

Toxicity of Deicing Salt Components to Early Amphibian Life Stages

By  
Sara Collins

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Approved: Dr. Ron Russell  
Supervisor

Approved: Dr. Susan Bjørnson  
Supervisory committee

Approved: Dr. Cory Pye  
Supervisory committee

Approved: Dr. Steve Mockford  
External examiner

Approved: Dr. Genlou Sun  
Program Representative

Approved: Dr. Pawan Lingras  
Graduate Studies  
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## **Abstract**

**Sara Collins**

### **Toxicity of Deicing Salt Components to Early Amphibian Life Stages**

**March 15, 2010**

Chemical contamination is a primary factor contributing to worldwide amphibian declines. Road salt is a major pollutant producing elevated chloride concentrations in freshwater systems. Additionally, ferrocyanide anti-caking agents are released from road salt and effects on the environment are poorly understood. Amphibians exhibit low tolerance to salt. Our purpose is to investigate chronic, sub-lethal NaCl exposure on developmental amphibian stages and determine acute toxicity of cyanide. Chronic toxicity experiments at environmentally significant salt concentrations were conducted on larvae of three species and embryos of five species in the laboratory. Species tested were spotted salamanders, American toads, spring peepers, green frogs and wood frogs. Cyanide acute toxicity experiments were performed on the five species. Chronic salt exposure reduced hatching, increased mortality, and induced developmental and behavioural anomalies. Median lethal concentration values were calculated from cyanide acute toxicity experiments. Results indicate that the chronic effects can inflict detrimental consequences to amphibian populations.

## **Introduction**

### ***Amphibian Population Declines***

Amphibian populations are declining at alarming rates globally (Alford and Richards, 1999; Baillie et al., 2004; Blaustein et al., 1994). In the midst of the current biodiversity crisis, amphibians are facing the greatest threats of extinction (Alford and Richards, 1999; Baillie et al., 2004). A recent assessment of global amphibian populations concluded that 43% of all populations are decreasing rapidly (Baillie et al., 2004). Amphibian populations are susceptible to decline due to sensitive physiological constraints, complex life cycles, high site fidelity, and low movement rates. Amphibians are thus extremely vulnerable to environmental disturbance (Alford and Richards, 1999; Blaustein et al., 1994). Often the most sensitive members in their communities, amphibians are considered by ecologists as valuable biological indicators of habitat quality (Alford and Richards, 1999; Blaustein et al., 1994). Worldwide population declines are concerning as they represent the current state of ecosystem health (Alford and Richards, 1999).

Amphibians are central organisms in the ecosystems they inhabit, playing key roles as both predators and prey (Blaustein, et al., 1994; Wilbur, 1997). Food webs have been described as the central theme in ecology, representing connections between taxa and energy flow within a system (Wilbur, 1997). A common misconception with the understanding of food webs is that they represent static relationships within a community (Wilbur, 1997). In actuality, food web structure is more fluid in its arrangement and this is especially striking when considering the structure of amphibian populations.



Amphibians change trophic positions throughout their life cycles, transforming from aquatic detritivores/omnivores to amphibious, carnivorous adults (Wilbur, 1997).

Class Amphibia is comprised of a group of ectothermic vertebrates that have smooth, glandular skin and exhibit semi-aquatic life histories (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). The word amphibian means “double life” referring to the biphasic aquatic and terrestrial life stages common to the members of this taxon (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). The nature of amphibian skin is a feature that plays a predominant role in the way these animals live (Pough et al., 2004; Tanara, 1975). This highly permeable organ is the site of water and gas exchange, and water can be lost quickly by evaporation (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). Amphibians are generally restricted to high moisture environments and are typically most active at night (Pough et al., 2004; Tanara, 1975). Eggs are laid in water or moist areas and are characterized by a covering of transparent, gelatinous layers (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). Following hatching, the aquatic larval stage undergoes transformation into the predominantly terrestrial adult phase through a process called metamorphosis (Gosner, 1960). Different species exhibit variations to this plan, with some primarily aquatic and others terrestrial (Pough et al., 2004; Tanara, 1975). Dependence upon both aquatic and terrestrial environments for completion of their life cycles render amphibians sensitive to habitat modification and changes to the chemistry of their environments (Blaustein et al., 1994; Pough et al., 2004). Chemical contamination of the environment has been identified as an important factor contributing to amphibian population declines (Alford and Richards, 1999; Blaustein et al., 1994; Hayes, 2000; Kiesecker, 2002; Pough et al., 2004). Amphibian skin and eggs are

permeable and readily absorb contaminants such as heavy metals, pesticides and other chemicals found in agricultural and industrial runoff (Pough et al., 2004). Atrazine, one of the most commonly applied herbicides worldwide, can cause feminization in male frogs, producing hermaphrodites (Hays, 2000). Pollutants may interact with other environmental stressors to produce synergistic effects (Blaustein et al., 2003; Relyea, 2003; Sih et al., 2004).

### ***Deicing Salt Contamination***

Road systems are sources of environmental pollution (Forman et. al., 2003). Exhaust fumes deposit metals, sulfur dioxide and nitrogen oxides into adjacent habitats (Ball et al., 1998; Forman et. al., 2003; Thunqvist, 2003). Road runoff from tars, oils, gasoline and vehicle residues contain various carcinogens and endocrine disrupters, hydrocarbons, polyaromatic hydrocarbons (PAH's), metals and deicing agents (Ball et al., 1998). Additional effects of road infrastructure and associated use include: direct mortality to wildlife, modification of animal behaviours, alteration of physical landscapes, population fragmentation, and facilitated spread of exotic species (Gibbs and Shriver, 2005; Forman et. al., 2003).

Road salts (primarily NaCl) are used extensively as deicing agents throughout the northern hemisphere due to their effectiveness, low cost, and easy dispersion (Jackson and Jobbágy, 2005; Kaushal et al., 2005; Paschka et al., 1999). In North America, 14 million tonnes of salt are applied annually to roads as deicing compounds (Environment Canada, 2001). Use has increased dramatically over the past 65 years (Jackson and Jobbágy, 2005; Kaushal et al., 2005). Human population pressures such as urban expansion and migration from urban areas to suburbs (thus increasing average commute

times) suggest that the demand for road salt will only continue to increase. Abrasives (sand, crushed stone) were used on roadways to facilitate automobile traffic prior to the introduction of deicing salts. Many highway departments adopted a “bare pavement” policy in the 1940’s requiring deicing salts, which function by lowering the freezing point of water (Wyant, 1998). When applied to asphalt, deicing salts melt ice and snow and form a barrier preventing bonding of ice to pavement (Wyant, 1998).

Natural background chloride concentrations in fresh water are generally only a few milligrams per liter, commonly ranging between 2-8 (Environment Canada, 2001; Kaushal et al., 2005). Chloride concentrations now exceed these natural background levels in many aquatic systems in Northern latitudes due to the introduction of millions of tonnes of road salt annually into the local environment (Godwin et al., 2003; Jackson and Jobbágy, 2005; Kaushal et al., 2005; Thunqvist, 2003). Environment Canada (2001) has reported chloride concentrations of 4,000 mg/L in ponds and wetlands and 5,000 mg/L in urban lakes, and over 18,000 mg/L have been measured in road runoff (Novotny et al. 1997). Kaushal et al. (2005) examined chloride levels in freshwater streams in northeastern United States, reporting peak concentrations of up to 5 g/L (25 % that of marine water). These levels remained elevated throughout the year, containing levels 100 times higher than pristine forest streams during summer months (Kaushal et al., 2005). A long term monitoring study of the ionic composition of a rural, upstate watershed in New York measured increases of sodium and chloride during the time span of 1952 to 1998 (Godwin et al., 2003). Sodium and chloride ions increased 130 and 243 %, respectively over this time period and these were directly attributed to the application of road deicers (Godwin et al., 2003)

### ***Effects of Wetland Degradation***

Wetlands are biologically rich habitats that serve as feeding and breeding habitat for large numbers of species (Brinson, 1993; Gibbs, 1993; Semlitsch and Bodie, 1998).

Wetlands facilitate essential roles in hydrological and chemical cycles, such as purifying water by filtering out sediments and contaminants (Brinson, 1993). For this reason, they have been elegantly described as the “kidneys of the land” (Brinson, 1993; Holland et al., 1990; Richardson, 1994; Semlitsch, 2000). Wetlands store excess water which is gradually released in groundwater aquifers, preventing over-flooding and reducing the impact of drought (Brinson, 1993; Holland et al., 1990; Richardson, 1994). The retention and slow release of nutrients helps prevent eutrophication. Wetland vegetation stabilizes the soil, reducing erosion rates and landslides (Brinson, 1993; Holland et al., 1990; Richardson, 1994). Wetland loss is currently a major issue in habitat conservation (Brinson, 1993; Richardson, 1994; Semlitsch, 2000; Semlitsch and Bodie, 1998).

Described as ecotones (a blend of two ecosystems), wetlands possess characteristics of both aquatic and terrestrial ecosystems (Holland et al., 1990). Defined as areas of land that are covered with water for a significant portion of the year, wetlands receive freshwater from inputs such as lakes, streams, tidal flows and groundwater connections (Brinson, 1993; Richardson, 1994; Semlitsch, 2000). Wetlands are areas comprised of numerous wetland “patches” connected through ground aquifers and specialized hydrological cycles which can vary greatly in both temporal and special scales. The interconnectedness of the patches is essential to understand in regards to conservation. The loss or contamination of a few wetland patches affects others and small wetland ponds are essential for maintaining functions over a greater land area (Gibbs, 1993;

Richardson, 1994; Semlitsch, 2000). These small patches are exceptionally vulnerable to contamination as they have low amounts of water to dilute polluted inflows. Additionally, their value is often underrated and they are seldom protected (Semlitsch and Bodie, 1998). Gibbs (1993) demonstrated that the loss of small wetland patches in a 600 km area of Maine reduced total wetland area by 19%, and total wetland numbers declined by 62%. Semlitsch and Bodie (1998) discovered that small wetland patches exhibit greater biodiversity than large wetlands. These small patches are often temporary ponds, experiencing annual cycles of filling and drying. The frequent drying cycles render them unable to support fish populations, therefore providing unique habitats free of fish predators and favored by organisms such as amphibians, which utilize them to complete aquatic stages in their lifecycles (Brinson, 1993; Gibbs, 1993; Semlitsch, 2000; Wilbur, 1997).

Salinization of wetlands is of considerable environmental concern. Excessive amounts of chloride and sodium ions disrupt the functioning of wetland systems by degrading habitat quality and having toxic effects on biological organisms (Environment Canada, 2001; Godwin et al., 2003; Jackson and Jobbágy, 2005; Thunqvist, 2003; Kaushal et al., 2005). Habitat quality becomes altered over time as sodium ions displace naturally occurring elements and protons in soils essential for plant growth, such as calcium, magnesium and potassium (Jackson & Jobbágy, 2005). Salt can mobilize certain deleterious elements such as toxic metals bound to sediments, making them available for biological uptake (Amrhein and Strong, 1990; Backstrom et al., 2004). Influx of salty runoff creates layers of different densities in a body of water, disrupting natural nutrient overturn, potentially leading to oxygen depletion (Jones and Jeffery, 1992). Chloride ions

can enter groundwater, contaminate wells, and pose a threat to people on sodium-restricted diets (Huling and Hollocher, 1972). The decision was made in August 2000 to include road salt on the Canadian Environmental Protection Act Toxic Substance List (Environment Canada, 2001).

When released into the aqueous environment, NaCl dissociates into chloride and sodium ions (Environment Canada, 2001; Jackson and Jobbágy, 2005; Kaushal, 2005). Positively charged sodium ions have the tendency to bind to negatively charged soil particles or be taken up in biological systems (Environment Canada, 2001; Jackson and Jobbágy, 2005; Kaushal, 2005). The chloride ion is highly soluble and disperses easily through water. It is conservative in aquatic systems and not subject to rapid loss or degradation (Godwin et al., 2003; Thunqvist, 2003). Road salts enter air, soil, ground water systems, and surface waters from direct runoff, snow melt, release from soils, or aerosolized spray (Environment Canada, 2001; Huling and Hollocher, 1972; Thunqvist, 2003).

Elevated levels of salt induce toxic effects to a variety of freshwater life (Environment Canada, 2001; Kaushal et al., 2005). Seventeen species of fish, amphibians and crustacea demonstrated a median acute toxicity ( $LC_{50}$ ) of 3,345 mg/L to chloride after 1 day of exposure (Environment Canada, 2001). Prolonged exposure to chloride concentrations above 220 mg/L is harmful to roughly 10% of aquatic species (Environment Canada, 2001; Kaushal et al., 2005). Concentrations below 220 mg/L may alter community structures and disrupt food chains by disrupting primary producer and invertebrate communities (Environment Canada, 2001). A concentration of 250 mg/L is considered the maximum limit for chloride in aquatic systems for the protection of freshwater biota

(Environment Canada, 2001). Many urban streams already exceed 250 mg/L, and if salinization continues to increase at its present linear rate, the majority of rural streams will reach these levels within the next century (Kaushal et al., 2005).

Five million tonnes of road salts are annually applied to Canadian roads (Environment Canada, 2001). Environment Canada (2001) considers urban areas in southern Ontario, Quebec, and the Atlantic provinces to have the greatest risks of groundwater chloride contamination due to high annual road salt loadings based on road length. Wetland salinization poses a particular threat to amphibian species because they exhibit low tolerance to saline environments (Chinathamby et al., 2006; Christy and Dickman, 2002; Gomez-Mestre et al., 2004; Sanzo and Hecnar, 2006). Virtually all species are restricted to freshwater, with a few exceptions adapted to brackish conditions (Gomez-Mestre et al., 2004; Pough et al., 2004; Smith et al., 2007). Ions readily diffuse through the permeable skin and eggs of amphibians and disrupt osmotic regulation (Gordon, 1962; Katz, 1973; Pough et al., 2004). Amphibian diversity in wetlands is negatively associated with salinity (Smith et al., 2007). Levels of approximately 12‰ seawater (Approximately 4.2 g/L NaCl) excluded virtually all amphibian larvae in a community (Smith et al., 2007). Because of their low tolerance to salt, increased salinization of freshwater habitats from road salts could contribute toward amphibian declines in northern and temperate regions (Chinathamby et al., 2006; Christy and Dickman, 2002; Gomez-Mestre et al., 2004; Sanzo and Hecnar, 2006).

### ***Salt Tolerance in Amphibians***

Tonicity is the measure of osmotic pressure between two solutions separated by a semi-permeable membrane. It has been suggested that blood hypertonicity built up by

electrolytes and urea, enables a few amphibian species to tolerate high saline environments (Katz, 1973; Liggins and Grigg, 1985). These mechanisms could explain adaptation of some amphibians to brackish conditions, and why variability exists in tolerance levels among many amphibian species. Vertebrates use ion transport to homeostatically regulate internal osmotic pressures (Yancey, 2005). The osmole (Osm) is a measure of the number of moles of a chemical compound that contribute to the osmotic pressure of a solution. Osmolarity is the measure of the osmotic pressure exerted by solution across a semi-permeable membrane and refers to the number of osmoles of solute per liter of solution (Yancey, 2005). Salts of ocean waters (mainly NaCl) yield an average osmotic concentration of around 1000 milliosmoles per liter (1000 mOsm). Most cells have between 300-400 mOsm by basic solutes such as potassium ions, metabolites and proteins (Yancey, 2005). Osmoregulators possess organs and systems (gills, kidneys) that function to maintain internal body fluids at around 400 mOsm, eliminating the need for organic osmolytes. However, there are major exceptions where some osmoregulators are known to use organic osmolytes in certain situations (Yancey, 2005). Organic osmolytes are small solutes used by cells of water-stressed tissues to maintain cell volume. Major chemical categories that osmolytes are divided into include: small carbohydrates (sugars), polyols (such as glycerol and sorbitol), amino acids (including glycine, proline and taurine), and methylamines and methylsulfonium solutes, including urea (Yancey, 2005). Some studies have investigated osmoregulation in species displaying high tolerance to salinity in an attempt to identify the exact mechanisms responsible. This has been accomplished by acclimating individuals to high salt environments and analyzing the solutes contributing to plasma osmotic pressure (Gordon,



1962; Katz, 1973; Konno, 2006; Liggins and Grigg, 1985).

### ***Ferrocyanide Anti-caking Agents***

Another potential hazard associated with the application of deicing salt is the release of ferrocyanide compounds into the environment. Iron cyanides are added to deicing salt as anti-caking agents (Eisler, 1991; Environmental Resource Management-New England, 1987; Paschka et al., 1999). Since the 1950s, these compounds have been applied to salt piles by spraying them or mixing them directly in brine solutions during processing (Paschka et al., 1999). In response to fluctuating humidity, salt naturally clumps together forming large aggregations of crystals. At high humidity, crystals form a brine solution that coats the surface of a salt pile (Environmental Resource Management-New England, 1987; Paschka et al., 1999). When humidity drops below 75%, water evaporates, leaving thick aggregations of recrystallised salt, referred to as “caking” (Environmental Resource Management-New England, 1987; Paschka et al., 1999). This is a significant problem because caking causes the uneven distribution of road salt, resulting in increased labour and greater quantities of salt needed for each application (Environmental Resource Management-New England, 1987). The most commonly used iron cyanide anti-caking agents is sodium ferrocyanide ( $\text{Na}_4\text{Fe}(\text{CN})_6$ ), also known as yellow prussiate of soda (YPS) and ferric ferrocyanide ( $\text{Fe}_4(\text{Fe}_3(\text{CN})_6)_3$ ) (Environmental Resource Management-New England, 1987). These additives decrease the solubility of sodium chloride in absorbed moisture and alter the crystallization structure of salt (Environmental Resource Management-New England, 1987; Paschka et al., 1999).

Cyanide compounds are classified as either simple or complex, with simple cyanide compounds including the following: sodium cyanide, potassium cyanide, and hydrogen

cyanide (Environmental Protection Agency, 1984; Paschka et al., 1999). Simple cyanides are toxic and readily dissociate in water, releasing highly toxic free cyanide which is defined as hydrogen cyanide (HCN) or the cyanide anion (CN<sup>-</sup>) (Environmental Protection Agency, 1984; Paschka et al., 1999). The concentrations of these two forms in the aquatic environment depend primarily on temperature and pH (Eisler, 1991; Environmental Protection Agency, 1984). The majority of free cyanide exists when pH is less than 8 and the temperature below 25 °C. A greater proportion of free cyanide exists as CN<sup>-</sup> when either or both pH and temperature are higher (Environmental Protection Agency, 1984; Lind et al., 1977). HCN is therefore the form that predominates under the majority of natural conditions. It also happens to be the more toxic of the two forms (Lind et al., 1977). YPS and ferric ferrocyanide are examples of complex cyanides, and exhibit low toxicity due binding of the cyanide molecule to a metal ion (Paschka et al., 1999). These molecules are extremely unstable under UV light exposure and will rapidly dissociate, releasing highly toxic free cyanide (Environmental Protection Agency, 1984; Meeussen et al., 1992; Paschka et al., 1999). The production of cyanide from this reaction is proportional to the duration and intensity of light exposure (Environmental Resource Management-New England, 1987). The rate and extent of ferrocyanide decomposition is directly related to sunlight intensity. Field studies have shown that these compounds become highly toxic to aquatic biota under sunny conditions, but not during heavily overcast days, indicating that UV-B exposure is required to induce toxicity (Calfee and Little, 2003b). Other important factors involved in the photodecomposition of cyanide compounds in water include water clarity and depth, initial ferrocyanide concentration, oxygen, temperature, and pH (Environmental Resource Management-New England,

1987; Environmental Protection Agency, 1984; Paschka et al., 1999). Decomposition generally occurs in shallow waters at depths estimated at approximately 50 to 100 cm (Environmental Resource Management-New England, 1987). The rate of this reaction may be reduced or halted in deep, turbid, shaded waters, because turbid water absorbs sunlight, decreasing the amount of light needed for the photodecomposition reaction to occur (Environmental Resource Management-New England, 1987). Additional factors influencing this reaction are water temperature and pH ; photodecomposition rate decreases with increasing pH, while increasing slightly at higher temperatures (Broderius, 1981).

