry, P. "Effects of Time and Dietary Cobalt Concentration on Tissue Cobalt Concentration." *Nutrition Research* 17.6 (1997): 947–955.

Hillman, Robert S. "Hematopoietic Agents." *Goodman & Gilman's the Pharmacological Basis of Therapeutics.* By Louis S. Goodman, Alfred Gilman, Joel G. Hardman, Alfred Goodman Gilman, and Lee E. Limbird. New York: McGraw-Hill, Health Professions Division, 1996. 1328-332.

Huck, Darrell. "The Study of Cobalt Toxicity in Pigs and Rats." Dissertation. North Carolina State University. 1975.

Jordan , C, RD Whitman, and M Harbut. Memory Deficits and Industrial Toxicant Exposure: A Comparative Study of Hard Metal, Solvent and Asbestos Workers." *International Journal of Neuroscience* 90 (1997): 113–128.

Kapu, MM, and DJ Schaeffer. "Planarians in Toxicology. Responses of Asexual Dugesia Dorotocephala to Selected Metals." *Bulletin for Environmental Contamination and Toxicology* 47 (1991): 302–307.

Karovic, O., I. Tonazzini, N. Rebola, E. Edstrom, C. Lovdahl, B. Fredholm, and E. Dare. "Toxic Effects of Cobalt in Primary Cultures of Mouse Astrocytes-Similarities with Hypoxia and

Role of HIF-1α." *Biochemical Pharmacology* 73.5 (2007): 694-708.

Kasprzak, K, TH Zastawny, SL North, CW Riggs, BA Diwan, JM Rice, and M Dizdaroglui. "Oxidative DNA Base Damage in Renal, Hepatic, and Pulmonary Chromatin of Rats After Intraperitoneal Injection of Cobalt (II) Acetate." *Chemical research in toxicology* 7 (1994): 329–335.

Kenaga, E. "Aquatic Test Organisms and Methods Useful for Assessment of Chronic Toxicity of Chemicals.*" Regulatory Toxicology and Pharmacology* 1 (1981): 277–292.

Kenaga, E. "Predictability of Chronic Toxicity from Acute Toxicity of Chemicals in Fish and Aquatic Invertebrates." *Environmental Toxicology and Chemistry* 1 (1982): 347–358.

Kenk, Roman. *Freshwater Planarians (Turbellaria) of North America*. Cincinnati: U. S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory, Biological Methods Branch, Aquatic Biology Section, 1980.

Kim, JH, HJ Gibb, P. Howe, and M. Sheffer. *Cobalt and Inorganic Cobalt Compounds*. Geneva: World Health Organization, 2006.

Klaassen, Curtis D., ed. *Casarett & Doull's Toxicology the Basic Science of Poisons*. New York: McGraw-Hill Medical, 2008.

Lehninger, Albert L, David L Nelson, and Michael M Cox. *Lehninger Principles of Biochemistry*. New York: Freeman, 2005.

Mahara, Y, and A Kudo. "Interaction and Mobility of Cobalt -60 Between Water and Sediments in Marine Environments Possible Effects by Acid Rain." *Water Research* 15.4 (1981): 413–419.

Marr, JCA, JA Hansen, JS Meyer, D Cacela, T Podrabsky, J Lipton, and HL Bergman. "Toxicity of Cobalt and Copper to Rainbow Trout: Application of a Mechanistic Model for Predicting Survival." *Aquatic Toxicology* 43.4 (1998): 225–238.

McDonald, D, J Reader, T Dalziel, R Morris, E Taylor, and D Brown. "The Combined Effects of PH and Trace Metals on Fish Ionoregulation." *Acid Toxicity and Aquatic Animals*. Cambridge: Cambridge University Press, 1989. 221–242.

Morita, M, and JB Best. "The Occurrence and Physiological Functions of Melatonin in the Most Primitive Eumetazoans, the Planarians." *Experientia* 49.8 (1993): 623–626.

Morita, M, and JB Best. "Effects of Photoperiods and Melatonin on Planarian Asexual Reproduction." *Journal of Experimental Biology* 231 (1984): 273–282.

Morvai V, Szakmary, E, Tatrai E, G Ungvary, and G Folly. "The Effects of Simultaneous Alcohol and Cobalt Chloride Administration on the Cardiovascular System of Rats." *Acta Physiologica Hungarica* 81 (1991): 253–261.

Nagpal, N. K. "Ambient Water Quality Guidelines for Cobalt - Overview." *BC Ministry of Environment*. Government of British Columbia, Sept. 2004. Web. 30 Oct. 2011. <http://www.env.gov.bc.ca/wat/wq/BCguidelines/cobalt/cobalt_over.html>.

