Acute toxicity of deicing compounds and personal care products to early amphibian life stages

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Abstract

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Chemical contamination of roadside vernal breeding pools threatens amphibian abundance and diversity. Deicing compounds from roads in northern latitudes are the primary cause of contamination in spring, coinciding with amphibian breeding. Triclosan, a personal care product, also negatively affects amphibians. My objective was to study the acute toxicity of deicers and triclosan on six species of Nova Scotian amphibian larvae: spotted salamanders (*Ambystoma maculatum*), wood frogs (*Lithobates sylvaticus*), spring peepers (*Pseudacris crucifer*), mink frogs (*Lithobates septentrionalis*), green frogs (*Lithobates clamitans*), and American toads (*Anaxyrus americanus*). Median lethal concentrations (LC_{50}) of all chemicals varied among species, but early-breeding species were consistently most sensitive. Early life stages were most sensitive to all chemicals, and median LC_{50} values increased throughout larval development. Larvae exposed at 22˚C were more sensitive than those exposed at 12˚C. Results indicate that synergism between environmental and developmental factors can lead to detrimental effects on amphibians.

June 30th, 2016

Introduction

Amphibian Population Declines

The widespread state of amphibian health has become cause for concern, particularly over the last decade (McCallum, 2007; Pough et al., 2004; Vitt and Caldwell, 2013). Amphibians are the most widely threatened group of vertebrates, with a global decline in species diversity and many species facing a steep decline in abundance (Altig, 2010; Collins, 2010; McCallum, 2007). This crisis even affects those species found in protected areas (Beebee and Griffiths, 2005; McCallum, 2007; Pough et al., 2004). McCallum (2007) suggests that the current rate of amphibian extinction far exceeds the background extinction rate, as estimated by the fossil record. The IUCN Red List (Baille et al., 2004; Williams et al., 2013) classified 43% of known amphibian species as threatened, endangered, or critically endangered. A recent review has shown that Canada is not immune to these declines, with 42% of amphibians at risk, as classified by COSEWIC (Lesbarrères et al., 2014).

A complex lifecycle with both aquatic and terrestrial stages, as well as precise environmental requirements make amphibians especially sensitive to changes in their habitat (Pough et al., 2004; Vitt and Caldwell, 2013; Wells, 2010). Amphibians are ectothermic vertebrates exhibiting a biphasic life history, with their aquatic and terrestrial life stages joined through metamorphosis (Gilhen, 1984; Gosner, 1960). Amphibian skin is a permeable and glandular organ that provides water and gas exchange essential to survival (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). This restricts amphibian habitat to moist environments, even during their primarily terrestrial stages (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). Their peak activity often occurs at night when lower temperatures minimize the rate of evaporation from their moist skin (Gilhen, 1984; Tanara, 1975). Amphibian eggs also readily absorb ambient water through their characteristic gelatinous covering, exposing these animals to an array of aquatic contaminants throughout their entire lifecycle (Gilhen, 1984; Pough et al., 2004). The nature of their development and physiology makes amphibians particularly vulnerable to environmental fluctuations in temperature and water chemistry (Pough et al., 2004; Vitt and Caldwell, 2013; Wells, 2010).

Amphibians are key organisms within the ecosystems they inhabit, acting as both predators and prey depending on their life history stage (McCallum, 2007; Pough et al., 2004; Vitt and Caldwell, 2013). Larvae contribute to nutrient and phytoplankton regulation, while forming an important element near the base of aquatic food webs (Blaustein, 2001; Tanara, 1975). Metamorphosis shifts amphibian trophic position from detritivore to carnivore and affects their impact on the food web in numerous ways (Blaustein, 2001; Tanara, 1975). Adult amphibians are typically among the top predators of their aquatic ecosystem, with a diet of primarily insects, while simultaneously serving as prey items for larger mammals and fish (Vitt and Caldwell, 2013). This wide range of interaction within the ecosystem, coupled with a natural sensitivity to disturbance, make amphibians prime indicators of environmental quality (Blaustein, 2001; Pough, 2004). Global decline in amphibian populations may represent an overall decline in ecosystem health, which signals a significant concern for environmental conditions across the world (Alford and Richards, 1999; Blaustein, 2001; Niemi and McDonald, 2004).

The cause of amphibian population decline is a complex issue that cannot be explained by a single factor, but rather by the dynamic interaction of multiple stressors (Fahrig et al., 1995; Kiesecker et al., 2001). Climate change, increased UV-B radiation, acidification, and pathogens are major contributing factors globally (Bancroft et al., 2008; Blaustein and Bancroft, 2007; Blaustein et al., 2010; Hof et al., 2011; Kiesecker et al., 2001). Habitat loss, species introduction, agrochemical application, as well as road development and runoff are other key factors affecting local populations, and are considered by many to be the most detrimental (Blaustein and Bancroft, 2007; Fahrig et al., 1995; Hof et al., 2011). It is necessary to recognize local and global factors, as well as the interaction between these factors that may produce detrimental synergistic effects.

Road Runoff and Water Quality

Roads can be especially harmful to populations of amphibians and pose a significant threat both directly and indirectly (Forman et al., 2003). Highway infrastructure in particular disrupts habitat structure via degradation and fragmentation (Andrews et al., 2008; Fahrig et al., 1995). Amphibians are often physically limited by roadways, whether by direct mortality from vehicle collisions or by altered movement patterns to compensate for the road obstruction (Beebee, 2013; Gibbs and Shriver, 2005; Hels and Buchwald, 2001). Roads that act as barriers through or between amphibian habitats have the potential to significantly decrease population numbers and diversity (Eigenbrod et al., 2008). Road development dramatically affects the quality of small ponds and wetlands in the vicinity, and in some cases obliterates them. These ponds are susceptible to an increased risk of flooding and landslides, as well as higher rates of erosion and sedimentation (Forman, 2000; Forman et al., 2003). Small seasonal habitats known as ephemeral ponds form in adjacent ditches; they are common breeding sites for the vast majority of amphibians (Forman, 2000; Hels and Buchwald, 2001). The shallow depth and roadside proximity of these sites increases their vulnerability to chemical contamination via road runoff (Norstrom and Jacks, 1998; Tromp et al., 2012).

Chemical runoff from roads may contain a number of harmful substances including hydrocarbons, metals, agrochemicals, combustion products and, in northern climates, de-icing compounds used to break down ice in winter months (Helmreich et al., 2010; Norstrom and Jacks, 1998; Tromp et al., 2012). Any of these pollutants can be detrimental to an ecosystem, whether via mortality or developmental defects, but pose a more significant threat when combined (Helmreich et al., 2010; Norstrom and Jacks, 1998; Tromp et al., 2012). Ecosystem health is characterized in part by species richness, which is considered to be closely linked with water quality (Alford and Richards, 1999; Houlahan and Findlay, 2003). Road runoff is a major contributor to poor water quality, and has been identified as one of the most important factors affecting amphibian population decline (Alford and Richards, 1999; Blaustein et al., 1994; Kiesecker, 2002; Pough et al., 2004).

Water quality is an important aspect of the entire ecosystem, but aquatic organisms are especially affected by even small shifts in chemistry and temperature. (Alford and Richards, 1999; Environment Canada, 2012; Pough et al., 2004). The permeable nature of amphibian eggs and skin subjects these animals to a variety of contaminants via their absorption of ambient water (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). Amphibians exhibit high site fidelity and low movement rates that restrict their available habitat and breeding sites to a small area (Gilhen, 1984; Pough et al., 2004; Tanara, 1975). Contaminated water sources cannot be avoided if the distance between sites is too great. Ephemeral ponds located along roadside ditches are well suited for breeding due to their shallowness and to the stagnant water that is clouded with sediment and organic material (Blaustein, 2001; Tanara, 1975). This protects amphibian eggs from the sun and scouting predators (Blaustein, 2001; Tanara, 1975). However, these conditions also significantly decrease oxygen levels and the rate of water turnover (Godwin et al., 2003; Jackson and Jobbagy, 2005), impacting their survival if these ponds become polluted (Blaustein, 2001).

Acute toxicity is lethal to amphibian eggs and larvae, but chronic toxicity can also lead to long-term physical or behavioural abnormalities (Christy and Dickman, 2002; Gomez-Mestre et al., 2004; Sanzo and Hecnar, 2006). Many agrochemicals (e.g. Atrazine) have been shown to produce deformities in aquatic organisms (Hays, 2000), while chloride based deicers can disrupt key behaviours such as predator avoidance mechanisms and swimming patterns (Rohr et al., 2003; Sanzo and Hecnar, 2006). Sub-lethal toxicity may still impede amphibian larval growth and successful metamorphosis

(Glennemeier and Denver, 2001; Rohr et al., 2003; Sanzo and Hecnar, 2006). Relyea and Edwards (2010) tested amphibian predator avoidance following pesticide exposure. Tests that did not result in mortality frequently exhibited significantly reduced activity and disregard of predator proximity (Relyea and Edwards, 2010). Baker et al. (2013) analyzed the sublethal effects of various pesticides and fertilizers on amphibian larval growth. It was found that most of these chemicals significantly reduced larval growth, an effect that was most drastic when two or more chemicals were used in tandem.

Both synergistic and combined effects of multiple chemicals makes it difficult to discern a primary contributor, and may also produce defects that would not otherwise occur in the presence of a single chemical (Hayes et al., 2006; Mann and Bidwell, 1999; Relyea, 2003). Additive toxicological effects are prevalent with the use of agrochemicals such as pesticides and herbicides that are most commonly applied together (Hayes et al., 2006; Mann and Bidwell, 1999). Many of these are commercially available in the form of solutions containing an array of chemical ingredients (Hayes et al., 2006). The effects of any one variable, chemical or otherwise, are shifted and often exacerbated in natural conditions where other environmental stressors are present (Blaustein et al., 2003; Relyea, 2003; Sih et al., 2004). This includes the combination of chemical and natural stressors, such as the presence of a predator. Relyea (2003) studied the sublethal effects of pesticides on six species of anuran tadpoles when paired with predator cues. Cabaryl, a commonly used commercial pesticide, was tested in the presence and absence of predatory stress. Exposure to high levels (6.5 mg/L) of cabaryl resulted in 95-100% mortality over a 16 day period, while low concentrations (0.3 mg/L) did not differ significantly from controls (Relyea, 2003). Additive predator stress exponentially increased lethality, both at lower concentrations and over shorter periods of time (Relyea, 2003). Levels of cabaryl that were previously considered safe became lethal in the presence of a hunting predator, making it difficult to discern a “safe” environmental level (Relyea, 2003). Therefore, a single contributing factor is not often the sole cause of negative effects.

Deicing Compounds and Salinization

In northern latitudes, road runoff includes deicing compounds that have been used in large quantities over the winter months (Environment Canada, 2001). The most common are salt-based deicers, referring to those using chloride, due to their low price and abundance (Environment Canada, 2012). Among these, NaCl is the most popular and is used most frequently (Environment Canada, 2012). North America applies approximately 14 billion tonnes of road salts per year (Environment Canada, 2012). Canada applies 5 million tonnes annually, which can amount to as much as 28.3 tonnes for each lane per kilometre of road (Environment Canada, 2012). Total use of NaCl in Nova Scotia alone has reached approximately 373, 956 tonnes annually, which falls within the second highest range of use in Canada (Environment Canada, 2012; Kaushal et al., 2005). Road salt use has risen exponentially over the past few decades, and there is no sign to suggest the current trend will decrease (Jackson and Jobbagy, 2005; Kaushal et al., 2005). With the expansion of urban areas and the staggering number of vehicles, road salt usage will be more likely to increase (Environment Canada, 2001; Environment Canada, 2012; Kaushal et al., 2005).