Once dissociated from the complex molecule, the environmental fate of free cyanide is poorly understood (Paschka et al., 1999). Once free or simple cyanides are formed by ferrocyanide decomposition, several environmental factors can reduce and eliminate it from the aquatic environment (Environmental Resource Management-New England, 1987). The volatile nature of HCN has led some to suspect that it rapidly leaves surface waters through this action (Environmental Resource Management-New England, 1987; Paschka et al., 1999). However, no consistent data exist to confirm this hypothesis (Paschka et al., 1999). It is suggested that wind, water turbulence and agitation accelerate the volatilization of cyanide into the atmosphere (Environmental Resource Management-New England, 1987). A proportion of cyanide in the aquatic environment has been shown to bind with sediments (Meeussen et al., 1992; Olson and Ohno, 1989). An abundance of iron or other metal cations will bind with free cyanide, recreating complex cyanide compounds with low toxicity (Environmental Resource Management-New England, 1987). Several species of bacteria and fungi biologically degrade cyanide into ammonia

and carbon dioxide (Environmental Resource Management-New England, 1987). The fate and action of cyanide compounds in ground water is even less understood. Upon entry of ground water systems, these compounds could conceivably undergo long range transport, surfacing at unpredictable locations and times.

Routes of exposure to cyanide can be through ingestion, dermal absorption and inhalation. Once cyanide has entered an organism, it is distributed throughout the body via the blood (Eisler, 1991; Environmental Resource Management-New England, 1987). Cyanide acts as a potent asphyxiant, inducing tissue anoxia through inactivation of cytochrome oxidase, the terminal oxidative enzyme in the microbial electron transport chain. This action blocks aerobic ATP synthesis (Environmental Resource Management-New England, 1987). Animals possess natural detoxifying mechanisms in the liver, which converts cyanide into excretable thiocyanate (Eisler, 1991). Mortality occurs when the detoxifying mechanisms become overloaded (Eisler, 1991; Environmental Resource Management-New England, 1987). The fatal oral dose for cyanide in humans is 0.57 mg/kg, and inhalation of air containing HCN gas at a concentration of 200 mg/m<sup>3</sup> is immediately fatal (Eisler, 1991; Environmental Resource Management-New England, 1987). Due to the detoxification in the liver, toxic effects of cyanide at low doses are reversible and biomagnification or accumulation in the environment is considered highly unlikely (Eisler, 1991; Environmental Resource Management-New England, 1987). Many aquatic organisms are considered much more sensitive to cyanide toxicity than humans (Eisler, 1991). Toxic effects occur in humans at around 10 parts per million (ppm; mg/L) compared to toxic effects reported to occur at 10 ppb (µg/L) for some aquatic biota, such as fish (Eisler, 1991). The U.S. Environmental Protection Agency's

maximum contaminant level for cyanide in drinking water is 0.2 mg/L as free cyanide, and the freshwater quality guidelines for cyanide are lower. Acute exposure limit is 22 µg/L free cyanide and criteria for chronic exposure is 5.2 µg/L. The criterion for the protection of marine life was determined to be 1 µg/L (Environmental Protection Agency, 1984). The Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life is 5 µg/L cyanide (Canadian Council of Ministers of the Environment, 2007). It has been reported that YPS concentration in road salt typically ranges between 50-100 ppm (mg/kg) (Environmental Resource Management-New England, 1987). A number of factors influence the degree of cyanide toxicity in aquatic organisms (Eisler, 1991). These include temperature, pH, oxygen concentration, the particular species and life stage exposed, the condition of exposed animals, and numbers of previous exposures (Eisler, 1991). Accidental spills of sodium cyanide (NaCN) and potassium cyanide (KCN) have resulted in massive kills of aquatic organisms including fish, amphibians, invertebrates and aquatic plants (Calfee and Little, 2003a; Eisler, 1991). Cyanide toxicity data has been reported for some fish species, resulting to the general consensus that fish are considered to be a sensitive animal group (Calfee and Little, 2003a; Calfee and Little, 2003b; Eisler, 1991; Leduc, 1978; Ruby et al., 1986; Smith et al., 1978). Lethal effects occur between 20-76 µg/L of free cyanide, and chronic effects on swimming ability and reproduction have been reported to occur at levels as low as 5 µg/L free cyanide (Eisler, 1991; Kimball et al., 1978).

Virtually no data exist on cyanide toxicity to amphibians. Only one study reports toxicity data for an amphibian species to the cyanide compound, YPS which is often added in fire fighting chemical mixtures. A variety of fire retardant chemicals are used by

forest fire managers and fire control agencies to fight wildland fires in North America (Calfee and Little, 2003b). Aerial applications of these chemicals have contaminated surface waters, resulting in massive fish kills (Calfee and Little, 2003b). The most common fire fighting chemical formulations are ammonia based, and often contain YPS as a corrosion inhibitor (Calfee and Little, 2003b). These researchers exposed tadpoles of the southern leopard frog (*Lithobates sphenoccephala*) as well as juvenile rainbow trout (*Oncorhynchus mykiss*) to six fire fighting chemicals both containing and not containing YPS, and YPS alone. This study was conducted for the purpose of investigating the interactive toxicity between YPS, ammonia, and simulated solar UV radiation to the two species tested. Additionally, preliminary tests were performed on tadpoles of the boreal toad (*Anaxyrus boreas*), to compare sensitivities between the two amphibian species. Mortality of trout and tadpoles was significantly increased in the presence of UV radiation when exposed to YPS both alone and when present in formulations (Calfee and Little, 2003b).

Studies of ferrocyanide effects on water quality are limited in number (Paschka et al., 1999). Levels of free cyanide measured at four salt distribution piles along the Mississippi River in Minnesota were as much as 40 times the maximum acute toxicity standard for the preservation of aquatic life (Oberts et al., 2000). Elevated concentrations of sodium chloride (NaCl) and total cyanide were measured in surface waters adjacent to four highway salt storage facilities in Maine (Olson and Ohno, 1989). Paschka et al. (1999) calculated a series of mass-balance equations in attempt to estimate the range of total cyanide concentrations in road runoff for some potential situations (taking into account different factors such as salt application and road length). Estimated total cyanide

concentrations ranged up to 6 mg/L, which greatly exceed the Canadian Water Quality Guidelines for the protection of aquatic life, the U.S. Environmental Protection Agency maximum contaminant level in drinking water, and the U.S. Environmental Protection Agency acute and chronic freshwater quality criteria (Paschka et al., 1999). The authors concluded that there are potentially negative effects on water quality arising from addition of iron cyanide complexes in road salt, and that extensive monitoring studies examining total, free, and complexed cyanides in surface and groundwater in all areas of potential contamination are required (Paschka et al., 1999).

### ***Ecological Implications of Habitat Contamination***

In ecotoxicological research, acute toxicity tests are conducted to determine adverse biological effects of a material through brief and intense exposure times, where mortality is the only measurable endpoint. From these experiments,  $LC_{50}$  values are determined, representing the lethal concentration to 50% of a given population of test subjects (Gad and Chengelis, 1988; Kooijman and Bedaux, 1996). These values are comparable and are used as a basis for risk assessments and environmental quality legislation (Gad and Chengelis, 1988). Sub-lethal contaminant exposure below the lethal concentration may be equally detrimental to the fitness of an individual or entire population (Sih et al., 2004).

Particular factors may act in unison to create synergistic effects on sensitive biota (Blaustein et al., 2003; Relyea, 2003; Sih et al., 2004). Degradation of habitat quality due to chemical contamination is a leading contributor to amphibian declines (Blaustein et al., 1994; Hays, 2000; Kiesecker, 2002) and is a factor shown to interact with natural stressors in ecosystems (Relyea, 2003). Interactions between anthropogenic (chemical) and natural (predator-prey interactions) stressors have been described by Relyea (2003)

and Relyea and Mills (2001). Non-lethal exposure to many toxins may disrupt normal activities (such as swimming and behaviour) and these alterations could result in greater predation risk. Salinity levels that are too low to cause direct mortality may still produce observable effects in some amphibian species, such as decreased survivorship and failure to reach metamorphosis (Christy and Dickman, 2002; Gomez-Mestre et al., 2004; Sanzo and Hecnar, 2006). Exposure to various agrochemicals at levels below lethal concentrations have altered larval amphibian survival, growth, development, and behaviour (Glennemeier and Denver, 2001; Rohr et al., 2003). Many pesticides are rarely applied alone in agricultural use, and are often used in combinations with various other chemicals and pesticide combinations (Hayes et al., 2006; Mann and Bidwell, 1999). Furthermore, commercial pesticide preparations incorporate various solvents, carriers or surfactants and these mixtures can have additive or synergistic toxicological effects (Mann and Bidwell, 1999).

In a recent study, Hayes et al. (2006) examined pesticide combinations on larvae of *Lithobates pipiens* and adult African clawed frog (*Xenopus laevis*) using combinations of pesticides regularly applied in combination. Four herbicides (atrazine, metolachlor, alachlor, and nicosulfuron), three insecticides (cyfluthrin, cyhalothrin, and tebupirimphos), and two fungicides (metalaxyl and propiconazole) were examined alone or in combinations (Hayes et al., 2006). Individual pesticides inhibited larval growth and development but pesticide combinations had greater effects. Larval growth and development were greatly reduced. Exposed larvae took longer to reach metamorphosis and were smaller than the controls that metamorphosed earlier (Hayes et al., 2006).

Effects of one variable described in the laboratory can fluctuate depending on



influences from biotic and abiotic factors in nature (Relyea, 2004; Relyea and Mills, 2001). Grey tree frog tadpoles (*Hyla versicolor*) were exposed to very low concentrations of the pesticide carbaryl in presence (versus absence) of chemical cues from predatory salamanders. The increased stress experienced by the tadpoles resulted in a 4-fold increase in carbaryl lethality (Relyea and Mills, 2001). When this experiment was repeated with *Lithobates clamitans* tadpoles, carbaryl was 8 times more lethal, and was 46 times more deadly to *Lithobates catesbeiana* tadpoles (Relyea, 2003). Increased stress levels caused by subjection to predator chemical cues resulted in greater contaminant lethality than previously determined when experienced alone. Lefcort et al. (1998) examined the effects of metal combinations on the survival, growth, metamorphosis and behaviour on Columbia spotted frog (*Lithobates luteiventris*) larvae. The interaction between Zn and Cd was found to be more toxic in combination together than when occurring alone (Lefcort et al., 1998). The results from these studies are compelling when one considers the numerous factors that could account for increases in stress.

Amphibians serve as prey for many animal species (including other amphibians) and the larval stages in particular are most susceptible to predation (Brodie and Formanowicz, 1983). Amphibian larvae have the ability to detect the presence of predators in their environments through the perception of pheromone-like chemical cues (Skelly and Werner, 1990; Van Buskirk and Schmidt, 2000). Tadpole physiology and behaviour change adaptively upon detection of these cues (McCollum and Leimberger, 1997; Van Buskirk and Schmidt, 2000; Wilson et al., 2005). Typical behavioral responses that tadpoles exhibit upon detection of a predation threat include: increasing physical distance from predator (Relyea, 2001), schooling (Wilbur, 1997), reduction in activity (Skelly and

Werner, 1990), and the occupation of safer microhabitats (Smith and Van Buskirk, 1995). Non-lethal exposure to other environmental contaminants has been shown to disrupt normal activities, altering tadpole behaviour and potentially increasing predation risks (Relyea, 2003; Sanzo and Hecnar, 2006). Bridges (1999) demonstrated that sub-lethal levels of the pesticide carbaryl affected activity levels and anti-predatory behaviour in *H. versicolor* tadpoles. It is suspected that sub-lethal salt exposure could elicit similar responses. Larval amphibians exhibited altered behaviours during salt exposure which could influence predator-prey interactions in the wild (Collins and Russell, 2009; Sanzo and Hecnar, 2006).

The cause of recent, rapidly increasing amphibian population declines is not a simple one. Multiple factors exist when complex relationships interact to affect animal populations and these relationships are specific to individual localities (Alford and Richards, 1999; Blaustein et al., 1994). We need to understand how individual environmental stressors interact to be able to extrapolate laboratory results to events occurring in natural ecosystems. Effects of one variable described in the laboratory could fluctuate depending on influences from biotic and abiotic factors in nature (Sih et al., 2004). Furthermore, recent studies have suggested that synergisms between multiple stressors may heighten negative effects of a single variable, such as toxicity (Relyea, 2003; Relyea and Mills, 2001). Contaminant levels that are declared “safe” in the laboratory setting may be lethal in nature when combined with other stresses (Sih et al., 2004).

Deicing salt contamination poses a threat to the health of freshwater ecosystems and could be a critical factor contributing to amphibian population declines in northern

latitudes (Collins and Russell, 2009). Human population trends indicate that the demand for salt will only increase as human pressures continue to escalate. There are many aspects of deicing salt contamination which have not been thoroughly investigated, in particular, sub-lethal effects resulting from chronic exposures. Acute toxicity experiments to NaCl have identified median lethal concentration ( $LC_{50}$ ) values to NaCl for the larvae of five amphibian species (Collins and Russell, 2009; Sanzo and Hecnar, 2006).

Identifying acute toxicity values of a substance is the first step in characterizing its effects in an ecosystem; however, a limitation of the acute toxicity testing approach is the inability to realistically extrapolate results to ecological systems in the presence of natural stressors (Relyea, 2003; Sih et al., 2004). In many habitats, it is probable that pollutants are inflicting fitness-reducing effects on biota at levels well below determined  $LC_{50}$  values (Sih et al., 2004). Another aspect of deicing salt contamination that has received very little attention is the fate and effects of ferrocyanide anti-caking agents in aquatic systems. Studies have shown that freshwater fish are sensitive to low concentrations of cyanide (Calfee and Little, 2003a; Calfee and Little, 2003b; Eisler, 1991; Leduc, 1978; Ruby et al., 1986; Smith et al., 1978) but virtually no toxicity data exists for amphibians.

### ***Objectives***

The objectives of this research are to investigate effects of chronic, sub-lethal NaCl exposure on sensitive amphibian life stages to determine possible effects on growth, development, and behaviour. The second objective is to identify additional sources of toxicity to amphibian larvae in deicing compounds. We identified cyanide as a compound of interest from this perspective. For these purposes, chronic toxicity tests in controlled laboratory conditions were performed on the larvae and embryonic stages of multiple

amphibian species to examine effects on hatching, growth, development, and behaviour. The ecological implications of these effects were considered to gain realistic insight into events occurring in nature. Behavioural experiments were conducted to examine how sub-lethal effects of deicing salt exposure may influence anti-predator behaviour in tadpoles and involved exposing salt-treated and non salt-treated wood frog (*L. sylvaticus*) larvae to predator chemical cues, and recording activity at set intervals throughout a period of time. To examine effects of cyanide exposure, the larvae of *L. sylvaticus*, green frogs (*Lithobates clamitans*), spring peepers (*Pseudacris crucifer*), American toads (*Anaxyrus americanus*), and spotted salamanders (*Ambystoma maculatum*) were exposed to a range of sodium cyanide (NaCN) concentrations, producing median lethal concentration (LC<sub>50</sub>) values.

This research provides relevant insight into events that may occur in wetlands. Identifying the sub-lethal effects of chronic Cl<sup>-</sup> exposure identifies realistic threats that these organisms experience. Determining the (LC<sub>50</sub>) values of HCN to five species provides the scientific community with primary data on cyanide toxicity to these amphibian species under laboratory conditions. Comparing the responses of multiple species and life stages determines which species are at greatest risk and offers insight into how communities are structured around environmental stressors, and identify differences in individual evolutionary life histories and physiological adaptations among amphibian species.

## Methods

### *Chronic Toxicity Experiments*

Larvae of *A. maculatum*, *L. sylvaticus*,

and *A. americanus* were exposed to salt solutions in the laboratory through chronic toxicity tests over the entire larval life stage. These species are common throughout Nova Scotia (Appendix I). Testing was conducted at three salt concentrations (8, 300, and 900 mg/L NaCl; corresponding to 5, 180, 550 mg/L Cl<sup>-</sup>). The chosen concentrations represent environmentally significant values that are well below previously determined lethal concentrations (Collins and Russell, 2009). Controls were represented by the 8 mg/L NaCl concentration (typical of fresh water). All salt solutions were made from coarse food-grade pickling salt (NaCl) and local pond water. Pickling salt is 100% NaCl and was used to eliminate potentially confounding effects of anti-caking agents and iodine present in other salts. For anuran larvae, each of the three salt concentrations consisted of four replicates, each containing 20 L of water and housing 15 individuals. Leaf litter was added to each tank to simulate the natural environment. All individuals selected had hatched from the same egg mass (to reduce genetic variability) and were at developmental stage 25 (the first feeding stage) (Gosner, 1962). Due to the highly cannibalistic behaviour of the caudate larvae, salamanders were housed individually in 2 L containers, with 10 replicates for each of the three salt treatments. Caudate larval development is not staged as with anuran larvae; therefore, similar sized salamanders of similar weights were selected for testing. Larvae were observed daily for physical and behavioural abnormalities and deceased individuals were removed. Frequent water tests for nitrogen, pH, and conductivity were performed to maintain water quality and consistency. Water changes were performed every 2-3 days. Larvae were fed *ad libitum*, and received a variety of food types (spinach, decayed leaf matter, egg whites, mosquito larvae, and earthworms) to meet nutritional requirements. Individuals were removed

immediately following metamorphosis, and the weight of each specimen was recorded. Experiments were terminated when all individuals from the controls had metamorphosed. Measured variables included larval period, weight at metamorphosis and mortality.

### ***Embryonic Toxicity Experiments***

To determine effects of salt exposure on embryonic stages of amphibian larvae, eggs of *L. sylvaticus*, *L. clamitans*, *A. maculatum*, *A. americanus*, and *P. crucifer* were exposed to a range of NaCl solutions until hatching. All salt solutions were made from pickling salt (NaCl) and local pond water (5-10 mg/L Cl<sup>-</sup>). Testing was conducted at the three salt concentrations described for the larval chronic toxicity experiments, with 4 replicates of each. Number of eggs per treatment varied between species, depending on numbers available (40 eggs per treatment for *L. sylvaticus* s, 30 per treatment for *L. clamitans*, 20 each for *A. maculatum*, 50 eggs per treatment for *A. americanus* and 30 each for *P. crucifer*). Testing was conducted in an environmental chamber programmed to simulate spring light and temperature conditions (12:12 L:D; 12:8°C). Measured variables included hatching success and the proportion of physical abnormalities in the resulting tadpoles.