Niyogi, S, and CM Wood. "Biotic Ligand Model, A Flexible Tool for Developing Site–Specific Water Quality Guidelines for Metals." *Environmental Science and Technology* 38.23 (2004): 6177–6192.

Nriagu, J. "A Global Assessment of Natural Sources of Atmospheric Trace Metals." *Nature* 338 (1989): 47–49.

Palit, Syamasri, Sharma Archana, and Talukder Geeta. "Effects of Cobalt on Plants." *The Botanical Review* 60.2 (1994): 149–181.

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org. Accessed 2011-07-08.

Rathore, R, and B Khangarot. "Effects of Temperature on the Sensitivity of Sludge Worm Tubifex Tubifex Muller to Selected Heavy Metals." *Ecotoxicology Contamination and Toxicology* 24 (2002): 16–20.

Richards, JG, and RC Playle. "Cobalt Binding to Gills of Rainbow Trout (Oncorhynchus mykiss): An Equilibrium Model. Comparative Biochemistry and Physiology, Part C, Pharmacology, Toxicology, and Endocrinology." *Aquatic Toxicology* 119.2 (1998): 185–197.

Salnikow, K, Donald S, Bruick R, Zhitkovich A, Phang J and K Kasprzak. "Depletion of Intracellular Ascorbate by the Carcinogenic Metals Nickel and Cobalt Results in the Induction of Hypoxic Stress." *Journal of Biological Chemistry* 40 (2004): 337–344.

Smith, Ivan C., and Bonnie L. Carson. "Cobalt: An Appraisal of Environmental Exposure." *Trace Metals in the Environment*. Ann Arbor: Ann Arbor Science, 1981. Vol. 6.

Southern, LL, and DH Baker. "The Effect of Methionine or Cysteine on Cobalt Toxicity in Chicks." *Poultry Science* 60.6 (1981): 1325–1332.

Standard Method 4500-H$^+$ B, 1996, "PH Value," Standard Methods For The Examination of Water and Wastewater. www.standardmethods.org

Standard Method 4500-O G, 1993, "Oxygen Dissolved," Standard Methods For The Examination of Water and Wastewater. www.standardmethods.org

Standard Method 4500-NH$_3$ D, 1997a, "Ammonia (Nitrogen)," Standard Methods For The Examination of Water and Wastewater. www.standardmethods.org

Standard Method 2320 B, 1997b, "Alkalinity," Standard Methods For The Examination of Water and Wastewater. www.standardmethods.org

Standard Method 2340 C, 1997c, "Hardness," Standard Methods For The Examination of Water and Wastewater. www.standardmethods.org

Stryer, Lubert. "Amino Acid Degredation and the Urea Cycle." *Biochemistry*. New York: Freeman, 1988. 1.

Swenson, M. *Physiology of Domestic Animals*. Ithaca: Comstock Pub Associates, 1977.

Szakmary, E, G Ungvary, A Hudak, M. Naray, and V. Morvai. "Effects of Cobalt Sulfate on Prenatal Development of Mice, Rats, and Rabbits and on Early Postnatal Development of Rats." *Toxicology and Environmental Health* 62 (2001): 367–386.

Thomson, ABR, LS Valberg, and DCG Sinclair. "Competitive Nature of the Intestinal Transport Mechanism for Cobalt and Iron in the Rat." *Journal of Clinical Investigation* 50.11 (1971): 384–394.

Tipping, E, S Lofts, and AJ Lawlor. "Modeling the Chemical Speciation of Trace Metals in the Surface Waters of the Humber System." *Science of the Total Environment* 210.211 (1998): 63–77.

"USGS Minerals Information: Statistical Compendium – COBALT." *USGS Mineral Resources Program*. United States Geological Survey, n.d. Web. 2 Oct. 2011. <http://minerals.usgs.gov/minerals/pubs/commodity/cobalt/stat/index.html>.

Van Soest, Peter. *Nutritional Ecology of the Ruminant*. 2 ed. Ithaca: Cornell University Press, 1994.

Van Vleet, JF, AH Rebar, and FJ Ferrans. "Acute Cobalt and Isoproterenol Cardiotoxicity in Swine: Protection by Selenium-vitamin E Supplementation and Potentiation by Stress-Susceptible Phenotype." *American Journal of Veterinary Research* 38 (1977): 991–1002.

Young, R. *Cobalt. American Chemical Society*. New York City: Reinhold, 1948.

Yuan Y, Hilliard G, Ferguson T, and D Millhorn. "Cobalt Inhibits the Interaction between Hypoxia-inducible Factor-α and von Hippel-Lindau Protein by Direct Binding to Hypoxia-inducible Factor-α. *Journal of Biological Chemistry* 278 (2003): 15911–15916.