The toxic effects of road salts in runoff have been shown for numerous amphibian species (Collins and Russell, 2009; Environment Canada, 2012). Ecologists consider it to be one of the leading anthropogenic factors acting against amphibians in northern climates (Environment Canada, 2001; Environment Canada, 2012). Salt-based deicers not only cause direct mortality, but also contribute to the degradation and salinization of the surrounding ecosystem (Environment Canada, 2001; Godwin et al., 2003). This increasing salinization of freshwater ponds and wetlands is becoming a major threat to amphibians by contaminating available habitats (Godwin et al., 2003; Kaushal et al., 2005).

Chloride is soluble and accumulative, meaning that it can move readily through water and has a tendency to build up (Environment Canada, 2001; Hill and Sadowski, 2015; Kaushal et al., 2005). Natural background levels of chloride in fresh water ranges from 2-8 mg/L and are considered safe, but the additive influx from anthropogenic sources has been shown to damage aquatic systems (Bernhardt-Romermann, et al., 2006; Godwin et al., 2003). The maximum “safe” limit for chloride in a freshwater system is documented as 250 mg/L (Environment Canada, 2001; Hill and Sadowski, 2015; Kaushal et al., 2005). However, approximately 10% of aquatic species are harmed by prolonged exposure to a chloride concentration of 220 mg/L, and there are sublethal effects resulting in decreased survivorship that are more difficult to measure (Collins and Russell, 2009; Environment Canada, 2001; Kaushal et al., 2005). Many streams and rivers located within urban boundaries exceed this limit, and there is a rising trend among rural streams as well (Hill and Sadowski, 2015; Kaushal et al., 2005). In Canada, Cl^- concentrations as high as 4000 mg/L have been reported in wetlands and 5000 mg/L in urban lakes (Environment Canada, 2012; Sadowski, 2002). Measurements taken directly from road runoff have been as high as 18,000 mg/L (Environment Canada, 2012).

Kaushal et al. (2005) examined freshwater ponds for changing chloride levels in rural New York and New Hampshire, United States. Most ponds exhibited chloride concentrations as high as 5 g/L, which is approximately 25% that of seawater (Kaushal et al., 2005). Godwin et al. (2003) documented the results of a long term study that monitored the sodium and chloride concentrations of a watershed in rural New York between 1952 and 1998. Sodium was recorded as increasing 130% over this time and chloride increased by 243%; a direct result of road salt application (Godwin et al., 2003). Novotny et al. (2008) examined the progression of sodium and chloride throughout 38 lakes in eastern Minnesota. Urban lakes had sodium concentrations 10 times higher than non-urban lakes, while chloride concentrations exceeded 25 times higher (Novotny et al., 2008). Both sodium and chloride were significantly higher during winter and spring when aquatic systems are receiving fresh runoff, rather than in summer and autumn when flushing via rainfall occurs (Novotny et al., 2008).

Aquatic organisms, primarily amphibians and fish, are especially vulnerable to chloride toxicity (Forman and Alexander, 1998). However, those are not the only groups exposed to potential harm. Road salt not only contributes to the salinization of surface water, but also leaches deep below the surface into ground water (Transportation

Research Board, 1991). This can have a number of detrimental effects on soil and roadside vegetation. Plants may absorb the contaminated water through their roots, or come into contact with it directly via splash or spray from the road (Transportation Research Board, 1991). The main concern for vegetation affected by salt is dehydration, resulting in stunted growth or death of the plant (Transportation Research Board, 1991). Once subsurface water systems are contaminated by road salts, the quality of soil is decreased dramatically (Norstrom and Jacks, 1998; Transportation Research Board, 1991).

Soil salinization makes an ecosystem uninhabitable for many species that are intolerant to high salt levels, including most amphibians (Environment Canada, 2001; Norstrom and Jacks, 1998; Transportation Research Board, 1991). Chloride can alter the concentrations of cation exchange sites, leading to changes in soil solution pH and disruption of nutrient cycling (Norstrom and Jacks, 1998; Transportation Research Board, 1991). Green et al. (2008) studied the effects of excessive chloride levels in soil on the nitrogen cycle. The resulting increase in soil acidity significantly decreased microbial activity, slowing the process of nitrification (Green et al., 2008).

Salinization is a particular concern for small ponds where there is little potential for dilution (Godwin et al., 2003; Jackson and Jobbagy, 2005). Salt has a tendency to degrade ecosystems by displacing natural elements with sodium as NaCl dissolves and breaks down into its ions (Jackson and Jobbagy, 2005). Toxic metals bound to sediment may also be mobilized by salt. Trace metals from vehicles, such as lead (Pb) and cadmium (Cd), are present in roadside soils (Amrhein and Strong, 1990). These metals may be displaced from cation-exchange sites by sodium (Na), making them available for absorption and consumption (Amrhein and Strong, 1990; Backstrom et al., 2004). The salinized water absorbed by the soil also causes varying groundwater densities, leading to upset within the natural nutrient cycling (Backstrom et al., 2004; Jones et al., 1992). This has the potential to deplete oxygen in ponds and other shallow water systems with slow overturn. More dissolved solids will lessen the ability for water to hold dissolved oxygen (Jones et al., 1992). It is because of this toxicity and ecosystem salinization that road salts were added to the Canadian Environmental Protection Act Toxic Substance List in 2000 (Environment Canada, 2001).

Amphibians are at particular risk due to their preference for these shallow, low oxygen ephemeral ponds (Collins and Russell, 2009; Environment Canada, 2012; Helmreich et al., 2010). Chloride ions easily pass through the permeable membrane of amphibian skin, as well as the gelatinous covering of their eggs, disrupting osmotic regulation (Gordon, 1962; Pough et al., 2004). As amphibian eggs grow and absorb water from a contaminated source, development of larvae may be adversely affected (Gilhen, 1984; Pough et al., 2004). Hatching success was found to be drastically lower in ponds with a high chloride concentration (Environment Canada, 2012). Chlorides also disrupt cutaneous respiration and inhibit the skin’s mucus layer, which functions as an antibacterial and antifungal layer (Pough et al., 2004).

Early breeding amphibians are subject to the highest toxicity because their breeding period coincides with melting snow and ice that has been contaminated throughout the winter season (Collins and Russell, 2009; Helmreich et al., 2010). In Nova

Scotia, this includes the spotted salamander and the wood frog, which have been known to lay eggs as early as March (Gilhen, 1984). Newly hatched larvae are subject to fresh runoff harbouring peak chloride concentrations at the most sensitive stages of their lifecycle (Collins and Russell, 2009; Helmreich et al., 2010). Karraker et al. (2008) found that spotted salamander and wood frog egg masses were twice as dense in forest ponds than roadside ponds. Late breeding species such as green frogs are not as heavily affected because runoff has been diluted by the time they lay their eggs (Collins and Russell, 2009; Helmreich et al., 2010). However, tadpoles that overwinter may be susceptible to fresh road runoff in early spring the following year as well (Helmreich et al., 2010).

Green Deicing Alternatives

The high toxicity of road salts prompted Environment Canada (2012) to release a Code of Practice in accordance with their five-year Review of Progress report. The code seeks to regulate the use of salt-based deicers, as well as to promote the use of "green" alternatives. This refers to chemicals such as urea, formates, and acetates that are considered more environmentally friendly than the road salts currently used in such abundance (Environment Canada, 2012). While the aforementioned substances exhibit lower toxicity than NaCl, they may have other detrimental effects that are not initially apparent (Environment Canada, 2012).

Urea, or $(NH_2)_2CO$, is a substance that naturally occurs in the urine of many vertebrates and has been assessed as relatively non-toxic (Corsi et al., 2012; Muthumani et al., 2014). However, it performs poorly as a deicer in temperatures under -4˚C and is more costly to produce artificially (Muthumani et al., 2014; Ramakrishna and Viraraghavan, 2005). Urea's high nitrogen content means that it will bond with oxygen as it degrades, thus decreasing the amount available to aquatic organisms (Corsi et al., 2012). Formate salts show more promise due to a balance of high performance and low toxicity, but cost has limited their use (Corsi et al., 2012; Muthumani et al., 2014). Potassium formate ($KHCO_2$) is the most widely used of the green alternatives, with a low rate of oxygen consumption and minimal known toxicity to aquatic ecosystems (Corsi et al., 2012; Muthumani et al., 2014). It functions as low as -6˚C, but chloride-based deicers exhibit a wider optimal range and are often preferred for this reason (Corsi et al., 2012). Potassium formate has been shown to damage artificial constructs such as concrete and landing gear in its limited use as an airport runway deicer, and is also known to damage galvanized steel (Hassan et al., 2002; Shi et al., 2009). However, NaCl is also corrosive and only performs slightly better, with a minimum effective temperature of -10˚C.

Of all the available alternatives, calcium-magnesium acetate (CMA) has sparked special interest because it has a comparatively low toxicity and breaks down more rapidly than most de-icing compounds (Environment Canada, 2012; Fay and Shi, 2012; Transportation Research Board, 1991). Its corrosive effects on concrete and metals have been compared to that of tap water and are more or less benign (Fay and Shi, 2012; Xu et al., 2010). Any toxicity toward aquatic life is considered negligible, and unlike salt-based deicers it exhibits poor mobility in soil (Fay and Shi, 2012; Xu et al., 2010). CMA is biodegradable and acetate breaks down into CO_2 and H_2O, but it does not begin to degrade until the temperature rises to approximately 20°C (Environment Canada, 2012; Fay and Shi, 2012; Transportation Research Board, 1991). This means that it will remain in surface and ground water systems throughout the long winter months, often ranging from November-April in cold northern climates, and into spring (Environment Canada, 2012; Transportation Research Board, 1991). CMA may pose a threat for early and intermediate breeding amphibians that lay their eggs between April and June (Collins and Russell, 2009; Helmreich et al., 2010). Although its toxicity is low, CMA has a high oxygen demand and will deplete the available resources in receiving waters (Environment Canada, 2012; Transportation Research Board, 1991). Amphibians are in particular danger due to this depletion, since the ephemeral ponds acting as their primary breeding sites are low oxygen habitats to begin with (Collins and Russell, 2009; Helmreich et al., 2010).

Personal Care Products

Although road salts and other deicers are potentially harmful, they are not the sole source of aquatic contamination (Helmreich et al., 2010; Tromp et al., 2012). Personal care products are becoming prevalent in road runoff and waste water in North America (Helmreich et al., 2010; Qui, 2012; Tromp et al., 2012). Some of these chemicals may have multiple uses, being utilized in cosmetics, medicines, automotive maintenance, and even as deicing compounds (Helmreich et al., 2010; Tromp et al., 2012). One of these most common chemicals is propylene glycol ($C_3H_8O_2$). Its use as an aircraft and runway deicer has led to concern regarding its environmental impact (Corsi et al., 2009; Ramakrishna and Viraraghavan, 2005). Salt-based deicers are prohibited in this setting due to the corrosive ability of chloride, but glycols are not corrosive; all are freezing-point depressants (Corsi et al., 2009; Bausmith and Neufeld, 1999). By lowering the freezing point of ice, they effectively clear surfaces without consequences to the equipment or runway pavement (Bausmith and Neufeld, 1999; Corsi et al., 2009).

Over the past two decades, propylene glycol has largely replaced ethylene glycol as the standard for aircraft deicers and antifreeze (Corsi et al., 2009; Pillard, 1995). While ethylene glycol is less costly, it has been found to be significantly more toxic to both the environment and its inhabitants, including humans (Corsi et al., 2009; Pillard, 1995). Propylene glycol is considered relatively nontoxic in terms of direct mortality (Bausmith and Neufeld, 1999; Corsi et al., 2009; Pillard, 1995). However, its sublethal effects have become a cause of concern due to a potential for endocrine disruption and a high level of biochemical oxygen demand (Bausmith and Neufeld, 1999; Corsi et al., 2009; Pillard, 1995). This potential toxicity can also be increased through interactions with supplementary chemicals used in deicing mixtures (Corsi et al., 2009; Pillard, 1995). Pillard (1995) found that both propylene and ethylene glycol were significantly more toxic to fleas and minnows when combined with common additives. These formulated mixtures were similar in composition to commercial airline products; therefore it is suspected that the additives contribute heavily to its toxicity (Pillard, 1995).