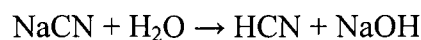
### ***Behaviour Experiments***

The behaviour of *L. sylvaticus* tadpoles that were hatched and reared in 8 mg/L NaCl (control) or 900 mg/L NaCl were examined in the presence and absence of an eastern newt predator (*Notophthalmus viridescens*). All salt solutions were made from pickling salt (NaCl) and local pond water. Testing chambers consisted of two 5.5 gallon glass tanks filled with 10 L of pond water (5-10 mg/L Cl<sup>-</sup>). In one tank, a predator was housed for a minimum of 48 hours. Each tank contained a small (approximately 12 cm long),

plastic aquarium plant approximately 10 cm from one end, submerged at the bottom of the tank. At the other end of each tank was a dome-shaped, transparent plastic cage. This testing procedure was derived from methods described from Bridges (1999). Trials consisted of placing five *L. sylvaticus* tadpoles in the container with a caged (but visible) predator in the center of the trial container. Tadpole movement (whether each tadpole was swimming or sessile) and the position of the tadpole (open or periphery) was recorded every 3 minutes for 60 minutes (20 observation periods). Trials consisted of a control (unexposed tadpole with no predator), unexposed tadpole + predator, salt exposed tadpole without predator, and salt exposed tadpole + predator. Three replicates of each treatment were performed for each trial.

### ***Cyanide Acute Toxicity Tests***

Larvae of five common of amphibian species (Appendix I) were exposed to hydrogen cyanide (HCN) concentrations in the laboratory to determine the median lethal concentration values (LC<sub>50</sub>s) of HCN on these amphibians. HCN was generated from the reaction of NaCN with H<sub>2</sub>O:



Species were selected on the basis of their abundance in ponds within the Halifax Regional Municipality. The five species tested were: the wood frog (*L. sylvaticus*), green frog (*L. clamitans*), spring peeper (*P. crucifer*), American toad (*A. americanus*) and spotted salamander (*A. maculatum*). Egg masses and recently hatched larvae were collected from local ponds and returned to the laboratory where they were raised to Gosner stages 25-28 (Gosner, 1960). Larvae were then subjected to 96 hour NaCN toxicity tests. Caudate larval development is not staged; therefore, salamanders weighing

approximately 0.020 g were selected for testing.

Acute, 96 hour LC<sub>50</sub> tests were conducted at a range of empirically derived HCN concentrations (Appendix II), and a control composed of Halifax tap water (0 mg/L HCN). Treatments ranged from 31.25 - 4000 µg/L for *L. sylvaticus*, 250 - 16 000 µg/L for *L. clamitans*, 250 - 8000 µg/L for *P. crucifer* and *A. americanus*, and 250 - 16 000 µg/L for *A. maculatum*. All cyanide solutions were made from crystalline NaCN and dechlorinated Halifax tap water. Cyanide concentrations (-CN) were verified before and after experiments with a Hach<sup>®</sup> portable spectrophotometer using the Pyridine-Pyrazalone method (Hach<sup>®</sup>, 2002). Six larvae were placed in each concentration (treatment), including the control, and there were 3 replicates per treatment. Experiments were checked daily and dead individuals were removed. At the end of 96 hours, testing was terminated and all specimens were removed. Tadpoles from all treatments were observed for physical or behavioral abnormalities during the course of the testing.

### ***Road Runoff Water***

Triplicate samples of road runoff were collected during the month of March 2009 at 11 sites within Halifax Regional Municipality, and returned to the lab for analysis. A Hydrolab<sup>®</sup> was used to determine pH, conductivity (µS/cm), and chloride (mg/L) for each sample. A Hach<sup>®</sup> portable spectrophotometer using the Pyridine-Pyrazalone method was utilized to test cyanide levels of the sample water. Site locations ranged in characteristics, from low to high use roads, as well as areas adjacent to a salt storage facility. Quality assurance/quality control (QA/QC) was accomplished by running a 0.200 mg/L HCN calibration standard before and after daily analyses and including a zeroing standard for each individual sample. Detection limits were 0.001 mg/L.



### ***Statistical Analysis***

Data analysis was performed using Systat® or Minitab®. Normality was assessed using a Ryan-Joiner test and equality of variances was tested by the D'Agostino and Pearson method. Differences among salt treatments in caudate and anuran larvae with larval period and weight at metamorphosis was tested with multivariate analysis of variance (MANOVA) and differences in hatching success and anatomical anomalies upon hatching among salt treatments in amphibian eggs were tested with a MANOVA. Differences in behaviour (swimming and open habitat use) were tested with 2 separate MANOVAs for unexposed (control) and salt exposed *L. sylvaticus* larvae. Mean chloride concentrations measured in road runoff water were power transformed ( $x^\lambda$ , where  $\lambda=-0.1$ ) and mean cyanide concentrations were also power transformed ( $x^\lambda$ , where  $\lambda=-0.2$ ) to achieve normality. Transformed cyanide concentrations in runoff water were regressed on transformed chloride concentrations.

## **Results**

### ***Chronic Toxicity Experiments***

*A. maculatum* mortality in salt treatments was 0, 40%, and 70% for 8 mg/L, 300 mg/L, and 900 mg/L treatments respectively (Fig. 1). Elevated salt treatments resulted in increased mortality in salamander larvae.

*A. americanus* mortality in salt treatments was 25%, 62%, and 57% for 8 mg/L, 300 mg/L, and 900 mg/L treatments respectively (Fig. 1). There was a trend in increased mortality in higher concentration salt treatments with *A. americanus* tadpoles.

*L. sylvaticus* mortality in salt treatments was 37%, 50%, and 53% for 8 mg/L, 300 mg/L, and 900 mg/L treatments respectively (Fig. 1). Elevated salt treatments resulted in

increased mortality in *L. sylvaticus* larvae.

*A. maculatum* weight at metamorphosis was  $0.48 \pm 0.02$  g for 8 mg/L  $\text{Cl}^-$  treatment,  $0.39 \pm 0.03$  g for 300 mg/L  $\text{Cl}^-$ , and  $0.38 \pm 0.04$  g for 900 mg/L  $\text{Cl}^-$  (Fig. 2). Significant differences in weight at metamorphosis were evident among salt treatments for *A. maculatum* larvae (MANOVA;  $F_{[2, 17]} = 6.7$ ,  $p = 0.007$ ). Pairwise comparisons of means show differences in salamander metamorphic weight between 8 mg/L and 300 mg/L treatments ( $p = 0.01$ ) and 8 mg/L and 900 mg/L treatments ( $p = 0.04$ ) (Fig. 2). 300 mg/L and 900 mg/L treatments did not differ significantly. Salamanders from high salt treatments were weighed less than those from low salt treatments.

*A. americanus* weight at metamorphosis was  $0.098 \pm 0.004$  g for 8 mg/L  $\text{Cl}^-$  treatment,  $0.078 \pm 0.005$  g for 300 mg/L  $\text{Cl}^-$ , and  $0.078 \pm 0.004$  g for 900 mg/L  $\text{Cl}^-$  (Fig. 2). Weight at metamorphosis did not differ significantly among salt treatments for *A. americanus* tadpoles ( $F_{[2, 91]} = 1.2$ ,  $p = 0.3$ ).

*L. sylvaticus* weight at metamorphosis was  $0.30 \pm 0.01$  g for 8 mg/L  $\text{Cl}^-$  treatment,  $0.32 \pm 0.01$  g for 300 mg/L  $\text{Cl}^-$ , and  $0.30 \pm 0.01$  g for 900 mg/L  $\text{Cl}^-$  (Fig. 2). Weight at metamorphosis did not differ significantly among *L. sylvaticus* in the various salt treatments ( $F_{[2, 93]} = 0.28$ ,  $p = 0.76$ ).

The *A. maculatum* larval period was  $34.2 \pm 2.4$  days for 8 mg/L  $\text{Cl}^-$  treatment,  $49.1 \pm 6.7$  days for 300 mg/L  $\text{Cl}^-$ , and  $73.7 \pm 28.0$  days for 900 mg/L  $\text{Cl}^-$  (Fig. 3). The larval period was significantly longer in the elevated salt treatments ( $F_{[2, 17]} = 4.5$ ,  $p = 0.03$ ) (Fig. 3). Tukey comparisons of means show differences in the *A. maculatum* larval period between 8 mg/L and 900 mg/L treatments ( $p = 0.02$ ) (Fig. 3). All other combinations

were not significantly different. High salt treatments extended *A. maculatum* larval times.

The *A. americanus* larval period was  $70.4 \pm 2.8$  days for 8 mg/L  $\text{Cl}^-$  treatment,  $81.3 \pm 4.4$  days for 300 mg/L  $\text{Cl}^-$ , and  $74.3 \pm 4.9$  days for 900 mg/L  $\text{Cl}^-$  (Fig. 3). Larval period was not significantly longer in the elevated salt treatments ( $F_{[2, 93]} = 0.83$ ,  $p = 0.44$ ). Salt did not affect *A. americanus* larval period.

The larval period for *L. sylvaticus* was  $89.5 \pm 3.1$  days for 8 mg/L  $\text{Cl}^-$  treatment,  $85.6 \pm 3.5$  days for 300 mg/L  $\text{Cl}^-$ , and  $92.1 \pm 3.9$  days for 900 mg/L  $\text{Cl}^-$  (Fig. 3). Larval period did not differ significantly among individuals within the elevated salt treatments ( $F_{[2, 93]} = 0.83$ ,  $p = 0.44$ ).

### ***Embryonic Toxicity Experiments***

Hatching success in *A. maculatum* eggs was 100% for 8 mg/L  $\text{Cl}^-$  treatment,  $96 \pm 4\%$  for 300 mg/L  $\text{Cl}^-$ , and  $95 \pm 2\%$  for 900 mg/L  $\text{Cl}^-$  (Fig. 4). These observations did not differ significantly ( $F_{[2, 9]} = 1.1$ ,  $p = 0.37$ ). Elevated salt concentrations did not affect *A. maculatum* egg hatching success and no anatomical abnormalities were observed.

Hatching success in *L. sylvaticus* eggs was  $93 \pm 1\%$  for 8 mg/L  $\text{Cl}^-$  treatment,  $87 \pm 5\%$  for 300 mg/L  $\text{Cl}^-$ , and  $71 \pm 3\%$  for 900 mg/L  $\text{Cl}^-$  (Fig. 4). Significant differences in hatching success were observed among the 3 salt treatments ( $F_{[2, 9]} = 10.6$ ,  $p = 0.004$ ). Tail anomalies were observed in *L. sylvaticus* tadpoles (Fig. 5). The proportion of bent tails in *L. sylvaticus* tadpoles was 0 in the 8 mg/L  $\text{Cl}^-$  treatment,  $1 \pm 1\%$  in the 300 mg/L  $\text{Cl}^-$ , and  $59 \pm 4\%$  in the 900 mg/L  $\text{Cl}^-$  (Fig. 6). Significant differences in tail abnormalities among the 3 salt treatments were observed ( $F_{[2, 9]} = 202.8$ ,  $p < 0.0001$ ).

Hatching success for *P. crucifer* eggs was 60 % for 8 mg/L  $\text{Cl}^-$  treatment,  $47 \pm 9\%$  for

300 mg/L  $\text{Cl}^-$ , and  $59 \pm 8\%$  for 900 mg/L  $\text{Cl}^-$  (Fig. 4). The proportion of bent tails in *P. crucifer* tadpoles was 0 in the 8 mg/L  $\text{Cl}^-$  treatment,  $15 \pm 3\%$  in the 300 mg/L  $\text{Cl}^-$ , and  $30 \pm 6\%$  in the 900 mg/L  $\text{Cl}^-$  (Fig. 6). The data for *P. crucifer* is included for discussion, but was not analyzed due to a problem with control treatments in the *P. crucifer* toxicity tests. Control treatments developed a fungal infection and were replaced with controls from a parallel experiment.

Hatching success in *L. clamitans* eggs was  $98 \pm 2\%$  for 8 mg/L  $\text{Cl}^-$  treatment,  $90 \pm 3\%$  for 300 mg/L  $\text{Cl}^-$ , and  $85 \pm 6\%$  for 900 mg/L  $\text{Cl}^-$  (Fig. 4). There were no differences in hatching success among the 3 salt treatments ( $F_{[2, 9]} = 2.5$ ,  $p = 0.13$ ). Elevated salt concentrations did not affect *L. clamitans* egg hatching success and no tadpole anatomical abnormalities were observed.

Hatching success for *A. americanus* eggs was  $85 \pm 6\%$  for 8 mg/L  $\text{Cl}^-$  treatment,  $53 \pm 13\%$  for 300 mg/L  $\text{Cl}^-$ , and  $77 \pm 14\%$  for 900 mg/L  $\text{Cl}^-$  (Fig. 4). Significant differences in hatching success were observed among the 3 salt treatments ( $F_{[2, 9]} = 2.0$ ,  $p = 0.2$ ). Tail anomalies were also observed in *A. americanus* tadpoles. The proportion of bent tails in *A. americanus* tadpoles was 0 in the 8 mg/L  $\text{Cl}^-$  treatment, 0 in the 300 mg/L  $\text{Cl}^-$ , and  $41 \pm 5\%$  in the 900 mg/L  $\text{Cl}^-$  (Fig. 6). The differences in tail abnormalities that were observed for *A. americanus* among the 3 salt treatments differed significantly ( $F_{[2, 9]} = 76.0$ ,  $p < 0.0001$ ).

### ***Behaviour Experiments***

Unexposed *L. sylvaticus* tadpoles decreased swimming activity and moved to less open habitat in the experimental vessel when confronted with the newt predator (Fig. 7).

The salt exposed *L. sylvaticus* tadpoles did not alter their swimming activity or move to less open habitat when confronted with a predator. MANOVA testing for the non-salt exposed tadpole experiment indicated a significant overall Hotelling test ( $F_{[2, 3]} = 9.6$ ,  $p = 0.049$ ) and significant differences in tadpole behaviour between the predator and non-predator trials for swimming ( $F_{[1, 4]} = 13.8$ ,  $p = 0.02$ ) and open habitat selection ( $F_{[1, 4]} = 12.6$ ,  $p = 0.02$ ). MANOVA testing for the salt exposed tadpole experiment indicated a non significant overall Hotelling test ( $F_{[2, 3]} = 0.003$ ,  $p = 0.99$ ) and non significant differences in tadpole behaviour between the predator and non-predator trials for swimming ( $F_{[1, 4]} = 0.002$ ,  $p = 0.94$ ) and open habitat selection ( $F_{[1, 4]} = 0.003$ ,  $p = 0.99$ ). Salt exposed *L. sylvaticus* tadpoles did not change their behaviour in the presence of a predator where non-salt exposed tadpoles decreased swimming and moved out of the open habitat.

### ***Cyanide Acute Toxicity Tests***

The trimmed Spearman-Kärber method was used to determine median lethal concentrations (96 hour  $LC_{50}$ ) (Fig. 8). No mortalities occurred in any of the controls for all five species. Median lethal concentration values with confidence intervals For *L. sylvaticus*, *L. clamitans*, *P. crucifer*, *A. americanus*, and *A. maculatum* were calculated as 1039.26  $\mu\text{g/L}$  (95% CI: 870.93- 1240.12), 2448.11  $\mu\text{g/L}$  (95% CI: 1884.15- 3180.87); 2566.85  $\mu\text{g/L}$  (95% CI: 1809.86- 3640.46), 4527.05  $\mu\text{g/L}$  (95% CI: 3630.42- 5645.13) and 11471.64  $\mu\text{g/L}$  (95% CI: 10173.79- 12935.06), respectively (Table 1). Tadpoles in the higher treatments displayed some degree of toxicity/abnormalities. Observed behavioral abnormalities included slower responses to stimuli (prodding) and overall reduction in activity levels such as swimming, as well as erratic circular swimming and

abnormal floating on back or sides. These effects became more pronounced with increasing cyanide concentrations.

### ***Road Runoff Water***

Elevated chloride concentrations were measured in runoff from each site. Concentrations ranged greatly from 90 to 44,479 mg/L (Table 2). The highest value was recorded from a sample of water that was adjacent to a salt storage facility and this level exceeds that of marine water by about 50%. This value is high enough to elicit an acute toxic response from most aquatic organisms (Adolph, 1925; Yancey, 2005).

Cyanide was also detected in runoff from all of the sites. Concentrations ranged from 3 to 52 µg/L (Table 2). Five out of 11 sites contained values that exceeded the Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life (5 µg/L cyanide) (Canadian Council of Ministers of the Environment, 2007). Seven of the 11 sites contained values high enough to impair reproductive functioning in freshwater fish (Kimball et al., 1978). There was a general increasing trend in cyanide concentration in runoff water with increasing chloride concentration (Table 2). Linear regression indicates a significant relationship between chloride and cyanide in runoff ( $F_{[1,9]} = 12.2$ ,  $p = 0.007$ ,  $r^2 = 0.53$ ).

## **Discussion**

### ***Chronic Toxicity Experiments***

#### ***Mortality***

Chronic exposure to ecologically relevant levels of NaCl reduced the survival of larval *A. maculatum* and *L. sylvaticus*. These two species experienced increased mortality at a level well below their determined median lethal concentration values (Collins and

Russell, 2009; Sanzo and Hecnar, 2006). Previous research has identified 96 hour LC<sub>50</sub> values of 2837.69 mg/L (Collins and Russell, 2009) and 2636.5 mg/L for *L. sylvaticus* tadpoles (Sanzo and Hecnar, 2006). *A. maculatum* has been identified as being even more sensitive, with 96 hour LC<sub>50</sub> value for larvae calculated to be 1942.15 mg/L (Collins and Russell, 2009). Rates of increased mortality for *A. maculatum* larvae were especially pronounced, from 0 in the 8 mg/L treatments to 40 and 70% mortality in the 300 mg/L and 900 mg/L treatments respectively. It is therefore expected that *A. maculatum* mortality due to road salt runoff is occurring in natural roadside ponds. *A. maculatum* and *L. sylvaticus* are known to be sensitive to elevated Cl<sup>-</sup> concentrations in ponds (Collins and Russell, 2009; Karraker, 2006; Turtle, 2000). Sanzo and Hecnar (2006) reported significantly reduced survival for *L. sylvaticus* exposed to chronic salt exposures ranging from 0 to 1030 mg/L NaCl. Embryonic survivorship of both *A. maculatum* and *L. sylvaticus* was impaired in vernal pools near roads in New York (Karraker, 2006; Turtle, 2000). *A. maculatum* and *L. sylvaticus* have been noted to be absent in ponds containing elevated chloride concentrations in Nova Scotia (Collins and Russell, 2009). Decreased survivorship for other amphibian species exposed to chronic salt experiments have been reported (Chinathamby et al., 2006; Christy and Dickman, 2002). Moderate levels of salinity significantly reduced survival of brown tree frog (*Litoria ewingii*) larvae; 39% survived to metamorphosis in 16% seawater compared to 92% in the control treatments (Chinathamby et al., 2006). Increased mortality was observed for tadpoles of the green and golden bell frog (*Litoria aurea*) when they were subjected to concentrations of 5.5% and greater of saltwater (Christy and Dickman, 2002).