Another versatile chemical is triclosan ($C_{12}H_7C_{l3}O_2$), which is present in many of the same products as propylene glycol (Bedoux et al., 2011; Dann and Hontela, 2011;

Kookana et al., 2011). While it is not used in deicing compounds, it is used in personal care products such as shampoo, soap and toothpaste, as well as various cleaning supplies (Bedoux et al., 2011; Dann and Hontela, 2011; Kookana et al., 2011). The primary function of triclosan is as an antibacterial and antifungal agent, but its effect on environmental health has recently been thrown into question (Bedoux et al., 2011; Dann and Hontela, 2011; Kookana et al., 2011). Its potential toxicity has raised concerns within the medical community and now within the ecological community as its significant presence in wastewater is considered more seriously (Bedoux et al., 2011; Dann and Hontela, 2011; Kookana et al., 2011).

The vast majority of triclosan content in sewage may be removed in treatment plants following disposal (Dann and Hontela, 2011). However, often a significant amount of triclosan is still present in effluent, where it poses a threat to aquatic ecosystems (Dann and Hontela, 2011). Kumar et al. (2010) examined the flow of triclosan into the Savannah River in Georgia, United States. They found a concentration of 5370 ng/L triclosan in the final effluent and, based on the mass flow estimate, concluded that 138 g/day of triclosan was being deposited into the river. This rate tends to fluctuate in each location, depending on the method of waste treatment that occurs at any particular plant (Dann and Hontela, 2011). Incomplete triclosan removal results in accumulation in the tissues of aquatic organisms via consumption or absorption (Dann and Hontela, 2011).

Freshwater snails in Denton, Texas were found to have concentrations of triclosan as high as 500 µg/kg in muscle tissue (Coogan and LaPoint, 2008). Caged rainbow trout in Sweden were examined by Adolfsson-Erici et al. (2002), at separate locations both upstream and downstream from a wastewater treatment plant. Those located upstream were found to have triclosan concentrations of 710 µg/kg in their bile, whereas those downstream had 17,000 µg/kg (Adolfsson-Erici et al., 2002). Amphibians exhibited sensitivity to acute triclosan toxicity as well as a number of adverse, sublethal effects: disproportional development, decreased body weight, increased hindlimb length, lower activity rates, loss of startle response, and altered thyroid hormone-associated gene expression (Frake and Smith, 2004; Smith and Burgett, 2005; Veldhoen et al., 2006). These results were often species-specific and dependent upon larval stage of development (Dann and Hontela, 2011). Triclosan further exhibited endocrine-disrupting effects on male western mosquitofish (*Gambusia affinis*) at concentrations of 101.3 µg/L (Raut and Angus, 2010). It is these sublethal effects that have been the primary cause for rising concern.

Nova Scotian Amphibians

All species caught for this study are native to Nova Scotia, and are widely distributed throughout the province in fair to high abundance. Sampling was no detriment to the local sustainability of these species. The IUCN lists all of the following species as "least concern".

The spotted salamander (*Ambystoma maculatum*) was the only species sampled of Order Urodela. They are a relatively large species that can exceed 20 cm in length and range from brown to black in colour, with yellow spots aligned in two rows along their backs (Gilhen, 1984; Stebbins and Cohen, 1995). Spotted salamanders can be found in hardwood forested areas with vernal pools suitable for breeding, and primarily forage at night (Gilhen, 1984; Stebbins and Cohen, 1995). Adults typically breed in early spring, between late March to late May, and lay large egg masses within the ponds themselves or against submerged vegetation (Gilhen, 1984). Larvae hatch and develop throughout the summer and early autumn months, occasionally overwintering to metamorphose the following year (Gilhen, 1984; Stebbins and Cohen, 1995).

Wood frogs (*Lithobates sylvaticus*), green frogs (*Lithobates clamitans*), and mink frogs (*Lithobates septentrionalis*) were sampled within Family Ranidae. Wood frogs range between 5-7 cm, and are primarily grey or rust-coloured with a characteristic dark mask around the eyes (Gilhen, 1984; Stebbins and Cohen, 1995). They inhabit a wide range, from forests to tundra, and can be found as far north as the Yukon Territory due to their unique freeze-thaw cycle (Gilhen, 1984; Stebbins and Cohen, 1995). Their cold tolerance allows wood frogs to breed earlier than other native anurans, between March and April (Gilhen, 1984; Stebbins and Cohen, 1995). Wood frog tadpoles typically metamorphose within a two month period of hatching. Green frogs exhibit highly variable skin colour and patterns in shades of green, brown, black, and occasionally bluish green (Gilhen, 1984; Tanara, 1975). Adults are fairly large bodied, between 5-10 cm, with long limbs and large tympanums (Gilhen, 1984; Tanara, 1975). Male tympanums are larger than those of females, and males also sport bright yellow throats to expose during breeding season (Gilhen, 1984). Green frogs are widely prevalent throughout Canada, and can be found in any habitats with fresh water bodies (Gilhen, 1984). Adults typically breed in June and July, producing floating egg mats, though tadpoles often overwinter and metamorphose the following year (Gilhen, 1984; Tanara 1975). Mink frogs are similar in size to wood frogs, but can be distinguished by their bright green and yellow colouring that is dorsally covered in dark spots (Gilhen, 1984). In this species, males also have larger tympanums and bright yellow throats (Gilhen, 1984). Mink frogs inhabit woodland ponds within mixed forests, and typically breed between late May and June (Gilhen, 1984; Stebbins and Cohen, 1995). Their large egg masses are laid in the deepest ponds available and hatch within a few days to a week, though tadpoles often overwinter (Gilhen, 1984; Stebbins and Cohen, 1995).

Spring peepers fall within Family Hylidae, the tree frogs. These small frogs range between 2.5-4 cm in length (Gilhen, 1984; Stebbins and Cohen, 1995). They are typically light brown or grey in colour, with a characteristic dark pattern on their backs in the shape of an 'x' (Gilhen, 1984). During breeding season, spring peepers inhabit wetlands and ponds adjacent to the woodlands where they return to for the rest of the year (Gilhen, 1984; Stebbins and Cohen, 1995). Breeding may take place anywhere from early April to mid-June, their small strings and clusters of eggs affixed to the vegetation within ponds (Gilhen, 1984; Stebbins and Cohen, 1995). Tadpoles develop throughout the summer and early autumn months to achieve metamorphosis the same year (Gilhen, 1984).

American toads belong to Family Bufonidae and may be anywhere from 2.5-10 cm in length (Gilhen, 1984; Tanara, 1975). They are dark in colour, typically brown or rust-coloured, and are covered in black spots with warts characteristic to their family (Gilhen, 1984). American toads can be found in variable freshwater habitats, but most often near woodlands. Their breeding season begins in May and continues throughout June (Gilhen, 1984; Tanara, 1975). Long strings of eggs are often laid over vegetation of ponds or in streams, and tadpoles hatch within a few days to a week (Gilhen, 1984). They tend to reach metamorphosis by the end of the summer (Gilhen, 1984; Tanara, 975).

Objectives

I had three main objectives that focus around the same centralized problems. The first was to examine the acute toxicity of chloride-based deicing agents on Nova Scotian amphibian larvae. NaCl was the primary focus of these tests, as it is the most commonly used deicing salt in Nova Scotia. Secondly, I investigated the toxic effects of green deicers for larvae of the same amphibian species to determine if they had fewer or reduced impacts. I used CMA as the primary agent because of its growing popularity on the market and its designation as one of the most "green" deicers available. My final objective was to determine the acute toxicity of personal care chemicals on amphibian larvae. Propylene glycol served as the bridge between deicing agents and personal care chemicals, as it is used in both. The primary focus of the latter was triclosan, due to both its popularity and its increasing notoriety as an ecological contaminant.

In addition, I wanted to test the acute toxicity of these chemicals across various weights and stages of larval development. This would show any trend which may occur in amphibian sensitivity to these chemicals during the early stages of their complex lifecycle. It would also determine whether such trends are species-specific or applicable to amphibian development as a whole. Amphibians are complex organisms that do not fit within rigid physical constraints. Their lifecycle is unlike most other vertebrates, involving numerous developmental stages. Each of these stages requires a different amount of time and energy expenditure. Many past studies in this area have not taken amphibian larval stage into account, or have only used those at intermediate levels. It is impossible to lump all amphibian larvae of a species into a single category, particularly when stages occur at different sizes and weights. I hypothesized that the tested amphibians would exhibit differing sensitivities depending on larval stage for all of the tested chemicals. I predicted that the earliest stages would be the most sensitive, while older stages that are more robust and well developed would be less sensitive. However, as larvae develop they tend to grow in size, which may confound the variables weight and larval stage.

I predicted that CMA would yield similar results to their salt-based alternatives when tested without aeration, making them comparable in toxicity. This is due to the high biochemical oxygen demand of CMA, which will deplete those available resources for the experimental tadpoles. The natural breeding ponds of most amphibian species are clouded and stagnant, having limited aeration and overturn. These experiments included egg exposure of early breeding amphibians in order to assess hatching success, considering that CMA will still be present in aquatic ecosystems during spring months.

Experiments across all objectives were conducted in varying temperature and lighting conditions. This simulated the shift in seasonal temperature and potential light exposure or restriction for different breeding sites. Many ephemeral ponds are clouded with sediment and organic material, thus inhibiting the light which penetrates the surface and reaches the egg masses. Breeding amphibians tend to prefer these low light conditions as it prevents high UVB exposure (Bancroft et al., 2008). Amphibians also exhibit a higher metabolic rate in higher temperatures, therefore metabolizing the potentially harmful chemicals faster. This may lead to increased acute toxicity at higher temperatures.

My research will provide insight into the acute toxicity of these chemicals, as well as the interaction of multiple variables. These comparisons will help to determine which amphibian species are at the greatest risk, and under what conditions. This will offer relevant insight into the complexity of environmental stressors and identify areas of high concern within the amphibian community and aquatic systems as a whole.

Methodology

Field Sampling and Storage

Six native species were obtained for testing to ensure a representation of variability among amphibian species: spotted salamanders (*Ambystoma maculatum*), wood frogs (*Lithobates sylvaticus*), spring peepers (*Pseudacris crucifer*), mink frogs (*Lithobates septentrionalis*), green frogs (*Lithobates clamitans*), and American toads (*Anaxyrus americanus*). Throughout the remainder of this thesis I will refer to these species by their common names. These are the most common species available in the province, thus they are the easiest to obtain and sampling is unlikely to adversely affect populations. Wood frogs and American toads were used in more individual experiments than the other species. These two species were sampled primarily from Devon and Sambro, N.S., respectively.

Sampling occurred from April to October of both 2013 and 2014. Overall sampling was done throughout Nova Scotia, with a focus on Halifax Regional Municipality. There were ten primary field sites, five of which were sampled in both 2013 and 2014 to provide more widely applicable results: Devon, Enfield, Harrietsfield, Ellershouse, and Sambro (Crystal Crescent Beach). The remaining sites had either dried up (e.g. ephemeral ponds), or did not have large amphibian populations and could not provide sufficient numbers of specimens at the time of sampling. Sampling methodology was consistent for each site, with egg masses and larvae collected via dip net. Specimens were temporarily stored in plastic bags filled with water from their respective site, and kept in a cooler for transportation. Ephemeral ponds located adjacent to roadways were the primary focus due to their high concentration of breeding amphibians, but larger ponds and wetlands were also sampled. All sites were within 100 metres of paved roadways, which typically functioned as secondary highways.

Sample specimens were stored in the Saint Mary's University Science Building; egg masses were stored at approximately 8˚C and larvae between 12˚C and 22˚C, depending on the seasonal norm for outside temperatures. Eggs and larvae were stored separately, and those from each site location were stored in individual one litre containers. The containers were filled with pond water from the specimen's field site.