Larval *A. americanus* mortality was not statistically significant, but exhibited an

increasing trend in the higher salt concentrations. Many toad species have exhibited exceptionally high tolerances to elevated salt concentrations (Collins and Russell, 2009; Dougherty and Smith, 2006; Gomez-Mestre et al., 2004; Gordon, 1962; Liggins and Grigg, 1985). The 96 hour LC<sub>50</sub> determined for *A. americanus* was 6471.5 mg/L NaCl (Collins and Russell, 2009). Some toad species such as the green toad (*Pseudepidalea viridis*), the natterjack toad (*Epidalea calamita*) and the cane toad (*Chaunus marinus*) are considered euryhaline (capable of breeding in brackish conditions) (Gomez-Mestre et al., 2004; Gordon, 1962; Liggins and Grigg, 1985). Adult *P. viridis* has been reported to prefer salinities below 8% but can tolerate salinities up to 19 and 23% (Gordon, 1962). Adult *C. marinus* tolerate up to 40% seawater (Liggins and Grigg, 1985). Dougherty and Smith (2006) found *A. americanus* to be unaffected by the salinity concentrations of NaCl and MgCl<sub>2</sub> in 7 day toxicity tests, while *L. clamitans* survivorship was found to be negatively correlated with concentrations of both type. The relatively high tolerance levels to salt reported in toad species could be caused by adaptation to occupying coastal habitats that receive inputs of salt from marine water; it could also be due to evolutionary adaptation to toad oviposition in highly ephemeral ponds (Gilhen, 1984).

The results of this research demonstrate that there are variations in tolerance levels and physiological responses to salt between different amphibian species. Variations in life histories and adaptations between species can account for these differences. Studies have investigated osmoregulation in amphibian species displaying high tolerance to salinity in an attempt to identify the mechanisms responsible. This has been accomplished by acclimating individuals to high salt environments and analyzing the solutes contributing to plasma osmotic pressure (Gordon, 1962; Katz, 1973; Konno et al.,



2006; Liggins and Grigg, 1985). Blood hypertonicity, enhanced by electrolytes and urea enables a few amphibian species to tolerate high saline environments. Experiments have shown that sodium, chloride and urea are the main solutes that contribute to plasma osmotic pressure (Katz, 1973; Liggins and Grigg, 1985). At high salinities, ionic concentrations in plasma rise with increasing environmental concentrations (Gordon, 1962). Additionally, the rate of urine production is considerably reduced (Liggins and Grigg, 1985). Immersion of *P. viridis* in high saline solutions (500 mOsm NaCl) following acclimation to tap water caused immediate cessation of urine flow; immersion of toads which had been adapted to the high saline environment to tap water caused a large increase in urine flow (Shpun and Katz, 1999). In another study, *P. viridis* was gradually adapted to high salinities, up to 800 mOsm NaCl solutions (animals were considered adapted after survival for 10 days); following adaptation, osmotic concentration of blood was analyzed. Blood was always hypertonic to external solutions and had high urea concentrations, urea and sodium concentrations in urine also increased in toads adapted to high salinities (Katz, 1973). Most larval anurans are considered strictly ammonotelic until completion of metamorphosis, because the thyroid hormone is required to activate the urea cycle at metamorphic climax (Gormez-Mestre et al., 2004). There are however, a few exceptions to this rule, and it is suggested that some species have been shown to use urea as an osmolyte throughout the larval phase (Gormez-Mestre et al., 2004). In a study investigating the effects of water salinity on *E. calamita* tadpoles, it was found that although these tadpoles are an exception in that they do produce urea, they do not use it as an osmolyte, and that the increase in osmolality observed is completely contributed to increased sodium and chloride body concentrations (Gormez-

Mestre et al., 2004).

Mechanisms that contribute to the build up of urea in plasma include increased urea synthesis (by increase of ornithine-urea cycle enzymes) and increased urea reabsorption in the kidney (Katz, 1973). These studies demonstrate the various physiological mechanisms some amphibian species have evolved to deal with osmotic stress in their environments. This variation in tolerance levels and adaptability which exists between species gives us insight into how communities are structured around such environmental stressors.

Toxicological research has often focused exclusively on determining the acute toxicity of a contaminant. Acute experiments are characterized by brief, elevated exposure times. In this study, *A. maculatum* and *L. sylvaticus* experienced higher mortality rates when exposed to 900 mg/L compared to controls. These results demonstrate that low levels of contamination, well below LC<sub>50</sub> values can inflict mortality when experienced chronically. This is an important consideration, since environmental safety standards regarding habitat contamination are based on LC<sub>50</sub> values of species (Gad and Chengelis, 1988).

#### *Length of Larval Period*

The developmental transformation of tadpole into adult is an intricate process, governed by complex interactions between an organism's endocrine system and environmental stimuli (Denver, 1997; Griffiths, 1997; Hayes, 2000). The growth and development of an organism is highly influenced by a variety of habitat characteristics (Griffiths, 1997). Not surprisingly, many environmental contaminants alter endocrine activities in amphibians, including developmental rates (Hayes, 2000).

In this study, *A. maculatum* larval period was significantly lengthened as salt concentrations increased but *L. sylvaticus* and *A. americanus* larval periods were not significantly affected by salt exposure. Other studies have described reduced developmental rates for other amphibians exposed to chronic salt toxicity tests (Chinathamby et al., 2006; Christy and Dickman, 2002; Gomez-Mestre et al., 2004). Tadpoles of *Litoria aurea* experienced mortality, decreased growth and inability to reach metamorphosis when subjected to concentrations of 5.5% and greater of saltwater (Christy and Dickman, 2002). Time taken to reach metamorphosis was greater for tadpoles of *Litoria ewingii* exposed to 16% seawater (84 days) than for control tadpoles (55 days) (Chinathamby et al., 2006). The natterjack toad (*E. calamita*) is capable of breeding in brackish waters; however, even these toads experienced delayed metamorphosis of between 4-9 days in high salinities compared to controls when subjected to elevated salt concentrations (Gomez-Mestre et al., 2004).

Delays in metamorphosis could be caused by disruptions to hormonal pathways (Gomez-Mestre et al., 2004). The amphibian endocrine system follows the general vertebrate pattern: peripheral endocrine tissues receive hormone messages from the pituitary, which is controlled by releasing hormones from the hypothalamus, controlled by the central nervous system (CNS), which is influenced by environmental stimuli (Hayes, 2000). The amphibian system does exhibit some unique qualities (Hayes, 2000). Throughout the process of metamorphosis, tissues and organs undergo modifications as larval structures are dissolved and reorganized to create new adult features (Shi, 2000). This sophisticated process is controlled predominantly by thyroid hormones (TH). Circulating plasma TH levels increase throughout development and peak at

metamorphosis (Gutleb et al., 2000; Shi, 2000). This system can be disrupted by environmental substances; for example, contaminants that mimic sex steroids inhibit thyroid hormonal functioning (Hayes, 2000). Research has shown that metamorphosis fails to occur when TH synthesis is blocked (Gutleb et al., 2000; Shi, 2000). Results from immunoassays of thyroid hormone content suggest that chloride may repress hormones in amphibians, disrupting normal development (Gomez-Mestre et al., 2004). Another explanation is that tadpoles under high salinities allocate greater energy towards osmoregulation (Gomez-Mestre et al., 2004). Larvae of *E. calamita* exposed to saline conditions had significantly lower levels of glucose and total proteins measured from whole animal extracts (Gomez-Mestre et al., 2004). The authors suggest this could be a result of either lower metabolism rates in stressful conditions, or because of direct allocation of energy expenses towards osmoregulation (Gomez-Mestre et al., 2004).

Prolonging time to reach metamorphosis can have catastrophic effects on amphibian populations (Brodie and Formanowicz, 1983; Denver, 1997). Timing of metamorphosis is highly structured around environmental characteristics such as temperature and pond desiccation. Prolonging developmental time could result in individuals not reaching metamorphosis before the habitat dries up, or before temperatures drop too low (Denver, 1997; Gilhen, 1984). Additionally, delayed metamorphosis increases the length of time spent during the animal's most vulnerable stage, as larval stages are more susceptible to predation and toxicity from aquatic pollution (Brodie and Formanowicz, 1983).

Amphibians often exhibit great variation in response to environmental stressors (Pfenning et al., 1990; Van Buskirk and Schmidt, 2000). As previously described, salt exposure has resulted in prolonging the larval period, thus delaying metamorphosis for *A.*

*maculatum* larvae. Previous research has described similar responses for other amphibian species (Chinathamby et al., 2006; Christy and Dickman, 2002; Gomez-Mestre et al., 2004). However, there have been some reports of chronic salt exposure reducing the length of the larval period, resulting in individuals reaching metamorphosis in decreased time periods (Sanzo and Hecnar, 2006). Sanzo and Hecnar (2006) found that individuals of larval *L. sylvaticus* that did reach metamorphosis in high NaCl treatments did so at increased rates. Different species may exhibit varied responses depending on adaptations to particular life histories. Many amphibian species exhibit high degrees of phenotypic plasticity influenced by environmental conditions during larval development (Denver, 1997; Griffiths, 1997; Pfennig et al., 1990). This characteristic allows populations to respond adaptively to unpredictable environments. Griffiths (1997) makes an interesting point that the amphibian biphasic life cycle is a characteristic that makes the group sensitive to environmental change, but also represents an adaptation allowing the exploitation of these unpredictable and ever-changing habitats. For example, predator stress in an environment has been shown to alter behaviour and tail morphology in some species (Van Buskirk and Schmidt, 2000). Some amphibian larvae have been shown to develop larger, more muscular tails when exposed to chemicals signaling predation risk and individuals possessing this characteristic were shown to have increased survivorship rates due to enhanced swimming performance (Van Buskirk and Schmidt, 2000).

*Scaphiopus multiplicatus* tadpoles are known to switch between alternative morphologies (carnivorous or non-carnivorous) dependent on resource limitations and habitat characteristics (Pfennig et al., 1990).

Seasonal drying and filling patterns of temporary wetlands fluctuate with

environmental conditions, making them unpredictable habitats (Denver, 1997; Griffiths, 1997; Wilbur, 1997). Many amphibian species breed in shallow ponds that are highly susceptible to desiccation and their larvae frequently experience catastrophic mortality in these temporary environments (Griffiths, 1997). *L. sylvaticus* is one amphibian species that is known to select temporary, vernal ponds for breeding and larval development (Gilhen, 1984). Local populations inhabiting temporary ponds are under time restrictions to reach metamorphosis before the natal pond completely evaporates. Many amphibian species have adapted to exploit these habitats by evolving flexible life histories that respond to the unpredictable patterns of these types of ponds (Griffiths, 1997). Some species have adapted to the threat of pond desiccation by accelerating development rates when experiencing osmotic stress (Smith, 1987). Pond desiccation results in enhanced ionic concentrations. In such situations, tadpoles probably have a physiological mechanism that associates osmotic stress with changes in water level (Sanzo and Hecnar, 2006). Development will be accelerated if desiccation risk is high, and may be prolonged if the risk is low (Denver, 1997; Griffiths, 1997). For example, spadefoot toads (*Scaphiopus* spp.) are known to develop in harsh environmental conditions and larva can accelerate metamorphosis as the habitat dries, thus escaping mortality (Denver, 1997; Griffiths, 1997). A link between the neurohormonal stress pathway and response to pond desiccation has been demonstrated (Denver, 1997). Tadpoles subjected to habitat desiccation exhibited elevated CRH levels. When tadpoles were experimentally administered CHR injections, whole body thyroid hormones and corticosterone (CORT) (metamorphosis regulators) were elevated and development was accelerated (Denver, 1997). As previously described, metamorphosis is controlled by circulating TH

hormones, which are derived from the amino acid tyrosine (Shi, 2000). There are two naturally occurring forms of TH hormones: thyroxine ( $T_4$ ) and converted from this, the more potent form, triiodothyronine ( $T_3$ ) (Raven and Johnson, 2002; Shi, 2000). CORT has been demonstrated to increase TH activity by enhancing  $T_3$  binding to its receptor (Hayes, 2000; Suzuki and Kikuyama, 1983). Thus, this system monitors both external and internal stimuli, transducing environmental information into a physiological response (Denver, 1997).

Populations of organisms that possess high degrees of developmental plasticity may consequently exhibit accelerated rates of local adaptation (Johansson et al., 2001). Amphibians commonly display high site fidelity and low movement rates throughout their lifecycles (Blaustein et al., 1994). It is therefore possible that over a species' entire range, local populations may develop slight alterations to lifecycle and physiological characteristics in response to distinct qualities of their habitats. This could explain why individuals of the same species from different localities exhibit varied responses to environmental stressors. In this study, *L. sylvaticus* larval period was not affected by salt exposure. These animals were collected from temporary ponds within the municipality of Halifax, Nova Scotia. Alternatively, Sanzo and Hecnar (2006) demonstrated that chronic salt exposure reduced the larval period for *L. sylvaticus*, thus accelerating the time to reach metamorphosis. Animals used in their study were collected from ponds in Thunder Bay, Ontario. It is probable that the two populations have historically encountered different degrees of particular environmental stressors, therefore shaping how individuals respond under certain conditions. An intriguing study conducted by Johansson et al. (2001) in Scandinavia compared nitrate tolerance between different populations of the

common frog (*Rana temporaria*). Northern Scandinavian populations were known to experience less exposure to anthropogenic nitrate sources compared to southern populations. When representatives from the two groups were exposed in the laboratory, high nitrate concentrations were found to reduce growth rates and metamorphic size in the northern populations, but not in the southern ones (Johansson et al., 2001). These results suggest that *R. temporaria* populations in the south have adapted greater tolerance to higher than natural nitrate levels. It is possible that Nova Scotian freshwater systems have naturally higher mineral or salt loads than systems around Thunder Bay, Ontario. Another explanation could be the possibility that temporary ponds in this region of Ontario are more prone to desiccation. The average annual precipitation for the Thunder Bay area is far less (711.6 mm) than that for Halifax municipality (1508 mm) (Environment Canada, 2009). In the case of the later suggestion, *L. sylvaticus* populations from Thunder Bay may have adapted to high desiccation risk by accelerating developmental time in order to escape the drying environment.

#### *Weight at Metamorphosis*

*A. maculatum* metamorphic weight decreased significantly as salt concentration increased. Although not statistically significant, *A. americanus* larvae exhibited a decreasing trend in metamorphic weight with increasing salt treatments. *L. sylvaticus* metamorphic weight was unaffected by salt concentration. Gomez-Mestre et al., (2004) also reported a significant reduction in weight of *E. calamita* larvae as salinity increased. This is an especially interesting effect because the larval period was also prolonged in the higher concentrations. As previously mentioned, it has been suggested that chloride may repress hormones in amphibians, disrupting normal development (Gomez-Mestre et al.,



2004). This could account for reduced size, despite an overly long larval period. Another explanation could be due to the devotion of more energy towards osmoregulation in elevated salt concentrations (Gomez-Mestre et al., 2004).

Chronic exposures to other types of chemical contaminants have decreased the weight of amphibian species (Howe et al., 2004; Loumbourdis et al., 1999). Cadmium exposure resulted in increased mortality and decreased mean body weight of *Rana ridibunda* larvae (Loumbourdis et al., 1999). In another study, chronic exposure to environmentally relative concentrations of the pesticide Roundup® (active ingredient: glyphosate, Monsanto) reduced the body size at metamorphosis, increased time to metamorphosis, and induced physiological and gonadal abnormalities in *L. pipiens* tadpoles (Howe et al., 2004).

Reductions in metamorphic size can significantly reduce an individuals' fitness in many ways (Semlitsch et al., 1988; Smith, 1987). Juveniles of large size at metamorphosis have been found to maintain their size advantage and become larger anuran and caudate adults (Semlitsch et al., 1988; Smith, 1987). Smaller individuals are competitively inferior to larger conspecifics, and are less likely to survive winter hibernation. Large body size increased survivorship to maturity for the chorus frog, *Pseudacris triseriata* (Smith, 1987). An eight year study investigating how timing and size at metamorphosis relates to adult fitness in the salamander *Ambystoma talpoideum* found that larger females reached sexual maturity and reproduced at a younger age than smaller individuals (Semlitsch et al., 1988). As an additional advantage, individuals of larger sizes have been shown to have decreased predation risks (Brodie and Formanowicz, 1983; Eklöv and Werner, 2000).

### ***Embryonic Toxicity Experiments***

*L. clamitans* and *A. maculatum* egg hatching success was not affected by exposure to the salt concentrations tested (Fig. 4). *L. clamitans* is known to be tolerant of Cl<sup>-</sup> (Dougherty and Smith, 2006). Chronic exposures of *L. clamitans* eggs to chloride in this study confirmed this finding. *A. maculatum* larvae are considered to be highly sensitive to salt exposure. Chronic Cl exposure increased mortality (Fig. 1), decreased the developmental rate (Fig. 3), and decreased the body weight at metamorphosis (Fig. 2). Acute toxicity testing identified *A. maculatum* as being the most sensitive species tested (Collins and Russell, 2009). *L. sylvaticus* eggs exhibited decreased hatchability (Fig. 4) and increased incidence of developmental anomalies with increasing salt concentrations (Fig. 5). *L. sylvaticus* larvae have exhibited sensitivity to salt exposure, experiencing increased mortality when raised in sub-lethal NaCl treatments (Fig. 1), and were the most sensitive anuran species tested in previous acute toxicity testing (Collins and Russell, 2009). Exposure to salt also reduced hatching success in *A. americanus* eggs (Fig. 4), and induced developmental malformations in hatched larvae (Fig. 5). *A. americanus* larvae are considered moderately tolerant of salt exposure compared to other anurans; they were the most tolerant out of five species tested in NaCl acute toxicity experiments (Collins and Russell, 2009). Other toad species have been reported to exhibit high salt tolerances (Dougherty and Smith, 2006; Liggins and Grigg, 1985). These results clearly demonstrate that there are different modes of salt toxicity for the amphibian species tested, with differences existing between distinct species and life stages. Variation in life stage sensitivity of a species have been reported in other toxicology studies (Buhl and Hamilton, 1998; Horne and Dunson, 1995; Koenst et al., 1977). A study by Horne and

Dunson (1995) investigating aluminum toxicity to salamanders discovered that 400µg/L of Al induced high embryonic mortality of Jefferson salamanders and late stage larvae, but found no toxic response to Al in early larval salamanders (Horne and Dunson, 1995). Such effects have also been noted in fish. Due to similarities in physiology and habitat use, responses of larval amphibians and fish can be comparable. Eggs of chinook salmon (*O. tshawytscha*) were more tolerant to acute toxicity of three fire retardant chemicals than fry and juvenile stages (Buhl and Hamilton, 1998). Growth of brook trout (*Salvelinus fontinalis*) was significantly reduced by cyanide exposure in early life stages, but not in adults (Koenst et al., 1977). During these early stages (embryo to juvenile), growth effects became progressively more influenced by cyanide concentration with increase in age, but only up until a certain point in development. The authors stated that this effect is associated with physiological differences in the different life stages (Koenst et al., 1977).