These conditions were designed to replicate those experienced by early life stage amphibians during the Nova Scotian spring and summer seasons. In preparation for laboratory experiments, 10-20 larvae from each sample were assessed for Gosner stage and weighed to produce an average weight range per larval stage. Staging involved examination under a dissecting microscope to assess the distribution of masses. In the case of anuran tadpoles, these observations are based from the detailed guide written by Gosner (1960). Those at Gosner stage 25 or above were fed regularly, as that marks the first feeding stage (Gosner, 1960). Spotted salamander larvae were instead grouped into small and large individuals based on weight, as they have no comparable staging process. Specimens were weighed to four decimal points using a digital scale.

Figure 1. Primary sample sites located across Nova Scotia. Blue sites indicate those sampled in both 2013 and 2014. Red sites were only sampled in 2013. From the top: 1. Mount Thom, 2. Trafalgar, 3. Ellershouse, 4. Enfield, 5. Devon, 6. Otter Lake, 7. Harrietsfield, 8. Bear Cove, 9. Peggy's Cove, 10. Crystal Crescent Beach.

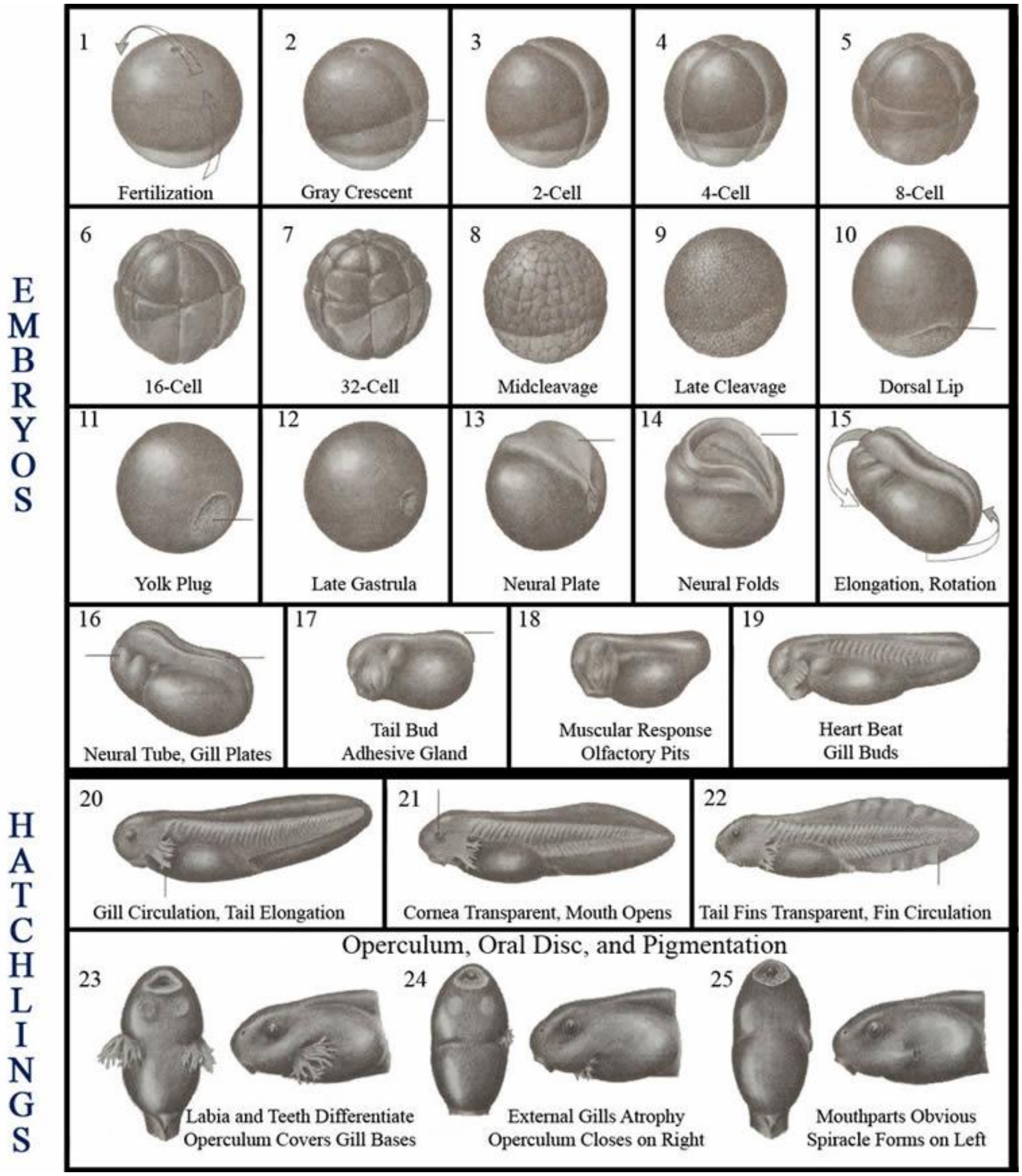

Figure 2a. The Gosner (1960) Staging System for anurans, depicting larval development from stages 1-25.

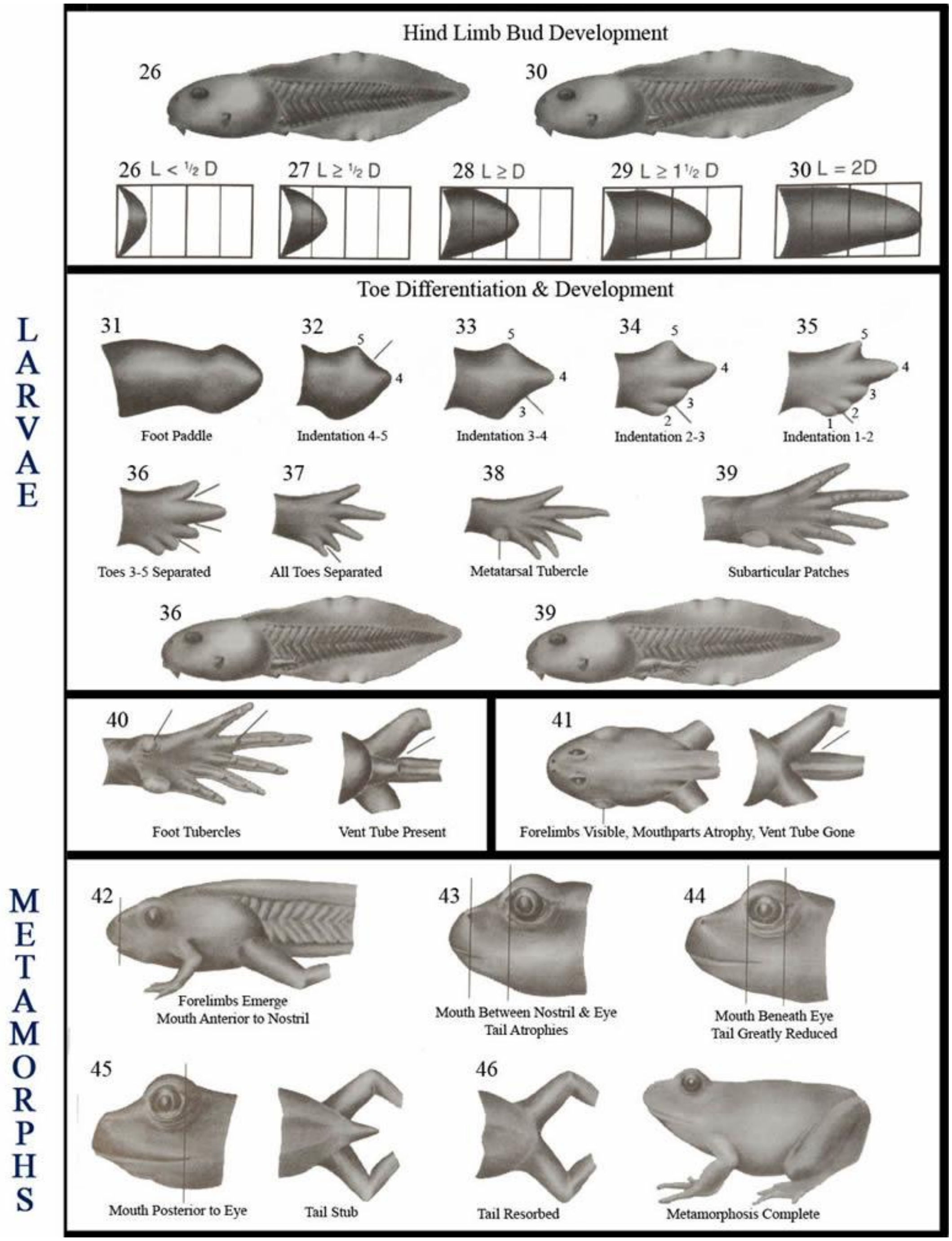

Figure 2b. The Gosner (1960) Staging System for anurans, depicting larval development from stages 26 to adulthood.

Acute Toxicity Tests

Lethal concentration (LC_{50}) tests were conducted on larvae at various developmental stages for exposure to NaCl, CMA, propylene glycol, and triclosan. The ranges of experimental concentrations were: NaCl from 50-12,000 mg/L, CMA from 50-4000 mg/L, propylene glycol from 1000-60,000 mg/L, and triclosan from 50-1000 µg/L. For each stage, the concentrations were increased incrementally by 200 mg/L or µg/L until 100% mortality occurred. Test solutions were mixed using dechlorinated water. For each test, six larvae were placed in a container containing 250ml of the specific solution; three replicates were done for each test (18 larvae total). Controls were 6 larvae in each of three replicates but the control contained dechlorinated water only. Triclosan experiments had a second set of controls composed of dechlorinated water and ethanol, as a small drop of ethanol was used in the test solutions to aid with the solubility of triclosan.

Only spotted salamander egg masses were used to determine hatching success when exposed to CMA. Ten eggs were separated from their egg masses and placed in a small, plastic container with a mix of dechlorinated Halifax tap water and CMA. This was done for all six concentrations ranging from 250-9000 mg/L CMA, with three replicates per concentration. Hatching success was recorded for each container. CMA experiments were conducted on intermediate developmental stages for larvae of spotted salamanders, wood frogs, and spring peepers. Larvae were divided into aerated and non-aerated treatments. This was due to the tendency of CMA to deplete oxygen, particularly in an enclosed container with a small volume.

Salt and triclosan treatments were tested in both cold and warm temperatures (on larvae of similar stage and weight) to compare varying environmental conditions across the spring and summer months. To test for potential triclosan breakdown, one experiment utilized two sets of triclosan experimental treatments in the light. The first, which was called Static, was set up as the other experimental conditions. The second, which was called Static Renewal, had each treatment solution remade and changed daily over the course of the 96 hour experiment. All experiments were observed daily over a 96 hour period, at which time dead larvae were removed from their containers, disposed of and any changes recorded. Results were analyzed using the trimmed Spearman-Karber method to calculate the median lethal concentrations and 95% confidence intervals.

Table 1. Breakdown of the amphibian species used for each experiment. G.S. = Gosner Stage.