The differences exhibited between species at the embryonic stage could be due to differences in the jelly coats which surround the developing embryo. The embryos of most animals are surrounded by a protective, extracellular matrix through which sperm must transverse (Pough et al., 2005). Amphibian embryos are surrounded by two kinds of this special matrix: the outer jelly layers and the inner vitelline envelope directly surrounding the embryo (Pough et al., 2005). Protection of the embryo is a major function of the jelly, but there are other very important functions as well (such as important roles in fertilization) (Elinson, 1974; Pough et al., 2005).

The form and thickness of amphibian jelly coats can vary widely. For example, many salamanders of the genus *Ambystoma* embed their eggs in thick, firm jelly masses attached to vegetation under the surface of a pond (Gilhen, 1982; Pinder and Friet, 1994).

Other amphibians including many of the anurans, have thinner, more loosely consolidated jelly coats which usually float on the surface or are loosely attached to vegetation (Pinder and Friet, 1994). Many eggs masses are cohabited by the symbiotic algae, *Oophila ambystomatis* found exclusively in amphibian egg masses and primarily in those of *A. maculatum* (Pinder and Friet, 1994). The relationship is clearly symbiotic, as amphibian embryos experience lower mortality, higher growth rates, and earlier hatching times with the algae compared to egg masses without (Hutchison and Hammen, 1958; Pinder and Friet, 1994). Newly hatched larvae reared with algae were found to be 1.3 mm greater in length and >2 developmental stages advanced compared to those reared without algae (Hutchison and Hammen, 1958). It is speculated that the algae benefit from higher CO<sub>2</sub> and ammonia concentrations found inside the egg capsules since the algae grows more vigorously when embryos are present in the jelly (Hutchison and Hammen, 1958; Pinder and Friet, 1994). It has been widely accepted that the advantage acquired by the amphibian embryos is due to increased oxygen supply produced by the algae, as oxygen delivery through the jelly covering can be restricted (Hutchison and Hammen, 1958; Pinder and Friet, 1994). This restriction of oxygen transfer is particularly consequential for species possessing thicker jelly coats. Pinder and Friet (1994) compared the potential for oxygen transport between *A. maculatum* egg jelly and *L. sylvaticus*. Dye was injected into the egg masses of each species to check for water channels (important routes for oxygen delivery). *L. sylvaticus* have loose egg masses, and there were numerous channels throughout the jelly. Within 30 seconds, the dye traveled through the egg mass and exited from the bottom. When dye was injected into *A. maculatum* egg masses, the dye remained fixed in place, and only spread 5-6 mm after six days, and

remained visible six weeks post injection (Pinder and Friet, 1994). The symbiotic relationship between *A. maculatum* egg jelly and *O. ambystomatis* algae must have allowed the salamanders to evolve a thicker, more defensive coat without suffering the consequence of oxygen deprivation. This feature of the *Ambystoma* jelly coat likely explains the tolerance exhibited by embryos to elevated NaCl concentrations, despite the confirmed sensitivity of the larva.

The tail malformation exhibited by *L. sylvaticus* and *A. americanus* (Fig. 5) consisted of a severe, lateral contort at the tail base. This deformity has been described in previous amphibian ecotoxicology studies, and is recognized as a sign of toxicity in larval amphibians (Hecnar, 1995; Karraker, 2007; Sanzo and Hecnar, 2006). The same physical effect was reported by Sanzo and Hecnar (2006) in *L. sylvaticus* from chronic salt exposure. Hecnar (1995) also reported the same anomaly with ammonium nitrate fertilizer. Karraker (2007) reported a similar abnormality in *L. clamitans* tadpoles hatched in salt solutions, and described disfigurations as “dorsal and lateral flexure of the tail”. In response to low pH, Dunson and Connell (1982) report a “curling defect” in embryonic amphibians. Exposure to a carbamate-based insecticide was reported to induce malformations in skeletal tissue of Iberian green frog (*L. perezii*) tadpoles, resulting in deformities to the spinal column and/or limbs (Alvarez et al., 1995). Our results, along with these various accounts, suggest that this particular malformation represents a general reaction to a chemical stressor, which implies that this particular region in a larval amphibian is especially sensitive to developmental disruption.

The majority of individuals that exhibited the tail malformation did not survive three weeks post hatching, and none survived to metamorphosis. Activity levels were greatly

reduced and swimming was exceedingly compromised. Deformed individuals swam in slower, uncoordinated movements and responded to physical stimuli by swimming in erratic, circular motions. Individuals possessing these abnormalities in the wild would likely be unable to forage adequately, and be immediate targets for predation.

### ***Behaviour Experiments***

In the presence of a newt predator, unexposed *L. sylvaticus* tadpoles decreased swimming activity and moved to less open habitat in the experimental chamber (Fig. 7). The behavior and physiology of a species represents adaptations to environmental stressors, and certain traits may be altered when exposed to particular situations. Some amphibian larvae develop large tails when exposed to chemicals signaling predation risk (Van Buskirk and Schmidt, 2000). Individuals possessing this characteristic have increased survivorship rates, probably due to enhanced swimming performance (Van Buskirk and Schmidt, 2000). When in ponds with invertebrate predators, tadpoles of *Hyla versicolor* develop large tails and smaller bodies and decrease time spent feeding and swimming (Van Buskirk and McCollum, 2000). Cope's grey tree frog (*H. chrysoscelis*) tadpoles exhibit developmental plasticity when reared with predatory dragonfly larvae (McCollum and Leimberger, 1997). Differences in *H. chrysoscelis* shape and colour when reared with or without predators were induced by non-contact chemical cues given off by the predators. Differences in morphology are thought to help lower the risk of predation (McCollum and Leimberger, 1997). When reared in the presence of a predator, pool frog (*Rana lessonae*) tadpoles developed larger and more muscular tails than did tadpoles that were raised in a non-predator environment. Predator-induced changes enhanced swimming performance: tadpoles of this species that were reared with a

predator displayed 9.5 and 14.6% faster swimming speeds compared to those raised without a predator (Wilson et al., 2005).

Typical behavioral responses that tadpoles exhibit upon detection of a predation threat include: increasing physical distance from the predator (Relyea, 2001), schooling (Wilbur, 1997), reduction in activity (Skelly and Werner, 1990), and the occupation of safer microhabitats (Smith and Van Buskirk, 1995). Like other animals, tadpoles face a fitness trade off between foraging and anti-predator behaviour. Increased foraging produces larger sized individuals and consequently higher adult fitness, but also results in higher predation risk (Wilbur, 1997). Potential costs of exhibiting anti-predator behaviour involve reduced foraging and feeding, causing slower development rates, which results in smaller sized individuals and/or a prolonged time to reach metamorphosis (Wilbur, 1997). Strong selective pressures are therefore at work to maximize foraging while minimizing predator exposure (Lefcort et al., 1998). It is expected that individual species and populations of amphibians will exhibit varied responses under certain situations, due to their unique evolutionary life histories. The extent that larval survival may compromise adult fitness is dependent on the strength and pattern of selective pressures that the particular population has encountered historically (Wilbur, 1997). If the chances of adult reproduction are greatly diminished by reduction of larval growth and development, then the risk of predation becomes less important (Wilbur, 1997).

Skelly and Werner (1990) investigated the response of *A. americanus* larvae to the presence of a caged odonate predator (*Anax junius*). Tadpoles were raised at four food rations crossed with the non-lethal presence and absence of the predator. Toads metamorphosed at smaller sizes when raised in the presence of *A. junius*, regardless of

food ration (Skelly and Werner, 1990). Tadpole behaviour in regards to spatial distribution and activity was also significantly altered. In presence of *A. junius*, larvae spent more time at the opposite end of container away from the caged predator, and overall activity levels were reduced by 41% (Skelly and Werner, 1990).

Abnormal behaviours were reported during the course of acute toxicity testing of sodium chloride on larval amphibians (Collins and Russell, 2009; Sanzo and Hecnar, 2006). During these experiments, behavioural changes were observed in tadpoles in all of the salt treatments (ranged between 500 to 10 000 mg/L NaCl). These behaviours included reduction in response times, and performance of abnormal activities as well as overall reduction in activity levels. Compared to controls, even tadpoles exposed to the lowest concentration (500 mg/l) displayed slightly altered behaviour (Collins and Russell, 2009). Sanzo and Hecnar (2006) reported similar responses in *L. sylvaticus* larvae exposed to sodium chloride. Effects were magnified as concentrations increased. Behavioural changes observed, such as reductions in activity levels and response time, have the potential to severely reduce the fitness of individuals in the wild. These sub-lethal effects could greatly increase an individual's vulnerability to predation by lowering the larvae's ability to detect and escape from a predator. As well as reducing the tadpole's chance of escape, some of the behaviours observed in the higher concentration treatments may have the potential to attract predators, such as when the animal floats on its back and sides, and the erratic, circular swimming that was observed. Reductions in activity result in decreased foraging and feeding behaviours, which produces smaller individuals. This effect would increase the length of time spent during the animal's most vulnerable stage, as larval stages are more susceptible to predation and toxicity from aquatic pollution



(Brodie and Formanowicz, 1983). In some species, reduced feeding may prolong larval development and result in smaller adults (Rohr et al., 2003). Individuals of smaller sizes have been shown to be more susceptible to predation (Brodie and Formanowicz, 1983). In this experiment, the salt-exposed *L. sylvaticus* tadpoles did not alter their swimming activity or move to less open habitat when confronted with a predator (Fig. 7).

Non-lethal exposure to other environmental contaminants has been shown to disrupt normal activities, altering tadpole behaviour and potentially increasing predation risks (Relyea, 2003; Sanzo and Hecnar, 2006). Lefcort et al. (1998) discovered that Columbia spotted frog (*R. luteiventris*) larvae exhibited decreased anti-predatory behaviour when exposed to various metals. Only the unexposed individuals altered (decreased) activity levels in presence of fish chemical cues. Additionally, the unexposed tadpoles displayed generally higher activity levels (foraging and feeding) than metal exposed tadpoles, both in presence and absence of predator cues (Lefcort et al., 1998). Lead has been reported to affect learning ability in *L. catesbeiana* tadpoles (Nixdorf et al., 1997). Similarly, sub-lethal exposures to carbamate-based insecticides have been shown to disrupt normal activities in plains leopard frog (*Lithobates blairi*) larvae (Bridges, 1997). The exact mechanisms of such pollutant- induced behavioural disruptions have not been identified and described. However, it has been suggested that in some cases alterations were induced in CNS monoamine neurotransmitter systems, with exposed larvae exhibiting significant reductions in serotonin levels compared to controls (Nixdorf et al., 1997).

Modification of predator-prey interactions can severely disrupt population and community dynamics in nature. Depending on predator densities, inhibition of anti-predatory behaviour in amphibian larvae could potentially result in the extirpation of

local populations within a few generations. Although amphibian predators occupy the same aquatic habitats and face the same abiotic environmental conditions as their prey, studies have shown that many amphibian predators possess higher tolerance levels to contamination, as amphibians are often the most sensitive members of a community (Blaustein et al., 1994). Many important amphibian fish predators may have greater salt tolerance than amphibian larvae. The  $LC_{50}$  value of sodium chloride to *O. mykiss* was found to be 11,112 mg/L (Environment Canada, 2001). An aquatic environment containing elevated salt concentrations could potentially reduce tadpole survival rates and severely alter development and behaviour while being relatively benign to their predators. This effect could have serious implications for many species since fish are considered to be a major predator on larval amphibians (Hecnar and M'Closkey, 1998).

Disruption of other normal behaviours can also carry high fitness costs to amphibian populations. Reduced feeding during larval stages results in developmental delays and/or smaller sized adults (Sinsch, 1997). Smaller individuals are associated with decreased adult fitness (Brodie and Formanowicz, 1983). Developmental delays increase the chance of predation and also threaten the ability for individuals to complete life cycles, as larval amphibians are often under time restrictions to escape the drying environment (Brodie and Formanowicz, 1983; Wilbur, 1997).

### ***Cyanide Toxicity Experiments***

*A. maculatum* was the most tolerant of the species tested to NaCN, exhibiting a considerably higher  $LC_{50}$  value (11471.64  $\mu\text{g/L}$ ) compared to any of the anurans. *A. americanus* had the next highest  $LC_{50}$  (4527.05  $\mu\text{g/L}$ ) and *L. sylvaticus* was the most sensitive species tested ( $LC_{50}$  1039.26  $\mu\text{g/L}$ ). The few existing studies on acute toxicity

of cyanide compounds to freshwater fish indicate that fish are much more sensitive than amphibians (Calfee and Little, 2003a; Calfee and Little, 2003b; Smith et al., 1978). A study examining toxic effects of a fire-retardant chemical (GTS-R) containing YPS determined that 128 mg/L of GTS-R released 273 µg/L cyanide within three hours under clear sky conditions, and resulted in 100% mortality of a fathead minnow test group (Calfee and Little, 2003a). Ninety six hour LC<sub>50</sub> concentration value of YPS under 4.0 uW/cm<sub>2</sub> UV light to *O. mykiss* was determined to be 200 µg/L, and was found to be 2600 µg/L for southern leopard frogs (*L. sphenoccephala*) (Calfee and Little, 2003b). This value is consistent with the values we determined for the amphibians tested in the present study, and indicates that *L. sphenoccephala* have slightly higher tolerance than *P. crucifer*. The amphibian species exhibited higher tolerance to the cyanide compound than did the fish species (Calfee and Little, 2003b).

The high tolerance of amphibians to cyanide compared previous studies on fish is an intriguing finding, as fish are generally known to be more resilient to environmental contamination. For example, acute toxicity of HCN varied from 57.00 µg/L for juvenile rainbow trout to 191.00 µg/L for juvenile fathead minnows (Smith et al., 1978). A possible explanation for the high tolerance exhibited by amphibians could be related to the adaptation of amphibians inhabiting bodies of water that have low oxygen conditions, or are prone to seasonal and diurnal hypoxia (Bickler and Buck, 2007; Ultsch et al., 1999). Since cyanide poisoning inhibits aerobic ATP synthesis through inactivation of cytochrome oxidase, its effects can be comparable to those experienced by an organism under hypoxic or anoxic conditions. Hypoxia tolerance in fish species varies greatly; for example, many species such as salmon which rely extensively on aerobic metabolism for

fast, sustained swimming are very sensitive to hypoxia. Alternatively, some fish such as carp and eels have evolved to withstand low oxygen conditions (Bickler and Buck, 2007). Smith and Heath (1979) investigated acute toxicity of four contaminants, one of which was a cyanide compound, to freshwater fish species. Goldfish (*Carassius auratus*) exhibited higher tolerance to cyanide than any of the other species tested (particularly in colder temperatures). The authors suggest this could be explained by the close relation of *C. auratus* to carp (*Carassius carassius*), known for the ability to metabolize anaerobically for long periods of time (Smith and Heath, 1979).

Many amphibian species breed in small, temporary wetland ponds which are devoid of fish predators (Hecnar and M'Closkey, 1998). In order to utilize these niches, amphibians have evolved physiological mechanisms which allow them to cope with the fluctuating (or depleted) oxygen levels typical of these habitats (Bickler and Buck, 2007). It has been suggested that the long term tolerance of fluctuating oxygen levels requires an ability to decrease metabolic rate in order to maintain brain ATP levels (Bickler and Buck, 2007). Amphibians exhibit moderately high tolerance to low oxygen conditions and many anuran species can withstand anoxic conditions for several days, and hypoxic conditions for several months (Bickler and Buck, 2007). Some amphibian species may also have evolved hypoxia tolerance when inhabiting larger ponds containing fish. Ultsch et al. (1999) studied the annual distributions of dissolved oxygen, temperature, fish, and tadpoles to characterize their interactions. They found that in summer, fish distribution was clearly related to oxygen availability, with fish avoiding hypoxic conditions >2 m below the ponds surface. Bullfrog (*L. catesbeiana*) tadpole distribution was not dependent upon oxygen availability. Tadpoles showed a preference for deep, hypoxic

regions of the pond and in these areas, tadpoles switched from facultative air breathing to being obligate air breathers, requiring trips up to the surface for air (Ultsch et al., 1999). The authors suggest that larval amphibians may use the deeper water as a refuge from predators (Ultsch et al., 1999).

As a group, amphibians are recognized for exhibiting great diversity in physiological adaptations and life cycles (Denver, 1997). Consequently, they often demonstrate varied responses to environmental stressors (Denver, 1997). The variability in tolerance levels to acute cyanide exposure between the species tested is a reflection of each species' unique evolutionary life history. It may have been adaptive for certain species to better withstand fluctuating oxygen levels, which consequently results in higher cyanide tolerance. *A. maculatum* was found to be the most tolerant species tested. Through personal observation, it was noted that *A. maculatum* larvae spend considerably more time resting on the bottom of ponds, compared to anuran tadpoles, which spend more time actively swimming and foraging. This observation could be an indication that salamander larvae may be better equipped for anaerobic metabolism due to decreased periods of prolonged swimming and increased time spent in the most potentially hypoxic area of an aquatic habitat. Additionally, larval salamanders possess external gills which could aid in the acquisition of oxygen under low conditions.

Determining the acute effects of a contaminant is the first step in characterizing its toxicity to an organism. However, it is only part of the story, and a comprehensive investigation of chronic, sub-lethal effects is essential. A range of sub-lethal effects produced from low or chronic cyanide exposure have been described for various freshwater fish species, and include alterations to survival rates, growth, development,

and reproduction (Eisler, 1991; Koenst, et al., 1977; Leduc, 1978; Lind et al., 1977; Ruby et al., 1986). Due to the lack of toxicity data on cyanide compounds to amphibian species, results from studies on fish are worthy of consideration since fish and larval amphibians often share similarities in physiology and habitat choice. Although our results suggest that amphibians are more tolerant than fish to acute cyanide exposures, chronic data from fish may still be relevant and comparable to the potential effects occurring to amphibians.

Reproductive impairments are devastating consequences of chronic cyanide toxicity. Reduced viability of eggs, disruption of essential reproductive process, inhibition of endocrine functions, and inducing developmental malformations are examples of reproductive effects experienced by some fish species exposed to sub-lethal cyanide concentrations (Eisler, 1991; Lind et al., 1977; Kimball et al., 1978; Ruby et al., 1986; Ruby et al., 1979; Szabo et al., 1991). Reproductive impairment was reported to occur in adult bluegill fish (*Lepomis macrochirus*) at levels of 5.2 µg/L CN/L in 289 days (Environmental Protection Agency, 1984). Spawning by *L. macrochirus* was inhibited by chronic exposure to levels as low as 5 µg/L (Kimball et al., 1978). Low levels of HCN (10 µg/L HCN for 12 days) decreased the number of eggs spawned and reduced egg viability in female *O. mykiss* (Ruby et al., 1986). An earlier experiment demonstrated that exposure of juvenile male *O. mykiss* to low levels of HCN (0.01 and 0.03 mg/L HCN for 18 days) inhibited spermatogenesis, thus suggesting that low cyanide exposure acts as an anti-mitotic agent (Ruby et al., 1979). Szabo et al. (1991) examined the interactions between brain hormone levels and gonadal development during sub-lethal cyanide exposure. Whole brain DA levels in *O. mykiss* exposed to 0.01 mg/L HCN for 12 days

were significantly higher than controls. Additionally, mean diameters of oocytes from exposed females were significantly decreased, and testes from males had significantly greater amounts of spermatogonial cysts (Szabo et al., 1991). It is known that gonadotropin hormones (GTH) are partly regulated by dopamine (DA), which acts as a gonadotropin release inhibitory factor (GRIF) (Chang and Peter, 1983). The researchers suggest that the higher brain DA levels brought on by low cyanide levels would result in reduced GTH release, and that lower plasma GTH could result in the ovarian structural abnormalities and delayed growth (Szabo et al., 1991). The increased spermatogonial cysts could be explained by the inhibition of the entry of the spermatogonia to the spermatocyte stage during maturation (Szabo et al., 1991). Lind et al., (1977) reported that the number of eggs produced per female of *P. promelas* was significantly reduced at 19.6 µg/L HCN and higher, and egg hatching success was significantly decreased at 44.2 µg/L. Developmental abnormalities occurred in embryos of *Salmo salar* exposed to 10 µg/L HCN/L, and included malformations in yolk sacs, eyes, mouth, and vertebral columns (Leduc, 1978). Spawning was delayed in *S. fontinalis* and number of eggs spawned per female was decreased with increasing HCN concentrations above 5.7 µg/L (Koenst, et al., 1977).