96h LC_{50} Experiments	Amphibian Species
Chloride over increasing G.S.	Wood frogs (G.S. 19-33)
Chloride at 12˚C and 22˚C	Wood frogs (G.S. 25)
CMA with aeration	Spotted salamanders, wood frogs, spring peepers (G.S. 25)
CMA without aeration	Spotted salamanders, wood frogs, spring peepers (G.S. 25)
CMA on hatching success	Spotted salamander eggs
Propylene glycol across species	Spotted salamanders, wood frogs, spring peepers, green frogs, American toads (G.S. 25)
Triclosan over increasing GS	American toads (G.S. 19 and 37)
Triclosan over increasing weight	Spotted salamanders (small and large)
Triclosan across species	Spring peepers, wood frogs, spotted salamanders, mink frogs, American toads, green frogs (G.S. 25)
Triclosan at 12˚C and 22˚C	Wood frogs and American toads (G.S. 19)
Static Renewal of Triclosan	Wood frogs (G.S. 19)

Results

Acute Chloride Toxicity

The lethal concentration of chloride differed among stages of wood frog development, with lethal concentrations being much lower at earlier Gosner stages (Figure 3a; Figure 3b). Median LC_{50} values follow a similar rising trend between Devon, N.S. (Table 2a) and Harrietsfield, N.S. (Table 2b). Devon stage 19 LC_{50} = 85.77 mg/L (95% CI: 67.88 – 100.38) and Harrietsfield stage 19 LC_{50} = 121.54 mg/L (95% CI: 90.08 – 144.72). Low LC_{50} values indicate that early life stages are highly susceptible to chloride toxicity. The rising trend in LC_{50} continues, increasing dramatically throughout tadpole development (stage 25+). Devon stage 33 LC_{50} = 1642.84 mg/L (95% CI: 1345.58 – 2005.76) and Harrietsfield stage 33 LC_{50} = 1406.96 mg/L (95% CI: 1185.48 – 1788.35). Chloride is less toxic to mature tadpoles approaching metamorphosis.

The wide range of LC_{50} values among Gosner stages 19, 22, 26, 29, and 33 can be further broken down into 24 hour segments. In general, tadpoles are more susceptible to chloride toxicity as the 96 hour period progresses (Figure 4; Table 3). Stage 19 progression: 24 hr LC_{50} = 354.44 mg/L (95% CI: 274.45 - 457.74), 48 hr LC_{50} = 147.09 mg/L (95% CI: 113.78 - 190.14), 72 hr LC_{50} = 112.33 mg/L (95% CI: 88.23 - 143.02). The same trend can be seen throughout development and in late stages as well. Stage 33 progression: 24 hr LC_{50} = 2280.82 mg/L (95% CI: 1935.56 - 2687.67), 48 hr LC_{50} =

2117.98 mg/L (95% CI: 1783.59 - 2515.05), 72 hr LC_{50} = 1812.07 mg/L (95% CI: 1492.76 - 2199.68).

Acute chloride toxicity differed for Gosner stage 19 wood frog tadpoles between 22˚C and 12˚C, over a 96 hour period (Figure 5). Room temperature yielded a low LC_{50}, while the colder temperature of 12˚C yielded a high LC_{50}. This illustrates that wood frogs tadpoles are more sensitive to chloride toxicity in a warm environment than in a cold environment. 22˚C 96 hour LC_{50} = 97.01 mg/L (95% CI: 69.22 – 110.14). 12˚C 96 hour LC_{50} = 972.27 mg/L (95% CI: 825.01 - 1145.81) (Table 4).

Acute CMA Toxicity

Larval acute CMA toxicity experiments done with aeration produced higher LC_{50} values than without aeration (Figure 6). This trend occurred in larvae of all three tested species (spotted salamanders, wood frogs, and spring peepers). Therefore, the toxicity of CMA when aeration is present is considerably lower (Table 5). Larvae are more susceptible to CMA toxicity without aeration, and a more distinct difference among species is evident. This response was also seen in spotted salamander eggs tested with aeration (Figure 7), showing that a high concentration of CMA is required to produce a negative effect on egg hatching success; hatching success in varying concentrations of CMA ranged from 84% to 23% (Figure 7).

Acute Propylene Glycol Toxicity

Propylene glycol had high LC_{50} values when acute toxicity tests (96 hour) were performed on the larvae of spotted salamanders, wood frogs, spring peepers, green frogs, and American toads (Figure 8). The lowest LC_{50} concentrations were for the early breeders (spotted salamanders and wood frogs), but the toxicity of propylene glycol was low to all species tested (Table 6).

Acute Triclosan Toxicity

Acute toxicity values of triclosan differed across the larval development of American toads and spotted salamanders. The lowest 96 hour LC_{50} values occur at stage 19 during early development, while the mature stage 37 exhibit higher LC_{50}s (Figure 9; Figure 10; Table 7). Early life stages of American toads are more susceptible to the toxic effects of triclosan than their older life stages. The same trend occurred for spotted salamanders, although they are separated by size rather than stage. Salamander samples were classified as either small, 0.0072g – 0.0191g, or large, 0.1621g – 0.3080g. The two groups were significantly different in weight ($t = 14.76$, $F = 226.5$, $P = < 0.0001$). Small spotted salamander larvae were more susceptible to triclosan toxicity than large larvae (Table 7; Figure 10). This relationship shows that susceptibility to triclosan toxicity decreases with Gosner stage and size.

Triclosan acute toxicity also differed across species (Figure 11). The six species of larvae were tested during early development to allow for comparability. Spring peepers, American toads, wood frogs, and spotted salamanders were the most susceptible to triclosan toxicity. Mink frogs and green frogs were less susceptible, with significantly higher 96 hour LC_{50}s (Table 8).

The acute toxicity of triclosan not only differed among species and developmental stage, but also between warm and cold temperatures. Wood frog and American toad tadpoles (Gosner stage 19) exhibited a decreasing trend in median LC_{50} values from 12˚C – 22˚C (Figure 12a; Figure 12b; Table 9). Both species of larvae were more susceptible to triclosan toxicity in a warm environment over a cold environment. Based on Figure 13, there was no evident difference in acute toxicity between static and static renewal treatments of triclosan. Static 96 hour LC_{50} = 133.05 µg/L (95% CI: 108.15 – 156.88), static renewal = 138.06 µg/L (95% CI: 115.27 – 160.45). This shows that the solutions remained intact over the experimental period.

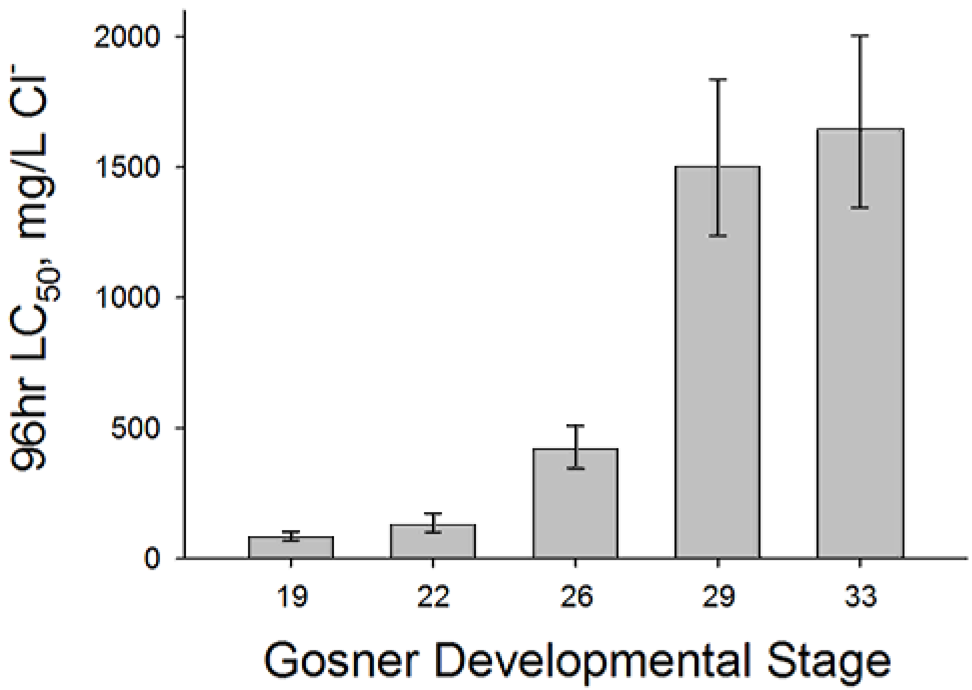

Figure 3a. Median LC_{50} and 95% CI (mg/L) of 96 hour chloride toxicity tests for increasing Gosner stage of wood frog tadpoles from Devon, N.S.

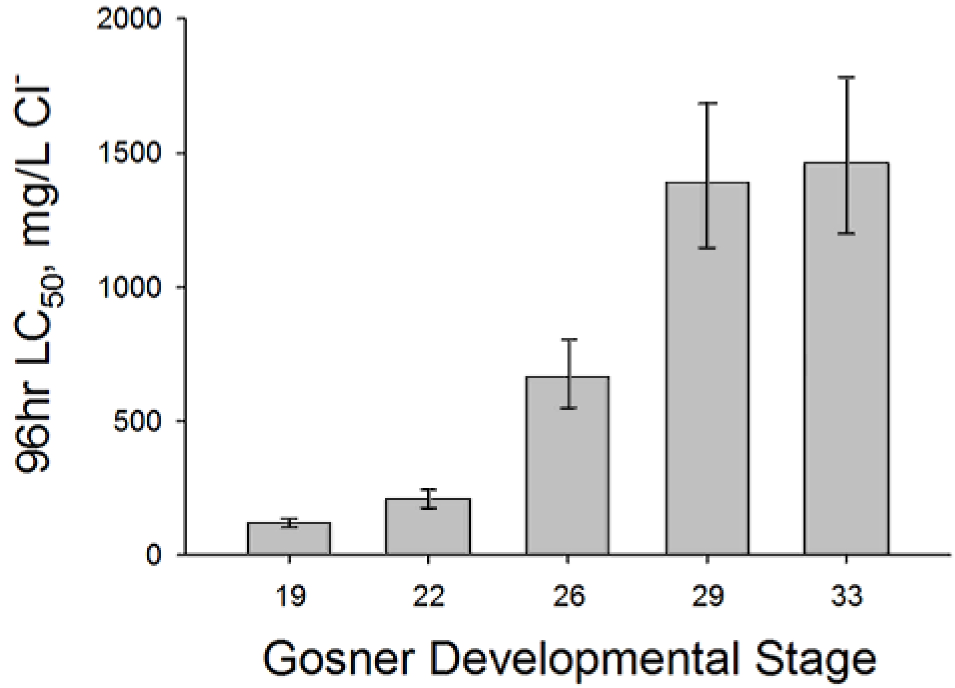

Figure 3b. Median LC_{50} and 95% CI (mg/L) of 96 hour chloride toxicity tests for increasing Gosner stage of wood frog tadpoles from Harrietsfield, N.S.

Table 2a. LC_{50} results (mg/L) of 96 hour chloride toxicity tests for increasing Gosner stage of wood frog tadpoles from Devon, N.S.

Gosner Stage	Median LC_{50}	Lower 95% CI	Upper 95% CI
19	85.77	67.88	100.38
22	132.96	101.87	173.53
26	421.25	345.10	514.20
29	1506.71	1235.08	1838.07
33	1642.84	1345.58	2005.76

Table 2b. LC_{50} results (mg/L) of 96 hour chloride toxicity tests for increasing Gosner stage of wood frog tadpoles from Harrietsfield, N.S.

Gosner Stage	Median LC_{50}	Lower 95% CI	Upper 95% CI
19	121.54	90.08	144.72
22	216.70	180.45	249.06
26	596.27	481.53	702.93
29	1342.68	1093.53	1626.41
33	1406.96	1185.48	1788.35

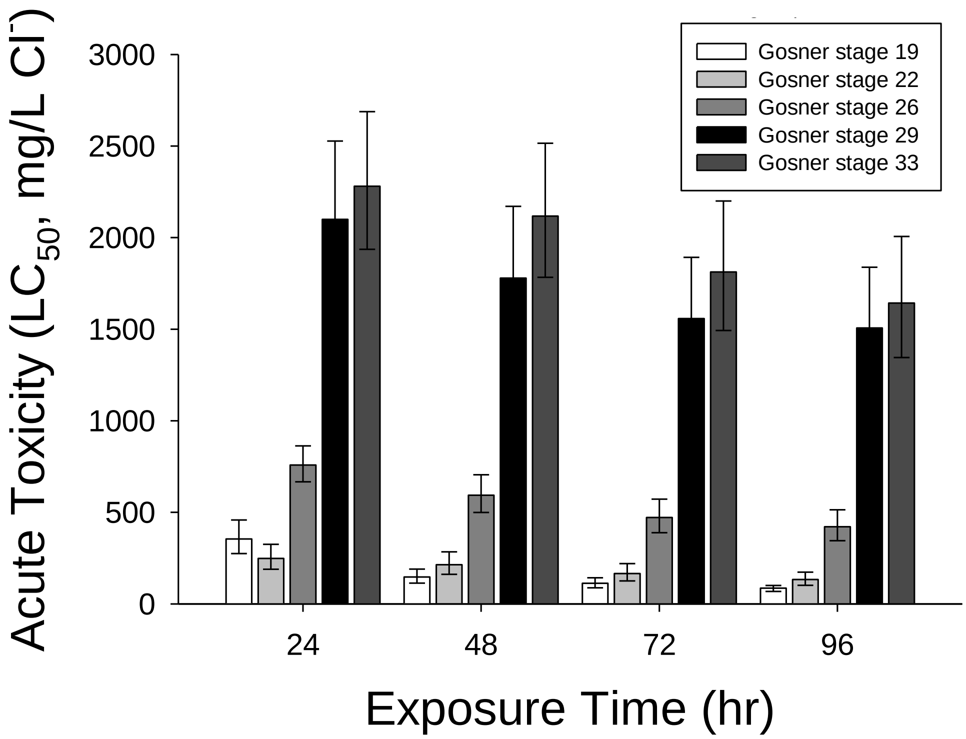

Figure 4. Median LC_{50} and 95% CI (mg/L) of chloride toxicity tests (24, 48, 72, 96 hr) across five Gosner stages of wood frog tadpoles from Devon, N.S.

Table 3. LC_{50} results of chloride toxicity tests (24, 48, 72, 96 hr) across five Gosner stages of wood frog tadpoles from Devon, N.S.

Time	Stage 19	Stage 22	Stage 26	Stage 29	Stage 33
24 hour					
Median LC_{50}	354.44	247.82	758.17	2099.2	2280.82
Lower 95% CI	274.45	188.71	666.31	1743.43	1935.56
Upper 95% CI	457.74	325.45	862.69	2527.57	2687.67
48 hour					
Median LC_{50}	147.09	214.15	593.32	1778.84	2117.98
Lower 95% CI	113.78	161.35	499.42	1457.74	1783.59
Upper 95% CI	190.14	284.22	704.86	2170.68	2515.05
72 hour					
Median LC_{50}	112.33	166.1	471.52	1558	1812.07
Lower 95% CI	88.23	125.65	388.46	1282.8	1492.76
Upper 95% CI	143.02	219.78	572.33	1892.24	2199.68

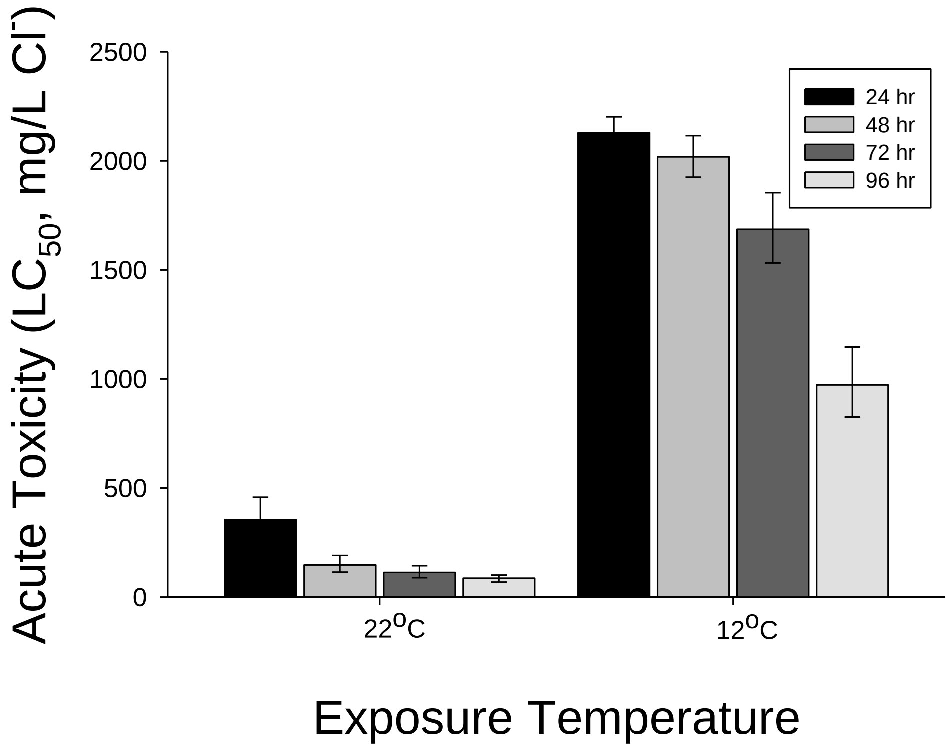

Figure 5. Median LC_{50} and 95% CI (mg/L) of chloride toxicity tests for wood frog tadpoles from Devon, N.S. (Gosner stage 19) at 22˚C and 12˚C.

Table 4. LC_{50} results of chloride toxicity tests for wood frog tadpoles from Devon, N.S. (Gosner stage 19) at 22˚C and 12˚C.

Temperature	Median LC_{50}	Lower 95% CI	Upper 95% CI
22	97.01	69.22	110.14
12	972.27	825.01	1145.81

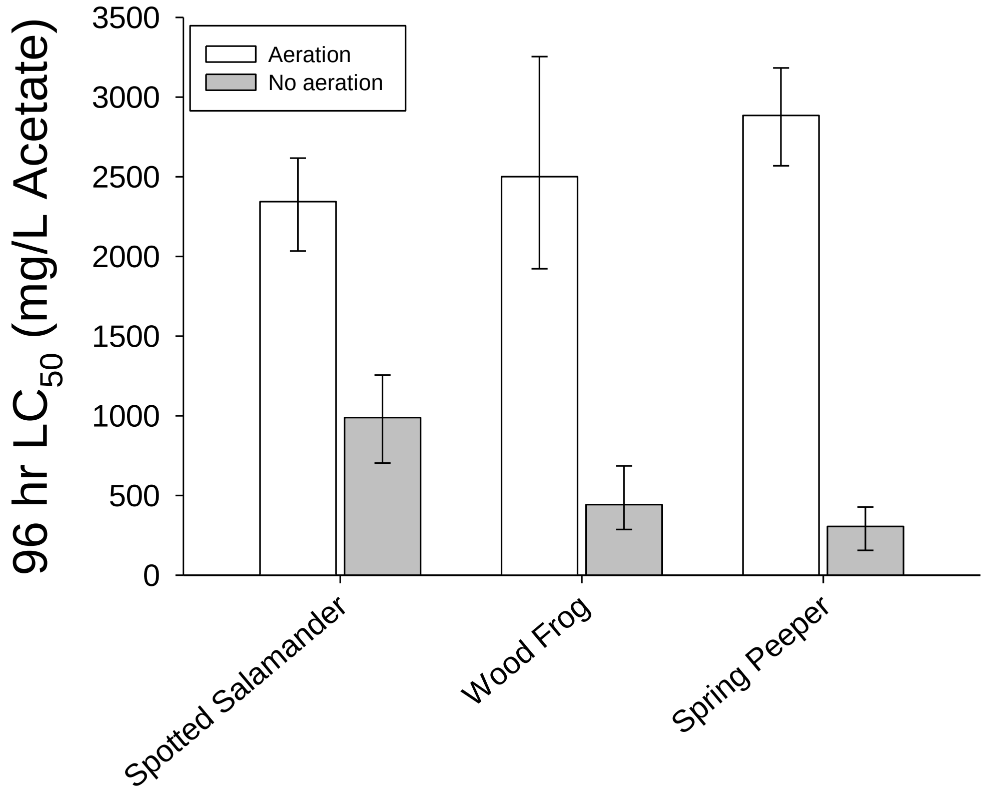

Figure 6. Median LC_{50} and 95% CI (mg/L) of 96 hour CMA toxicity tests (with and without aeration) for spotted salamander, wood frog, and spring peeper larvae at Gosner stage 25.

Table 5. LC_{50} results (mg/L) of 96 hour CMA toxicity tests (with and without aeration) for spotted salamander, wood frog, and spring peeper larvae.

	Median LC_{50}	Lower 95% CI	Upper 95% CI
<u>Aerated</u>			
Spotted salamander	2376.06	2045.14	2688.21
Wood frog	2588.45	1972.83	3089.48
Spring peeper	2831	2518.93	3196.55
<u>Not Aerated</u>			
Spotted salamander	982.06	606.21	1380.42
Wood frog	465.17	278.08	614.54
Spring peeper	268.40	191.76	488.16

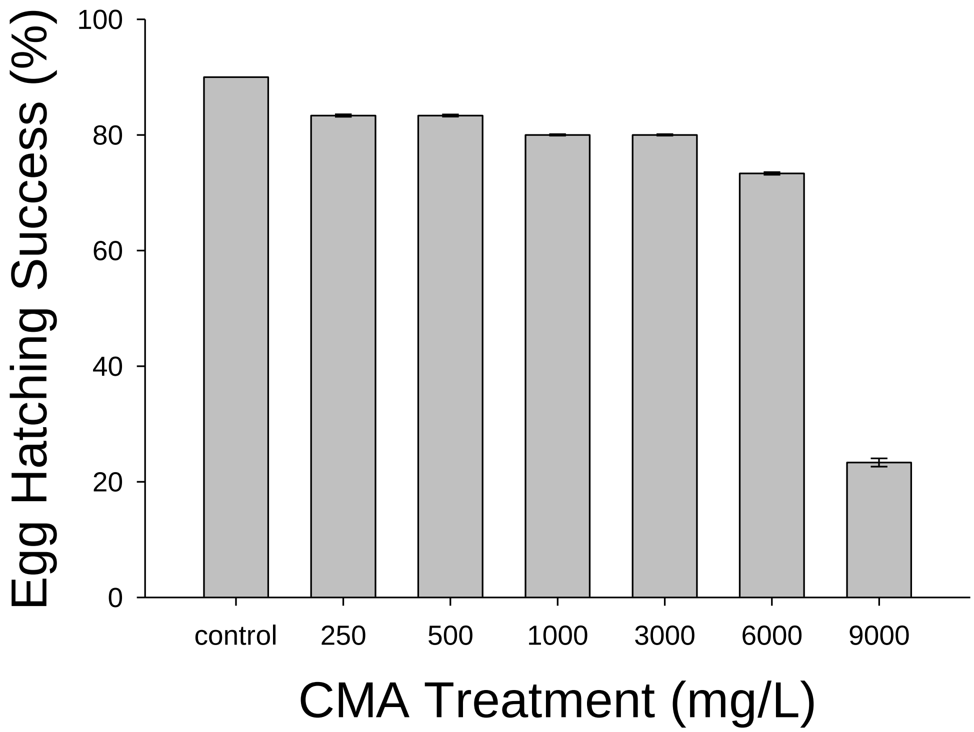

Figure 7. Egg hatching success for spotted salamanders from Enfield, N.S. exposed to varying concentrations of CMA (mg/L).