It is clear from these studies that very low concentrations of cyanide can inflict devastating effects to fish populations. It is known that amphibians are sensitive to physiological and endocrinological disturbances (Hayes, 2000; Hayes and Menendez, 1999). Since there have been no studies conducted on chronic toxicity of cyanide compounds to amphibian species, it is important to consider the effects reported to occur in various fish species. Tolerance levels to environmental pollutants often vary between

and within these two animal classes, but modes of toxicity and potential population effects may be comparable in certain situations.

Evaluating the threat of cyanide contamination in the aquatic environment is a complex endeavor. Numerous factors influence photo-enhanced toxicity in the natural environment, such as: solar angle, time of day, season, air pollution, clouds; surface reflection and water quality (Calfee and Little, 2003b). Under dark conditions, YPS produced LC<sub>50</sub> values of 2.40 mg/L for *O. mykiss* and 99.3 mg/L for *L. sphenoccephala* tadpoles, compared to the LC<sub>50</sub> values produced under UV conditions of 0.200 mg/L and 2.60 mg/L, respectively (Calfee and Little, 2003a). Lethality of HCN to juvenile *O. mykiss* was shown to vary seasonally and with exercise (swimming at one body length/second) (McGeachy and Leduc, 1988). During summer, non-exercised trout had 96 hour LC<sub>50</sub> values of 0.055 mg/L HCN, which dropped during the winter by greater than 20% to 0.043 mg/L HCN for non-exercised. Exercised fish during the winter survived twice as long as the non-exercised, producing an LC<sub>50</sub> value of 0.052 mg/L HCN (not significantly different from non-exercised summer fish LC<sub>50</sub>s). Smith et al. (1978) found HCN to be more toxic to fish under lower temperatures and oxygen conditions; however, Smith and Heath (1979) found that goldfish exhibited higher tolerance to cyanide under lower temperatures.

What is apparent is the lack of research on effects of cyanide anti-caking agents in the environment, especially in regards to effects on amphibian populations. Given the large and increasing use of road salt in North American, the environmental fate and effects of ferrocyanide in road salt clearly merits further study.

### ***Road Runoff***



Chloride in road runoff samples collected in March ranged between 85 to 46,716 mg/L. The latter value was obtained from a sample adjacent to a salt storage facility. This chloride concentration exceeds the levels of marine water by about 50% and could elicit a lethal response from virtually any freshwater organism (Adolph, 1925). Salt pathways are not well studied or quantified (Buttle, 1999). Ohno (1990) measured Cl, Na, and CN levels in surface waters adjacent to sand/deicing salt storage facilities, and reported Cl concentrations ranging up to 13 500 mg/L. Environment Canada (2001) has reported chloride concentrations of 4000 mg/L in ponds and wetlands and 5000 mg/L in urban lakes. Novotny et al. (1999) reported over 18 000 mg/L of chloride measured in road runoff. The authors concluded that the flood of such highly concentrated runoff into freshwater systems could elicit toxic shock responses in aquatic organisms (Novotny et al., 1999). Most of the deicing salt applied to roads reaches adjacent habitats through direct runoff or snowmelt. Peak Na<sup>+</sup> concentrations were measured at 6506 mg/L<sup>-1</sup> in snow banks adjacent to a highway in southern Ontario, and Cl<sup>-</sup> was measured up to 9916 mg/L<sup>-1</sup> (Buttle, 1999). Effects of such highly concentrated runoff are system dependent. The larger the body of water the runoff enters, the more diluted it will become. Alternatively, many small temporary ponds are formed directly from runoff of snowmelt (Mozley, 1932; Williams, 2006). In a study investigating effects of salty runoff into nearby streams, elevated Cl levels were measured at 31 times the amounts measured in upstream samples (for both 50 and 100 m downstream sampling locations), indicating a much larger effect zone than previously assumed (Demers and Sage, 1990). Additionally, levels remained elevated six months following the termination of winter deicing salt (Demers and Sage, 1990). Another factor worthy of consideration is that saline water is

denser than freshwater (Adolph, 1925). Highly saline runoff entering a freshwater body may then, be expected to sink. Indeed, some studies have reported salt-induced stratification in lakes due to deicing salt application (Jones and Jeffery, 1992). Salt contaminated runoff alters the physical and ecological characteristics of a lake (Ramakrishna and Viraraghavan, 2005). Salty runoff entering a lake changes the density of the inflow, so that inflow appears at the lakes bottom, resulting in an interruption of natural overturn. In a normal lake ecosystem, the mixing of water layers is essential for the distribution of oxygen and nutrients (Jones and Jeffery, 1992; Ramakrishna and Viraraghavan, 2005). The incomplete mixing of salt layers in a lake creates layers of different densities causing oxygen depletion (Jones and Jeffery; Judd, 1970). Salt induced stratification in a lake in Michigan created hypoxia in deeper areas of the lake, posing a hazard to plant and animal life (Judd, 1970; Ramakrishna and Viraraghavan, 2005).

Inflows of NaCl can become involved in ion exchange with certain compounds and elements that are bound to sediments, resulting in the mobilization of many undesirable elements (Amrhein and Strong, 1990; Backstrom et al., 2004). When in the absorbed state and bound to soil particles, toxic metals (such as mercury) are biologically unavailable (Backstrom et al., 2004). In the dissolved state, metals are bioavailable and exhibit toxicity to organisms (Amrhein and Strong, 1990; Backstrom et al., 2004). Chloride ions influence the state of absorbed and dissolved metals, increasing freely dissolved metal concentrations and thus bioavailability of these compounds (Backstrom et al., 2004).

Free cyanide concentrations measured at the runoff sites ranged from 2 to 71 µg/L. Some sites contained levels high enough to induce acute and chronic on freshwater fish

species (Eisler, 1991; Lind et al., 1977; Kimball et al., 1978; Ruby et al., 1986; Ruby et al., 1979; Szabo et al., 1991). Thirty percent of the samples collected contained free CN concentrations above Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life (5 µg/L cyanide) (Canadian Council of Ministers of the Environment, 2007). Ohno (1990) measured total cyanide concentrations ranging up to 200 µg/L CN in surface waters adjacent to sand/deicing salt storage facilities in Maine.

Studies of ferrocyanide effects on water quality are limited (Paschka et al., 1999). Levels of free cyanide measured at four salt distribution piles along the Mississippi River in Minnesota were as much as 40 times the maximum acute toxicity standard for the preservation of aquatic life (Oberts et al., 2000). Elevated concentrations of sodium chloride (NaCl) and total cyanide were measured in surface waters adjacent to four highway salt storage facilities in Maine. Sampling locations chosen consisted of channels or bogs adjacent to the salt piles, and upstream and downstream locations in brooks running by. Samples were collected monthly for one year. Significant levels of total cyanide were measured in adjacent surface waters at all sites, and it was concluded that HCN levels resulting from photodecomposition would exceed the US Environmental Protection Agency guidelines for the protection of aquatic life and drinking water quality (Olson and Ohno, 1989). Interestingly, although significantly elevated, the observed cyanide concentrations were less than predicted based on observed sodium concentrations. For comparison, it had been determined that there is 11.2 µg cyanide/g of road salt (Olson and Ohno, 1989). This observation was attributed to the suspected binding of cyanide to sediments. Due to elevated levels of free and total cyanide levels detected, it was advised that salt piles be covered to prevent leaching into nearby water

systems (Olson and Ohno, 1989).

As part of an investigation into the effects of winter snow melt in urban areas, Novotny et al. (1997) reported cyanide concentrations in water samples obtained from a watershed in Milwaukee. The Lincoln creek watershed lies within the city of Milwaukee and has over 200 stormwater sewer outfalls running into it. Total cyanide levels were measured as high as 130  $\mu\text{g/L}$ , and was determined that enough free cyanide could be generated through photodecomposition to exceed US Environmental Protection Agency acute and chronic water quality criteria (Novotny et al., 1997).

Paschka et al. (1999) calculated a series of mass-balance equations in attempt to estimate the range of total cyanide concentrations in road runoff for a range of potential situations, taking into account various factors such as salt application rates, cyanide concentrations in salt, and snowfall amounts. Estimated total cyanide concentrations ranged up to 6 mg/L, which greatly exceed the U.S. Environmental Protection Agency maximum contaminant level in drinking water (0.2 mg/L), and U.S. Environmental Protection Agency acute (22  $\mu\text{g/L}$ ) and chronic (5.2  $\mu\text{g/L}$ ) freshwater quality criteria (Paschka et al., 1999). Even low application rates of 80 kg/km produced estimates of 600  $\mu\text{g/L}$  total cyanide concentrations, and when taking into consideration the lowest possible amount YPS added to road salt to prevent caking, cyanide concentrations still exceeded 200  $\mu\text{g/L}$  (Paschka et al., 1999). The authors concluded that there are potentially negative effects on water quality arising from addition of iron cyanide complexes in road salt, and that extensive monitoring studies examining total, free, and complexed cyanides in surface and groundwater in all areas of potential contamination are required.

The degree of deicing salt impact on freshwater systems varies between and within

areas of contamination due to numerous factors besides the characteristics of the receiving body of water. The amount of salt applied, timing of salt application, the length of and type road, discharge of receiving stream, degree of urbanization, temperature, precipitation, dilution, and potential soil adsorption, are all factors which play roles in the contamination impacts (Ramakrishna and Viraraghavan, 2005). Further research on impacts on water quality and transportation paths of cyanide is needed.

### ***Effects on Community Structure***

The proximity of viable wetland patches is essential for the persistence of animal populations that function in metapopulation structures, such as amphibians (Brinson, 1993; Semlitsch, 2000; Semlitsch and Bodie, 1998). Metapopulations can be viewed as an interacting group of spatially separated populations, or a “population of populations” that are maintained by recolonizations from sub-populations (Levins, 1969; Sinsch, 1992). Sub-populations exist in continued balance between local extinction and recolonization events. Population connectivity therefore, is essential for maintaining the greater metapopulation structure (Hanski et. al., 1996). Models have shown that sub-populations exist in continuous balance between decline and increase within a network of breeding ponds and are prone to population decrease more often than increase because of high adult mortality and variable juvenile recruitment (Hanski et al., 1996; Levins, 1969; Marsh and Trenham, 2001; Semlitsch, 2000). The endurance of amphibian populations requires that individuals migrate freely between patches, as isolated sub-populations ultimately become extinct (Alford and Richards, 1999; Hanski et al., 1996; Levins, 1969). Amphibian species richness has been shown to be negatively correlated with increasing wetland isolation (Parris, 2006; Semlitsch, 2000; Semlitsch and Bodie, 1998).

Pond isolation was identified as the most important factor in determining number of amphibian species present in a wetland system (Parris, 2006). Gibbs (1993) found that after the loss of small wetlands, only 54% of the landscape was within maximum migration distance of amphibian species in the area. Population connectivity is largely dependent on juvenile dispersal; high levels of post-metamorphic survival are required to maintain populations (Cushman, 2006). Contamination may be an isolating factor between sub-populations by reducing the total number of suitable habitat patches in an area.

Metacommunity theory is an extension of metapopulation theory, and is often applied to communities in fragmented landscapes (Parris, 2006). Similar to metapopulations but on a larger ecological scale, metacommunities can be defined as a collection of local communities inhabiting a group of habitat patches, which are linked together through dispersal of interacting species (Parris, 2006).

Chemical contamination can be viewed as a stressor that affects the functioning of metapopulations and communities. If ponds become contaminated within a habitat matrix, they will act as population sinks within the metacommunity, contributing to the decline of total population numbers. If key source ponds (those which act as continuous source populations for the whole matrix) become contaminated, the entire system could deteriorate, resulting in local extinction of the community. Metacommunity structure can also become altered when members of a community are removed from the system, since variability in tolerance levels to stressors exist between species. The results of this study indicate that differences exist in tolerance levels between species, with some exhibiting greater sensitivity to deicing salt contamination than others. Some ponds may contain

levels of contamination which are eliminating the most sensitive species in a community. Amphibians play critical roles in food webs of their ecological communities, and the removal of a particular species can interfere with numerous ecological relationships, as some species have co-evolved with each other (Wilbur, 1997). For example, *L. palustris* and *A. americanus* are known to frequently occur together in ponds in North Carolina. A study demonstrated that the two species in this area had differentiated in such a way as to greatly reduce interspecific competition compared to the competitive effects measured between other species (Wilbur, 1997). Density dependence plays a strong role to influence metamorphic timing and body size. For example, due to reduction in competition, low density allows individuals to acquire the resources to increase growth rate and size at metamorphosis (Wilbur, 1997). Predators have great influence in communities. Predacious amphibians such as newts of the genus *Notophthalmus* and larval *Ambystomoid* salamanders commonly prey upon tadpoles, and *Notophthalmus* are actually considered keystone predators in many areas (Wilbur, 1997). Not only does their presence control population numbers of prey species, but they can also influence the behaviour and morphology of prey, as well as reduce competition between prey species (Wilbur, 1997). It has been suggested that in some cases, predators may have a net positive effect on a prey population through reducing competition, as density reduction results in individuals completing metamorphosis at increased times and larger sizes (Wilbur, 1997). Chronic NaCl toxicity experiments of this research, and previous acute toxicity tests (Collins and Russell, 2009) have identified *A. maculatum* as having low tolerance to salt. Additionally, this species was found to be absent in ponds containing elevated chloride concentrations (Collins and Russell, 2009). The loss of *A. maculatum*

due to deicing salt contamination has likely altered intra and interspecific competitive interactions in these systems.

Temporal scales are also important to recognize when considering the effects of contamination. Aquatic systems receive the highest amounts, and most contaminated road runoff during the spring. Small, temporary ponds adjacent to roads are frequently composed almost entirely from runoff (Forman et al., 2003; Williams, 2006). Early spring breeders are therefore at particular risk of facing the highest degrees of contamination, and for those with sensitive early life stages, the peril will be intensified. Such is the case for *A. maculatum* and *L. sylvaticus*; in Nova Scotia, these species are amongst the earliest spring breeders (Gilhen, 1984). Previous acute toxicity experiments have identified these species as being the most sensitive to elevated NaCl exposure (Collins and Russell, 2009), and this research has identified these two species as having sensitive early life stages to sub-lethal NaCl exposure.

### **Conclusion**

Results of this study indicate that deicing salt contamination of roadside wetlands can result in negative effects on early amphibian life stages at levels below lethal concentrations. These results exemplify the importance of considering sub-lethal effects of pollutants in an ecosystem. Effects on growth, development, hatching success, and behaviour clearly have the potential to reduce population viability by interacting with natural stressors in nature, and render individuals susceptible to predation. These effects are likely producing changes to amphibian metacommunity structure through reducing the number of suitable habitat patches, and excluding the most sensitive members of a community. Removal of certain amphibian species in an ecosystem have been shown to



alter community dynamics (Wilbur, 1997). Our results demonstrate that *A. maculatum* and *L. sylvaticus* are species which are likely facing the greatest impacts of deicing salt contamination in this region. These species are the earliest spring breeders in Nova Scotia, and have strong tendencies to oviposit in small roadside ponds (Gilhen, 1984). Larvae of *A. maculatum* were the most sensitive species to chronic salt exposures, with exposures resulting in mortality, and altered development and growth. *L. sylvaticus* was shown to be very sensitive during the embryonic stage. Salt exposures reduced hatching, produced developmental malformations, and altered behaviour. It is important to identify the most vulnerable species in a community. To preserve these sensitive populations, current deicing salt application could be reduced, or switched to an alternative in important breeding areas of these species.

The cyanide acute toxicity tests performed provide essential basic data on cyanide toxicity to amphibians. Results indicate that amphibians possess high tolerance to this toxin (compared to fish species). Clearly, the potential environmental hazards associated with ferrocyanide anti-caking agents in road salt are poorly understood and merit further study, especially in understanding transport paths and the ultimate environmental fate of these compounds.

Many regions in Northern localities do not routinely apply salts to roads. Often, this is due to environmental concerns over ground water pollution. In these locales, abrasives are primarily relied upon for winter transportation safety. Until effective, environmentally friendly deicing alternatives are developed, recommendations of this research include greatly reducing the amounts of de-icing salts applied and improving application procedures to minimize offsite movement of deicing compounds.

## **Recommendations**

- Environmental assessments should take sublethal effects of contamination into account when creating environmental quality regulations
- Further research towards determining transport and environmental fate of cyanide in aquatic systems
- Initiate research on chronic effects of cyanide exposure to amphibians
- Reduce road salt use, develop more efficient salt application procedures, switch to alternative deicing agents, and educate public on hazards of salt in the environment

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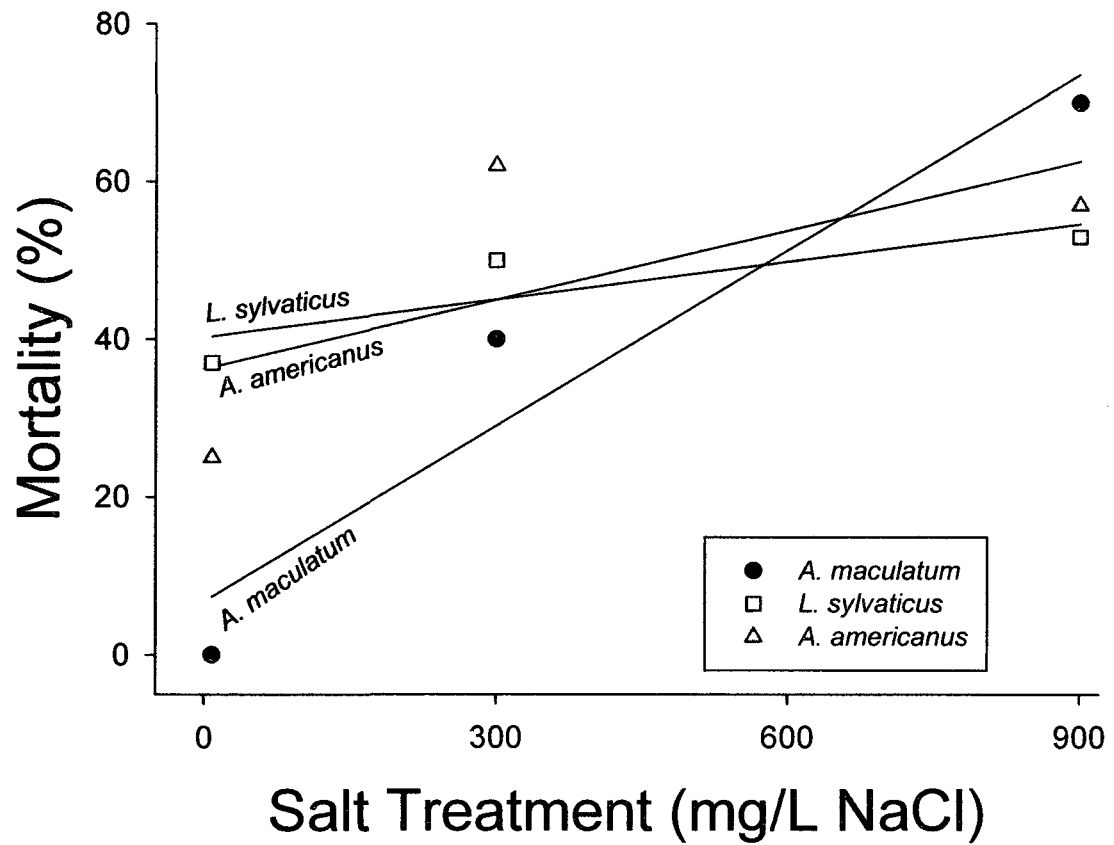
**Table 1:** LC<sub>50</sub> results and confidence intervals (µg/L) from cyanide 96hr acute toxicity tests.