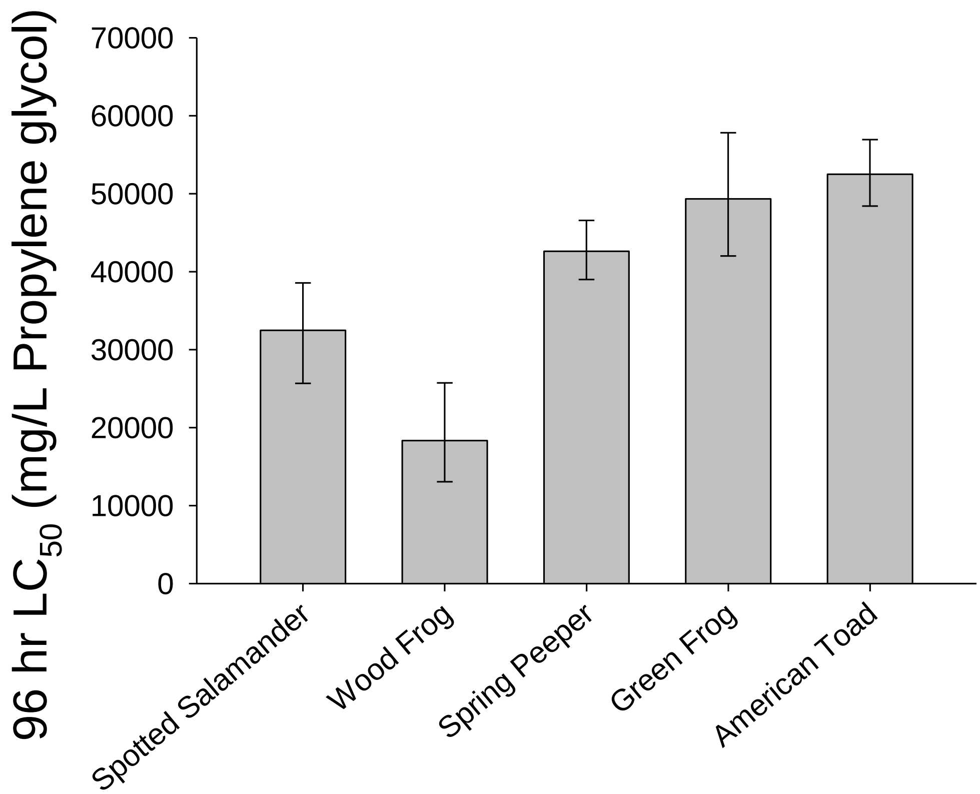

Figure 8. Median LC_{50} and 95% CI (mg/L) of 96 hour propylene glycol toxicity tests across five species of N.S. amphibian larvae.

Table 6. LC_{50} results (mg/L) of 96 hour propylene glycol toxicity tests across five species of N.S. amphibian larvae.

	Median LC_{50}	Lower 95% CI	Upper 95% CI
Spotted salamander	31,558.05	26,852.21	39,874.22
Wood frog	18,068.47	12,910.83	26,107.58
Spring peeper	42,405.65	38,288.76	47,518.04
Green frog	50,092.14	42,716.08	57,182.35
American toad	53,168.26	48,952.69	56,185.40

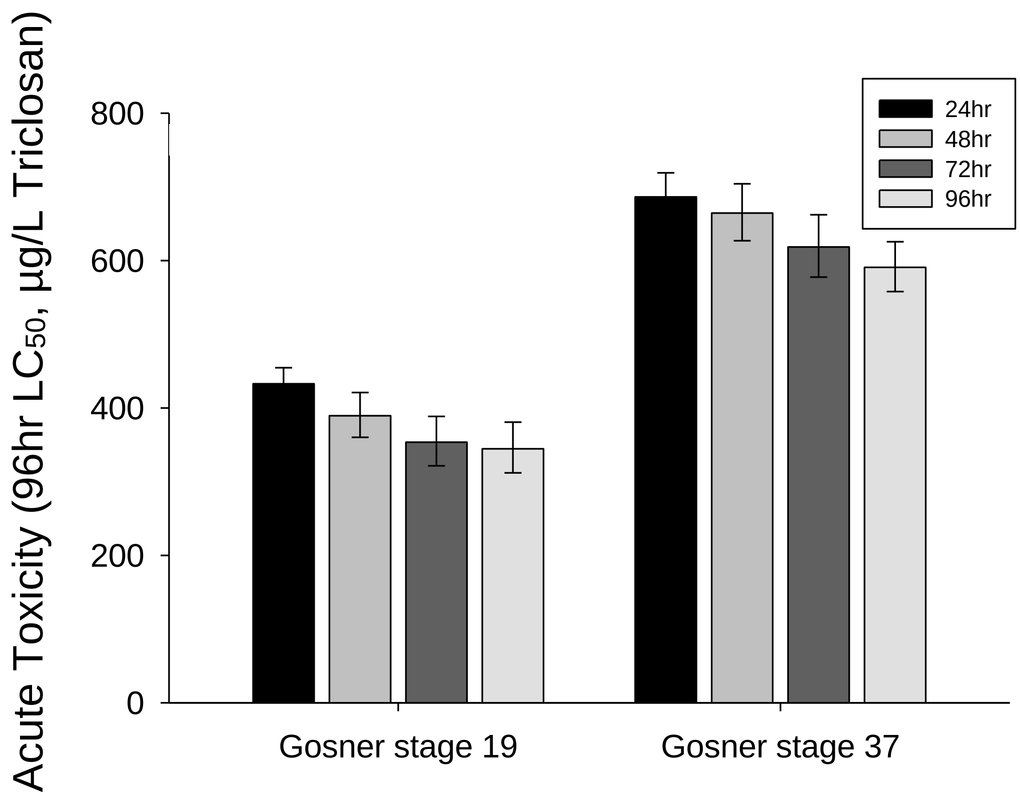

Figure 9. Median LC_{50} and 95% CI (µg/L) of triclosan toxicity tests for American toads at Gosner stage 19 and 37, from Sambro, N.S.

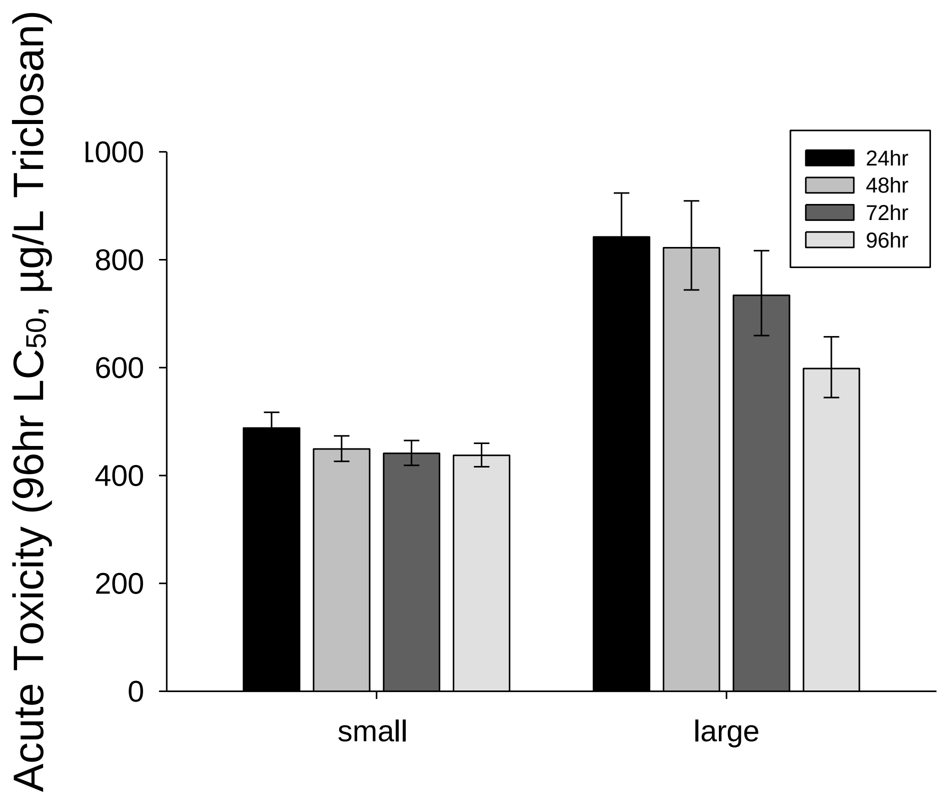

Figure 10. Median LC_{50} and 95% CI (µg/L) of triclosan toxicity tests for small and large spotted salamanders, from Enfield, N.S.

Table 7. LC_{50} results (µg/L) of triclosan toxicity tests for American toads (Gosner stage 19 and 37) and spotted salamanders (small and large), from Enfield, N.S.

	Median LC_{50}	Lower 95% CI	Upper 95% CI
American toad GS 19	361.02	345.44	390.76
American toad GS 37	592.18	571.06	612.85
Spotted salamander Small	417	401.66	430.58
Spotted salamander Large	608.41	579.26	633.18

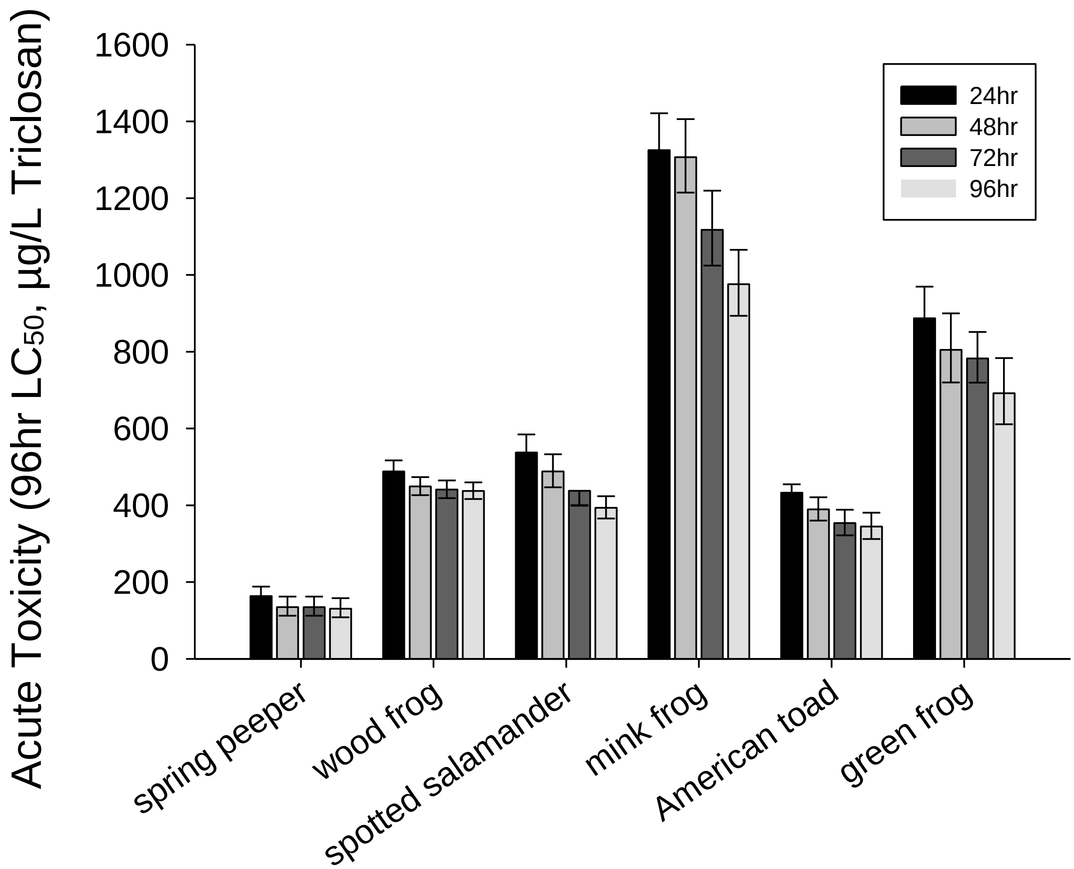

Figure 11. Median LC_{50} and 95% CI (µg/L) of triclosan toxicity tests across six species of N.S. amphibian larvae.

Table 8. LC_{50} results (μg/L) of triclosan toxicity tests across six species of N.S. amphibian larvae.