Species	LC <sub>50</sub>	Confidence	Interval
		lower 95%	upper 95%
<i>Lithobates sylvaticus</i>	1039.26	870.93	1240.12
<i>Lithobates clamitans</i>	2448.11	1884.15	3180.87
<i>Pseudacris crucifer</i>	2566.85	1809.86	3640.46
<i>Anaxyrus americanus</i>	4527.05	3630.42	5645.13
<i>Ambystoma maculatum</i>	11471.64	10173.79	12935.06

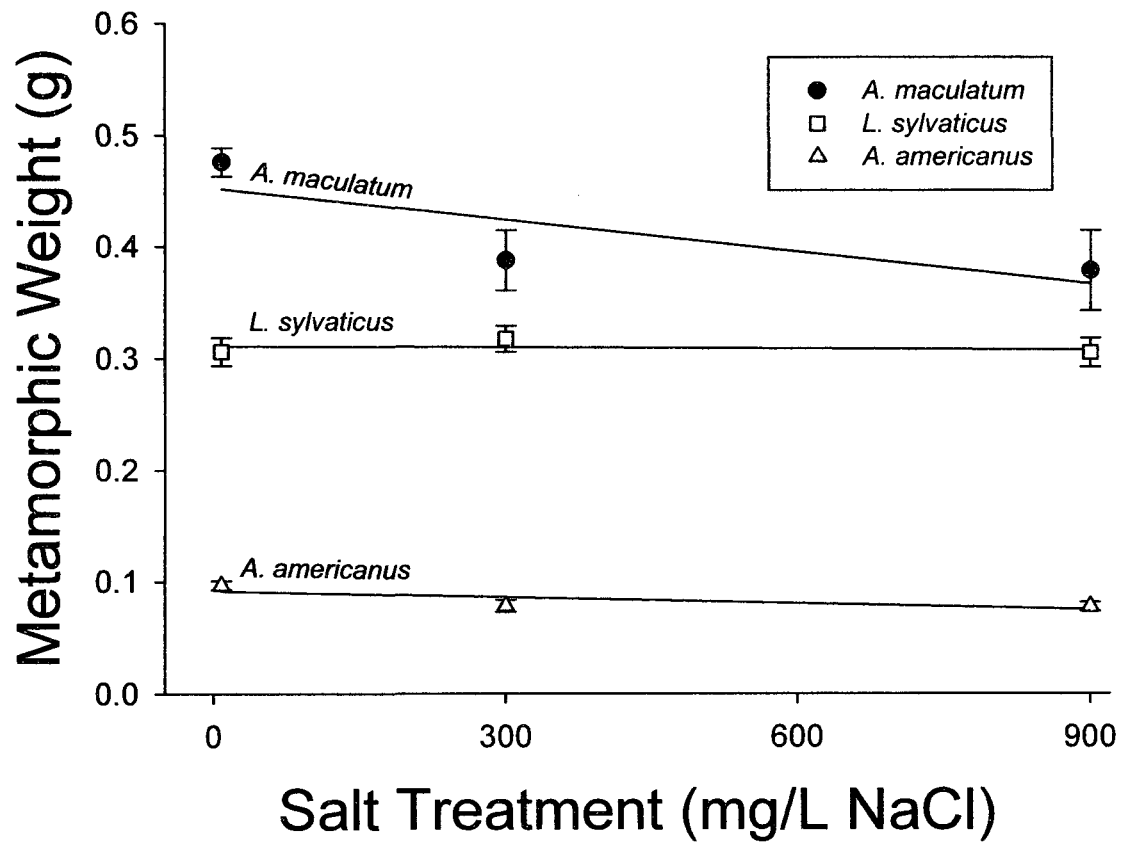
**Table 2:** Means and standard errors ( $\pm 1$  SE) of water quality components measured in road runoff water. Conductivity is expressed in  $\mu\text{S}/\text{cm}$ . Chloride and cyanide concentrations are in  $\text{mg}/\text{L}$ .

Location	pH	Conductivity	Chloride	Cyanide
Hwy. 102, Stanfield Airport	7.2 $\pm$ 0.10	1843 $\pm$ 752	752 $\pm$ 534	0.003 $\pm$ 0.0009
Hwy. 212, Antrim	5.8 $\pm$ 0.22	362 $\pm$ 16	90 $\pm$ 5	0.003 $\pm$ 0.0009
Hwy. 212, Goffs	7.2 $\pm$ 0.15	5918 $\pm$ 2295	2036 $\pm$ 897	0.009 $\pm$ 0.0012
Hwy. 306, Long Lake Park	6.9 $\pm$ 0.09	1674 $\pm$ 52	426 $\pm$ 21	0.003 $\pm$ 0.0006
Hwy. 349, Herring Cove	6.4 $\pm$ 0.06	1284 $\pm$ 150	244 $\pm$ 40	0.003 $\pm$ 0.0007
Hwy. 253, Ferguson's Cove	7.4 $\pm$ 0.04	4923 $\pm$ 650	1591 $\pm$ 241	0.005 $\pm$ 0
Hwy. 253, Purcell's Cove	6.6 $\pm$ 0.21	3537 $\pm$ 19	1089 $\pm$ 4	0.010 $\pm$ 0.0052
Chain Lake Rd., above Salt Dome	6.4 $\pm$ 0.06	1700 $\pm$ 322	493 $\pm$ 104	0.010 $\pm$ 0.0023
Chain Lake Rd., at Salt Dome	6.5 $\pm$ 0.07	4957 $\pm$ 59	1188 $\pm$ 105	0.032 $\pm$ 0.0043
Chain Lake Rd., below Salt Dome	7.3 $\pm$ 0.10	159587 $\pm$ 3695	44479 $\pm$ 1703	0.052 $\pm$ 0.0134
Otter Lake Rd.	6.6 $\pm$ 0.06	1240 $\pm$ 92	379 $\pm$ 13	0.005 $\pm$ 0.0052

**Figure 1:** Mortality for 3 amphibian species exposed to 8, 300, and 900 mg/L salt treatments.

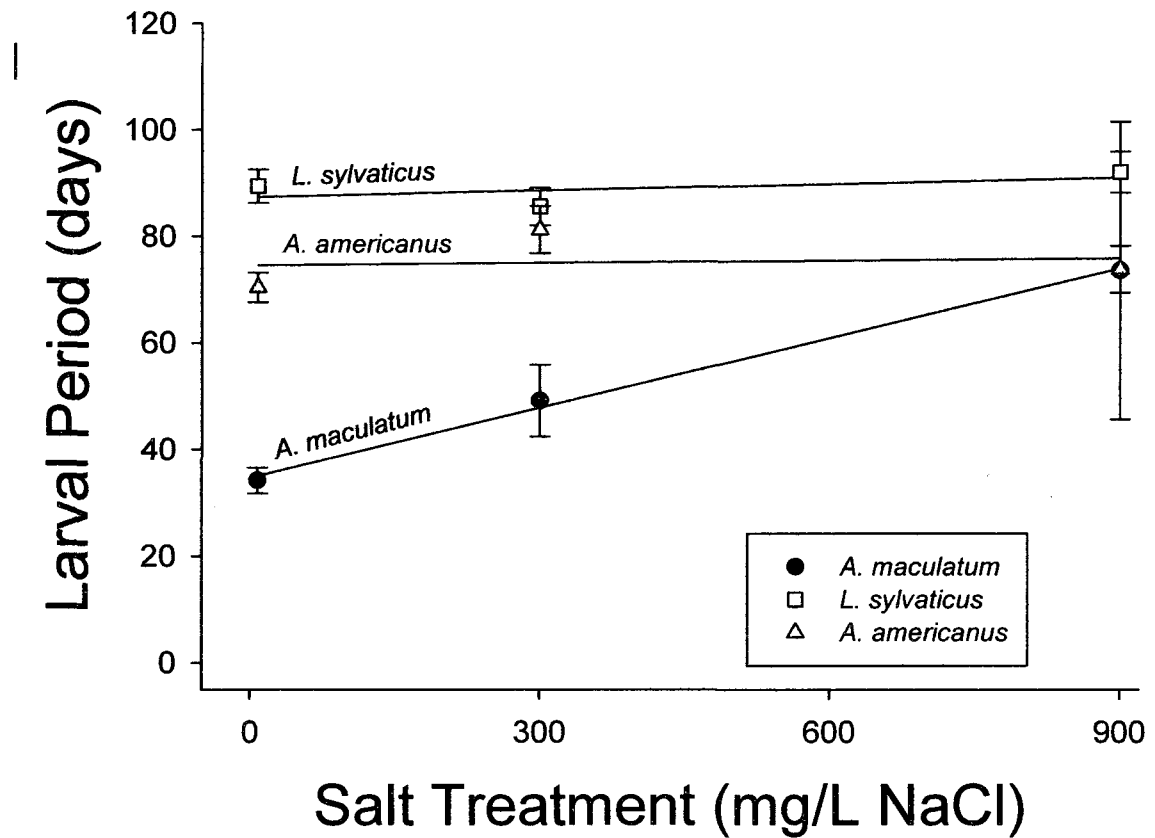


**Figure 2:** Weight at metamorphosis for 3 amphibian species exposed to 8, 300, and 900 mg/L salt treatments. Error bars represent  $\pm 1$  standard error.

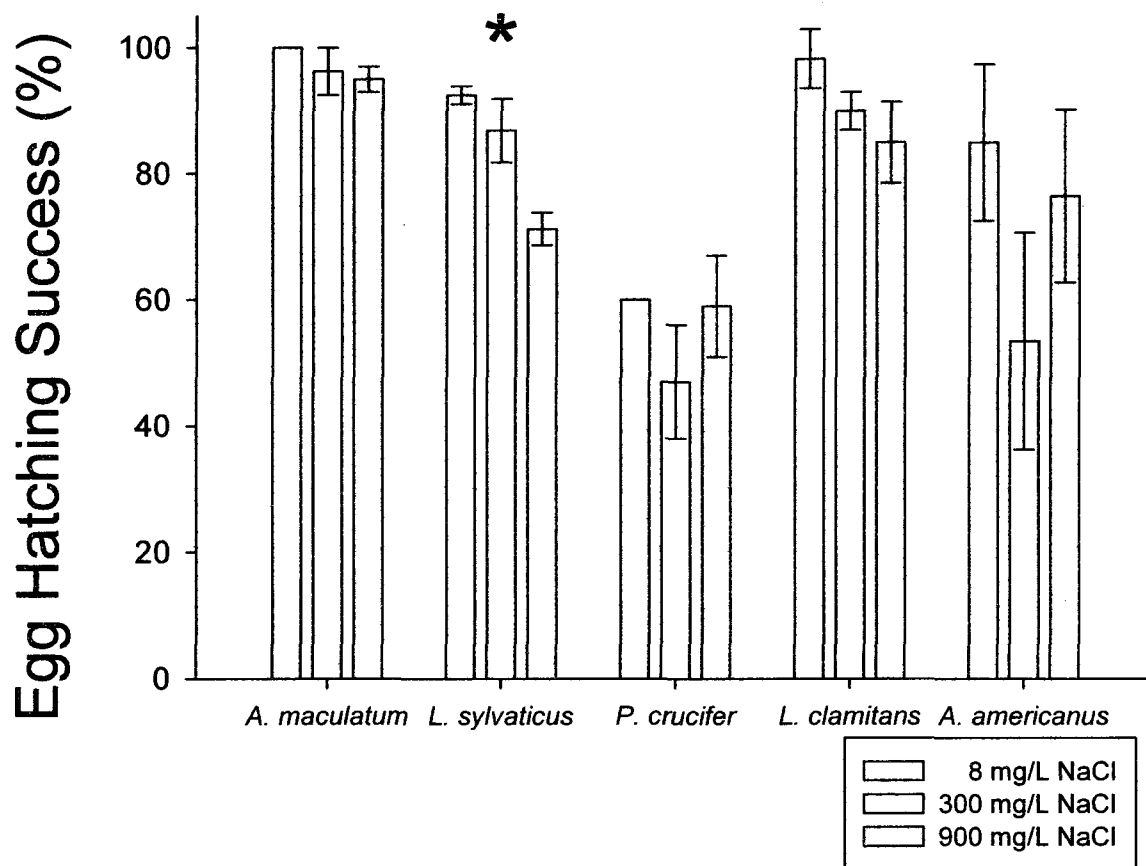




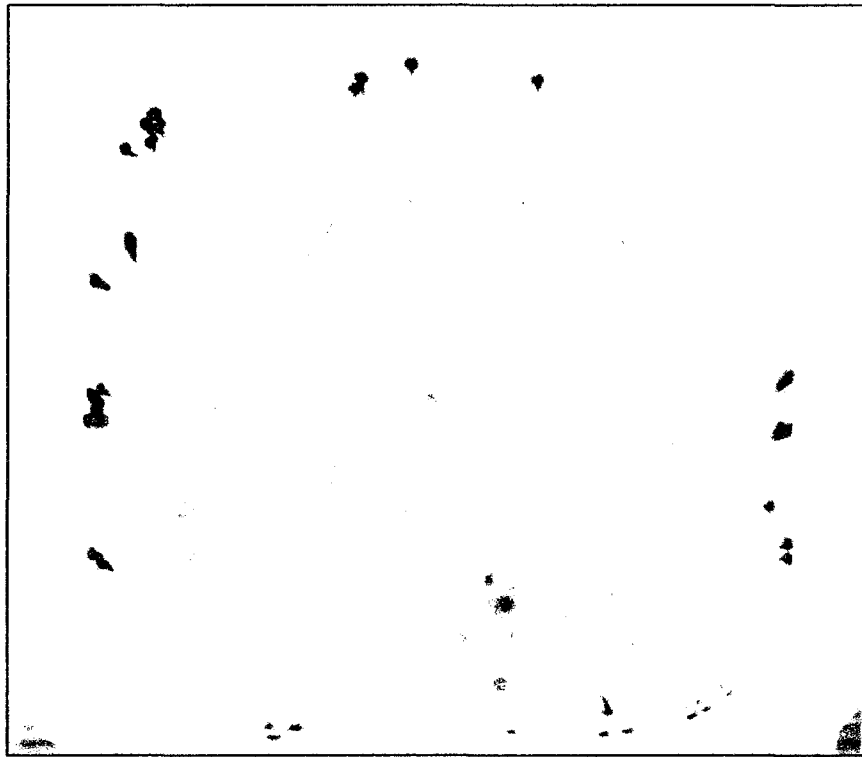
**Figure 3:** Larval period for 3 amphibian species exposed to salt. Error bars represent  $\pm 1$  standard error.



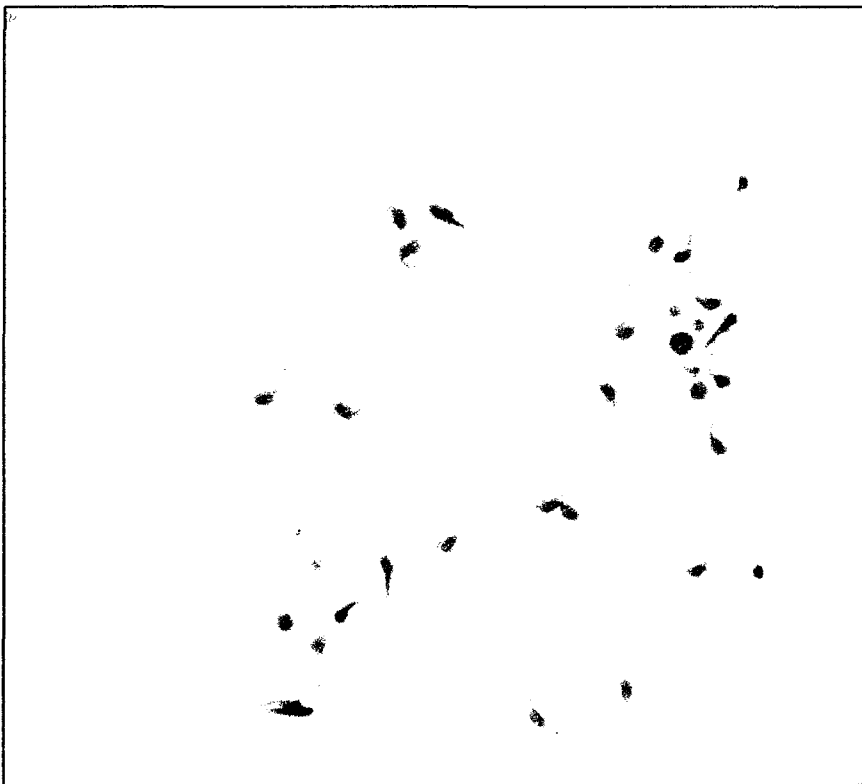
**Figure 4:** Hatching success of amphibian eggs exposed to salt. Error bars represent  $\pm 1$  standard error. Asterisk indicates significant differences. Spring peeper (*P. crucifer*) data is included but not analyzed.



**Figure 5:** Tail anomalies in *L. sylvaticus* tadpoles recently hatched from high chloride treatments.

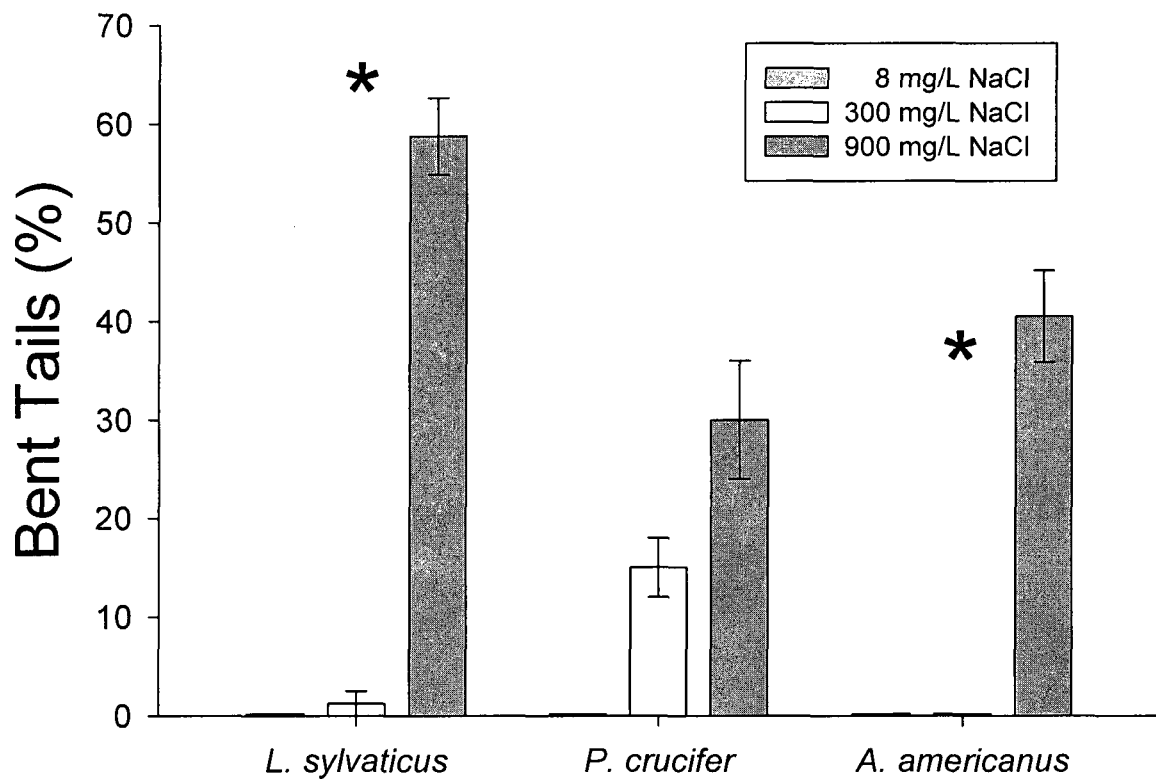


8 mg/L NaCl

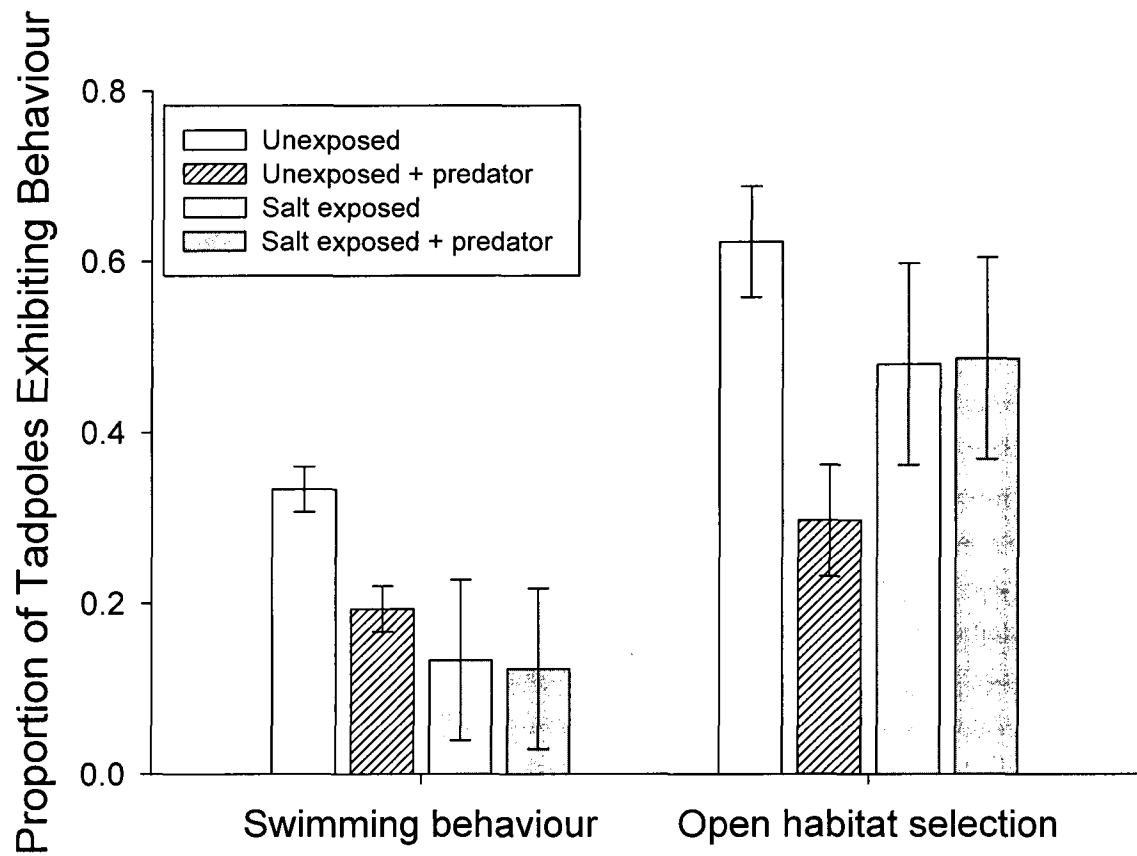


900 mg/L NaCl

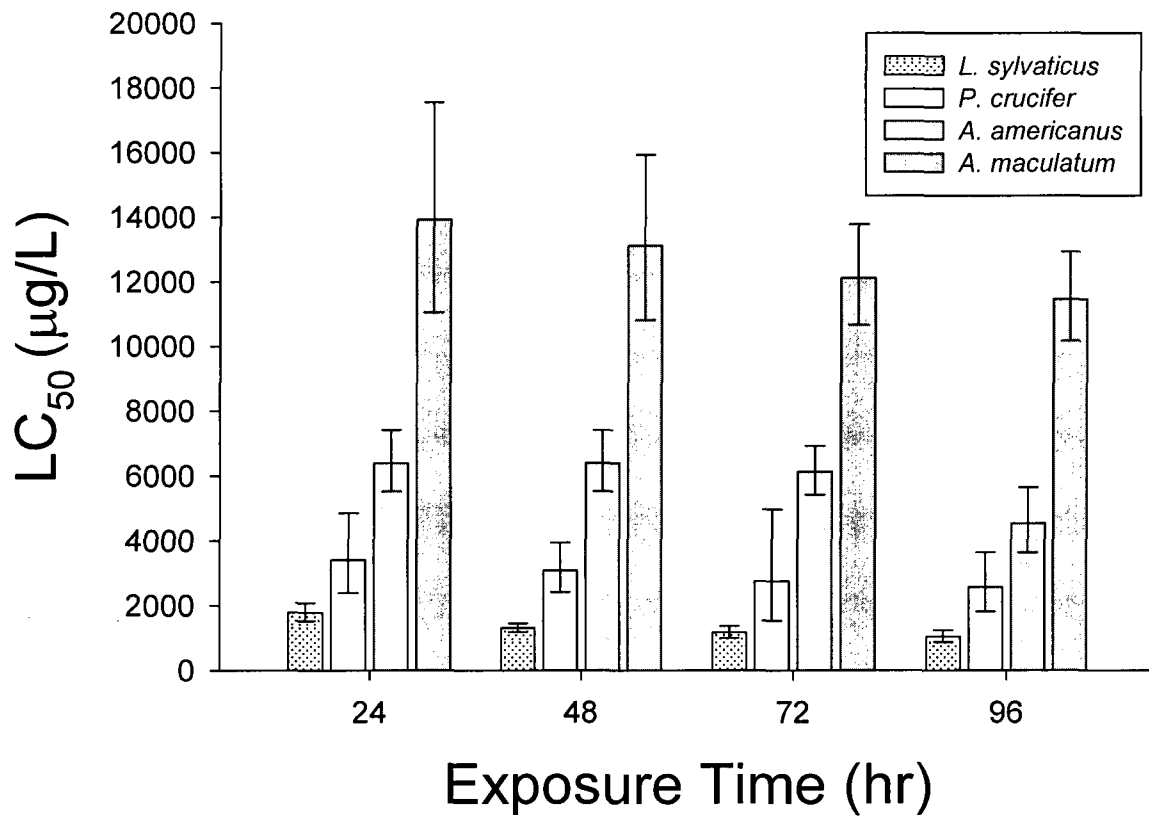
**Figure 6:** Percentage of tail abnormalities in tadpoles exposed to salt as eggs. Error bars represent  $\pm 1$  standard error. Asterisk indicates significant differences. Spring peeper (*P. crucifer*) data is included but not analyzed.



**Figure 7:** Differences in swimming and open habitat use behaviour between unexposed and salt exposed wood frog (*L. sylvaticus*) tadpoles in the presence and absence of a newt predator.



**Figure 8:** LC<sub>50</sub>s for acute cyanide toxicity tests (96hr) for 4 larval amphibian species and mortalities at 24, 48, and 72 hr.



## **Appendix I – Species Accounts**

Families represented in the study ponds were Salamandridae, Ambystomidae, Bufonidae, Hylidae, and Ranidae.

### **Family Salamandridae**

#### **Red- spotted Newt (*Notophthalmus viridescens*)**

In Canada, the red spotted newt is abundant from the Maritimes through to Northwestern Ontario (Gilhen, 1984). Newts have rougher skins than most salamanders, and the costal grooves (prominent in most other salamanders) are indistinct (Conant and Collins, 1991). Newts are primarily aquatic except for a few species such as the eastern red-spotted newt, which has a terrestrial juvenile stage, called a red eft (Conant and Collins, 1991). Aquatic larvae transform into efts, which migrate to damp, wooded areas for 1-3 years; after this period, they return to water and transform into sexually mature, aquatic adults (Conant and Collins, 1991; Gilhen, 1984). Life cycle forms include aquatic eggs and larvae, terrestrial juveniles and aquatic adults (Conant and Collins, 1991; Gilhen, 1984). Potential habitats include quiet, permanent or semi-permanent bodies of water such as: swamps, small lakes, shallow ponds and streams with submerged vegetation and absence of fish predators (Gilhen, 1984). Adults often remain active all winter, and can sometimes be observed through the ice (Conant and Collins, 1991). Efts are reddish- orange coloured and covered with red spots with black borders and adults are olive green-brown with yellow on the sides and undersides, and can reach 5-10 cm in length (Gilhen, 1984). Toxic secretions are produced by the skin to deter predation; the eft stage is the most toxic (Gilhen, 1984). Efts actively forage damp soils for a variety of

small invertebrates; adults consume a wide variety of prey including insects, worms, leeches, mollusks, crustaceans, and early life stages of other amphibians (Conant and Collins, 1991; Gilhen, 1984). The eastern spotted newt has even been described as a keystone predator in these habitats (Wilbur, 1997). Breeding occurs from late March – late May; during this period males develop a high wavy crest on the tail (Gilhen, 1984). Females lay between 200- 375 eggs singly on aquatic plants where they will hatch within 3-5 weeks (Gilhen, 1984). Larvae develop within three months, transforming into efts during August and September (Gilhen, 1984).

### **Family Ambystomidae**

#### **Spotted Salamander (*Ambystoma maculatum*)**

This group of mole salamanders is widely distributed in Nova Scotia (Gilhen, 1984). They are a large species that can grow over 20cm in length (Gilhen, 1984). Colour is a range of bluish/ black with two irregular rows of up to 50 yellow spots down their backs (Conant and Collins, 1991; Gilhen, 1984). In Canada, they occur in forested areas from Nova Scotia to Northwestern Ontario (Gilhen, 1984). Daytime is spent under rocks or damp forest debris and on damp or rainy nights they forage for a variety of invertebrates along the forest floor (Conant and Collins, 1991; Gilhen, 1984). Breeding occurs in quiet, vegetated areas of woodland ponds in early spring (Gilhen, 1984; Vogt, 1981). Eggs may be laid as one large mass, or clusters of smaller, either fixed to submerged vegetation or to the bottom of the pond (Gilhen, 1984; Vogt, 1981). Eggs masses develop a green colour, ranging from light to dark, due to the presence of a symbiotic alga (Pinder and Friet, 1994). Females produce 81-293 eggs per year and after hatching, larvae develop quickly in shallow areas transforming through July, August, and September (Gilhen,



1984; Vogt, 1981). If temperatures are low, individuals may over winter as larvae.

### **Family Bufonidae**

#### **Eastern American toad (*Anaxyrus americanus*)**

Belonging to the family Bufonidae, The Eastern American toad is widespread through Nova Scotia (Gilhen, 1984). They are a small species, ranging between 1-4 inches in length (Conant and Collins, 1991; Gilhen, 1984; Vogt, 1981). Colour is predominantly brownish, but can vary considerably from olive, brick red, or greyish black, particularly for females. Undersides are often in varying tones of green/ brown and white, often accompanied with a variable number of black spots containing warts (Conant and Collins, 1991; Gilhen, 1984). In Canada, the species can be found dispersed from Newfoundland and the Maritime provinces through to eastern Manitoba (Gilhen, 1984). Preferred habitats include the shores of freshwater ecosystems near woodlands (Gilhen, 1984). In spring they emerge from hibernation, and males begin their high-pitched, trill-sounding call (Gilhen, 1984). The height of breeding season is in May (Gilhen, 1984; Vogt, 1981). Eggs are released along the bottom of ponds in long streams containing 4,000- 8,000 eggs (Conant and Collins, 1991; Gilhen, 1984; Vogt, 1981). The small, black, tadpoles hatch from eggs within 2-8 days and develop in warm shallows through July and September (Gilhen 1984). Two- three years are required to reach maturity (Gilhen, 1984; Vogt, 1981). Juveniles and adults prey on a large variety of invertebrates; however, the main dietary source consists specifically on ants, beetles and spiders (Gilhen, 1984). Eastern American toads are one of the last amphibians in this region to enter hibernation (Gilhen, 1984; Vogt, 1981).

### **Family Hylidae.**

### **Spring peeper (*Pseudacris crucifer*)**

Family Hylidae, the tree-frogs, are represented in the Maritime region by the widespread spring peeper (*Pseudacris crucifer*) (Gilhen, 1984). These tiny frogs are variable in pattern and colour, but are generally shades of light brown- yellow or grey with dark bands and spots on areas such as the legs and on the side of the head (Gilhen, 1984). Their name “crucifer” originates from the “X” shaped pattern found on their backs in varying degrees of bands and spots (in some areas this pattern can be somewhat distorted.) (Conant and Collins, 1991; Gilhen, 1984; Vogt, 1981). They inhabit ponds, lakes and aquatic areas adjacent woodlands from Nova Scotia to eastern Manitoba (Conant and Collins, 1991; Gilhen, 1984). Breeding occurs between early April to June, often in temporary ponds such as those adjacent to roadsides (Gilhen, 1984). The males call is a distinguishable high pitched, repetitive “peep, peep” (Gilhen, 1984). Egg masses contain 800-1,000 small eggs, and are laid around vegetation and aquatic debris (Gilhen, 1984; Vogt, 1981). After breeding, males and females separate and return to woodlands, males often continuing to call as they disperse (Gilhen, 1984). Eggs hatch within 2-3 days and juveniles develop and transform from July through September (Gilhen, 1984; Vogt, 1981). Diets consist of soft bodied invertebrates- spiders being most common, and small bits of vegetation (Gilhen, 1984).

### **Family Ranidae**

#### **Green frog (*Lithobates clamitans*)**

Members of this genus are characterized by smooth skin, long legs and streamlined bodies (Gilhen, 1984; Tanara, 1975). They generally have large eyes and slightly pointed faces (Gilhen, 1984; Tanara, 1975). Males and females of a species may differ

morphologically through differences in colour and tympanum size (Gilhen, 1984; Tanara, 1975). During breeding season, the males develop a swollen thumb base on the forelegs (Gilhen, 1984; Tanara, 1975).

Colour and skin patterns can be highly variable in green frogs, ranging from green to combinations of green/brown and black, or bluish green, with black spots (Gilhen, 1984). The undersides range from white- grayish/white and the underside of the adult males chin is bright yellow (Conant and Collins, 1991; Gilhen, 1984). Maximum adult body size is around 10cm (Gilhen, 1984). Almost any body of fresh water can be potential habitat for Green frogs (Conant and Collins, 1991; Gilhen, 1984). In Canada, populations are distributed through Newfoundland and the Maritime provinces, to Ontario, Manitoba and southern British Columbia and they are especially widespread through Nova Scotia (Gilhen, 1984). Hibernation sites are aquatic; individuals bury themselves into the mud in the bottom of a permanent water body (Gilhen, 1984). Green frogs emerge from hibernation typically through April and May and breed in June and July (Gilhen, 1984). The male's "twang" –like call can be heard through day and night (Gilhen, 1984). Between 1000- 5000 eggs are laid in thin, filmy masses which float around the surface of aquatic vegetation (Gilhen, 1984; Vogt, 1981). Tadpoles over winter in their aquatic habitat before transforming the following summer (Gilhen, 1984; Vogt, 1981). Green frogs consume a large variety of aquatic and terrestrial invertebrates (Gilhen, 1984). They retire to hibernation in early autumn (Gilhen, 1984).

### **Wood frog (*Lithobates sylvaticus*)**

Wood frogs are found in variable shades of brown, red, or grey, with dark spots, and they are recognized for the "mask"- like spot around the eyes (Conant and Collins, 1991;

Gilhen, 1984). The wood frog occurs farthest north of any species of amphibian or reptile in North America (Conant and Collins, 1991; Gilhen, 1984). In Canada, they can be found as far as the treelines from Labrador to the Yukon territory (Gilhen, 1984). They are widely distributed throughout Nova Scotia (Gilhen, 1984). Inhabitants of damp woodlands of deciduous and mixed forests, they emerge from hibernation in early spring and are one of the earliest amphibians to breed, usually around late March to early April, sometimes while ice is still on the ponds (Conant and Collins, 1991; Gilhen, 1984). Males migrate to ponds and produce duck-like calls in surrounding vegetation and are mostly vocal at night (Conant and Collins, 1991; Gilhen, 1984). Large globular egg masses contain 500-800 darkly coloured eggs that hatch within 15-20 days and tadpoles transform within 1-2 months (Gilhen, 1984; Vogt, 1981). Wood frogs consume a great variety of insects, spiders and other invertebrates (Gilhen, 1984).

## Appendix II – Acute Cyanide Toxicity Testing Results

All individual treatments were composed of 18 individuals (3 replicates X 6 test larvae).

HCN Treatment (µg/L)	Mortalities				
	<i>L. sylvaticus</i>	<i>P. crucifer</i>	<i>A. americanus</i>	<i>L. clamitans</i>	<i>A. maculatum</i>
Control	0	0	0	0	0
31.25	0				
62.5	1				
125	0				
250	0	0	3	1	
500	0	0	0	2	1
1000	7	1	0	2	0
2000	18	6	0	4	0
4000	18	14	0	15	3
8000		14	16	18	2
16000				18	15