	Median LC_{50}	Lower 95% CI	Upper 95% CI
Spotted salamander	400.58	382.67	419.38
Wood frog	427.88	406.15	441.26
Spring peeper	180.76	172.04	191.45
Mink frog	981.23	915.06	194.42
Green frog	651.38	585.66	729.01
American toad	389.72	360.46	408.91

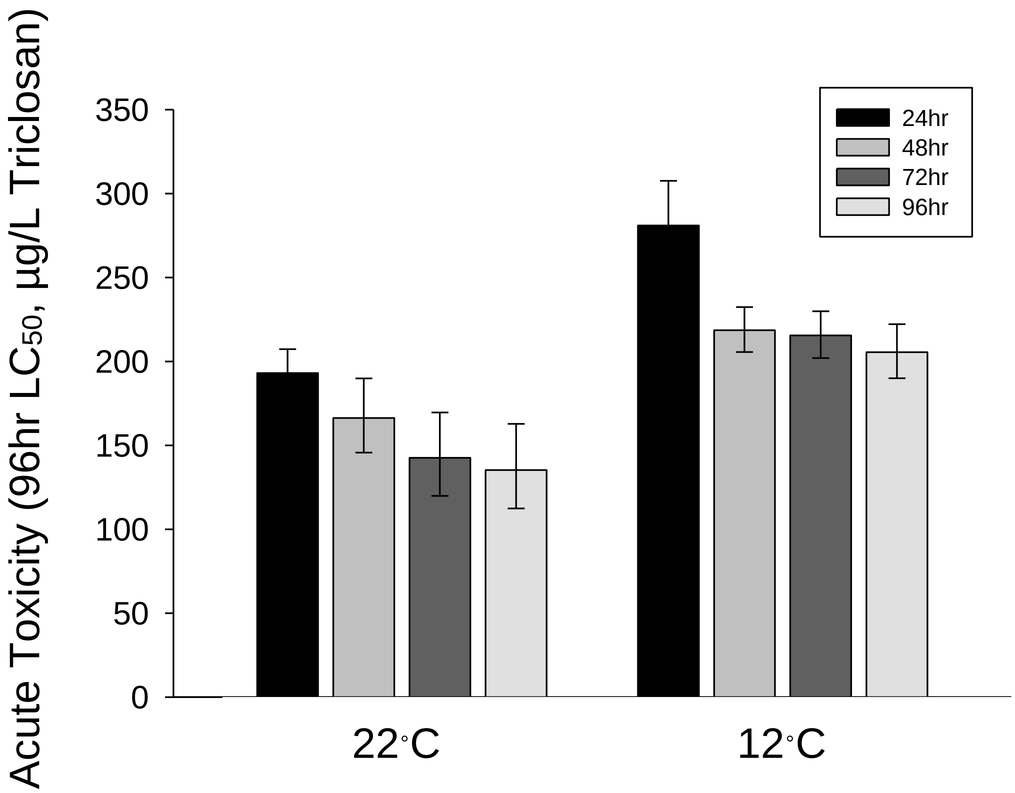

Figure 12a. Median LC_{50} and 95% CI (µg/L) of triclosan toxicity tests for wood frogs from Devon, N.S. (Gosner stage 19) at 22˚C and 12˚C.

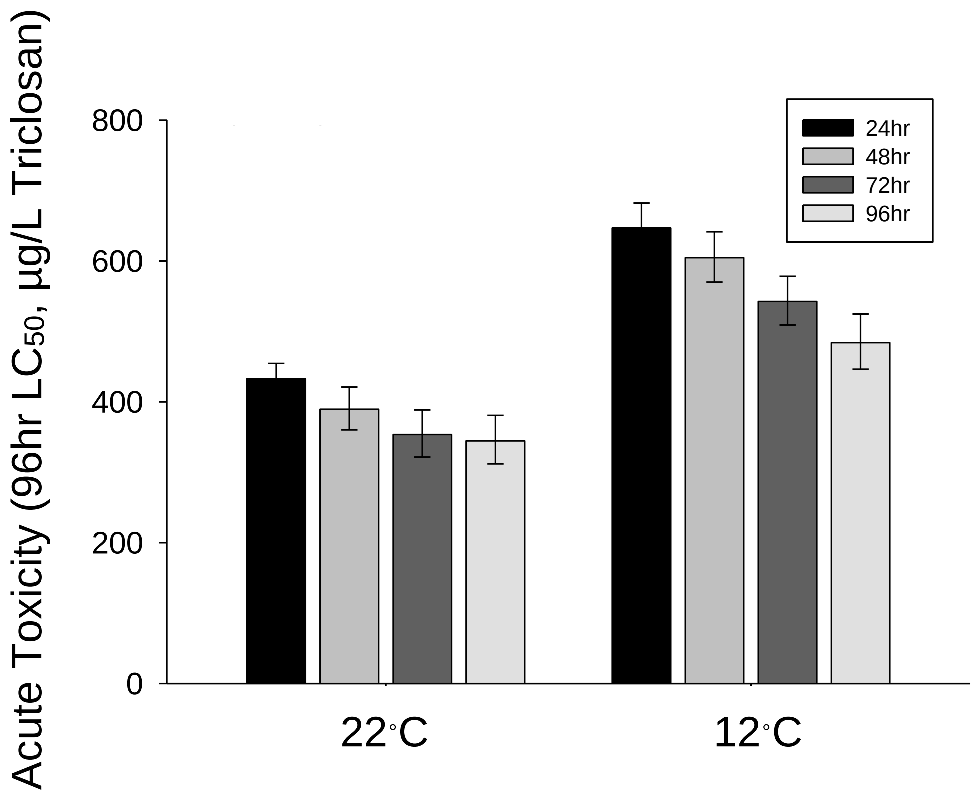

Figure 12b. Median LC_{50} and 95% CI (µg/L) of triclosan toxicity tests for American toad tadpoles from Sambro, N.S. (Gosner stage 19) at 22˚C and 12˚C.

Table 9. LC_{50} results (µg/L) of triclosan toxicity tests for wood frog and American toad tadpoles (Gosner stage 19) at 22˚C and 12˚C.

22˚C, 12˚C	Median LC_{50}	Lower 95% CI	Upper 95% CI
Wood frog	138.72	106.17	166.83
American toad	374.18	316.23	395.62
Wood frog	219.08	190.54	227.06
American toad	466.07	429.56	514.72

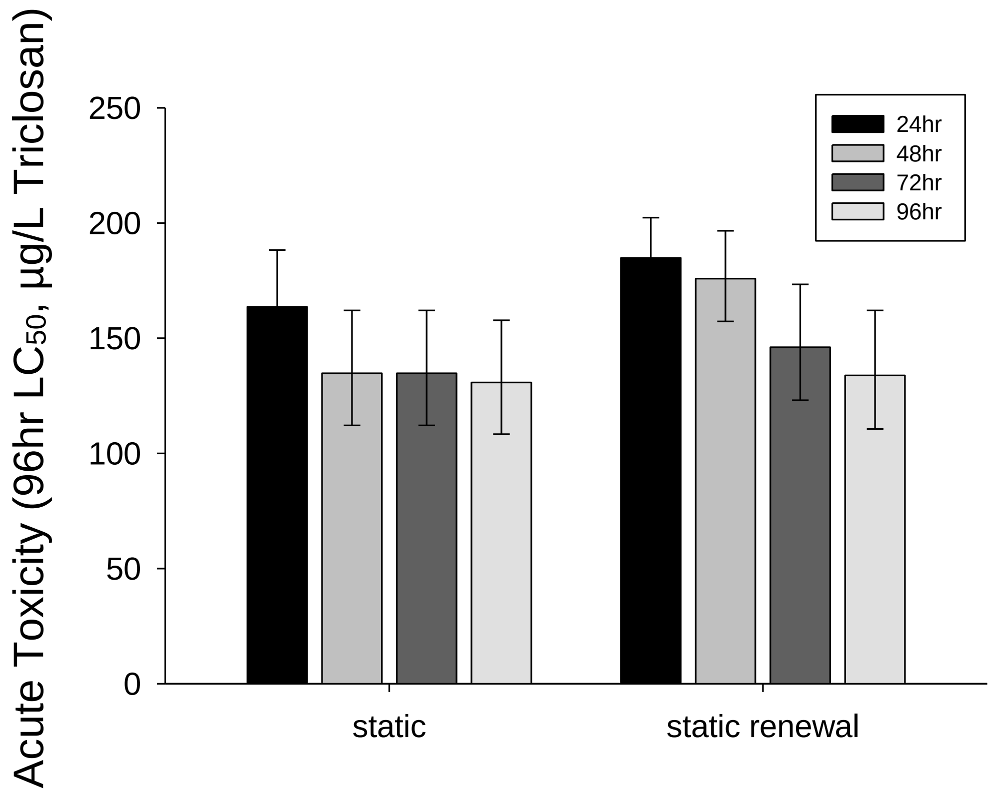

Figure 13. Median LC_{50} and 95% CI (µg/L) of triclosan toxicity tests for wood frogs from Devon, N.S. (Gosner stage 19) with both static and static renewal of solutions.

Discussion

Acute Chloride Toxicity

Acute exposure to ecologically relevant levels of NaCl was detrimental to the survival of wood frog tadpoles. The median lethal concentrations calculated were well below the values previously determined in other studies (Collins and Russell, 2009; Sanzo and Hecnar, 2006). Collins and Russell (2009) found a 96 hour LC_{50} value of 1721.4 mg/L for wood frog tadpoles, while Sanzo and Hecnar (2006) found LC_{50} to be 2636.50 mg/L. Collins and Russell (2009) also calculated a 96 hour LC_{50} value of 1178.20 mg/L for spotted salamanders larvae, making them even more sensitive than wood frog tadpoles. All specimens used in both studies were Gosner stage 25-26, the first feeding stage and an intermediate stage in development. My study found wood frog tadpoles at Gosner stage 26 to be more sensitive, with 96 hour LC_{50} results of 421.25 mg/L and 596.27 mg/L, in Devon and Harrietsfield, N.S. respectively. Runoff during spring and early summer months often exceeds the maximum “safe” limit for chloride, previously identified as approximately 250 mg/L (Environment Canada, 2001; Hill and Sadowski, 2015; Kaushal et al., 2005). Sanzo and Hecnar (2006) reported a range of 0.39-1030.00 mg/L chloride in many Ontario wetlands. An extensive study performed by Environment Canada (2001) reported an increase in chloride concentrations for ponds less than 50 metres from roadsides, with many exceeding 4000 mg/L. Based on this range, it is expected that mortality of wood frog tadpoles occurs in natural ponds and wetlands due to elevated chloride concentrations in road runoff.

The potential for chloride related mortality becomes more disconcerting when considering the entire span of tadpole development. Previous research in this field focuses solely on Gosner stage 25-26 as an acceptable standard, being an approximate intermediate point between hatching and metamorphosis (Gilhen, 1984; Gosner, 1960). However, in this study I found an inverse relationship between Gosner stage and chloride sensitivity. Younger wood frog tadpoles were significantly more sensitive to chloride than older tadpoles. The 96 hour LC_{50} calculations for individuals at Gosner stage 19 were 85.77 mg/L and 121.54 mg/L from Devon and Harrietsfield, N.S. respectively. The results for those at Gosner stage 22 were 132.96 mg/L and 216.70 mg/L, respectively. By Gosner stage 33, LC_{50} results have risen to 1642.84 mg/L and 1406.96 mg/L respectively. There is an increasing trend in median lethal concentrations as the wood frog tadpoles develop, meaning that their chloride sensitivity is decreasing with age. Nearly identical physiology makes it likely this trend extends in varying degrees to other amphibian larvae, particularly those also native to Nova Scotia.

This may pose a significant threat to early breeding amphibian species. Elevated chloride concentrations are most prevalent in road runoff during early spring months, from March to early May (Environment Canada, 2001; Helmreich et al., 2010). Fresh meltwater from snow and ice is highly contaminated with road salt that has been generously applied throughout the winter months, with chloride concentrations from runoff samples often exceeding 18,000 mg/L (Environment Canada, 2001; Helmriech et al., 2010). Early breeding amphibians, such as wood frogs and spotted salamanders (Gilhen, 1984), are therefore the most heavily exposed. This highly contaminated meltwater runs directly into the ephemeral ponds which line many roadsides and serve as primary breeding sites for amphibians (Helmreich et al., 2010; Karraker et. al, 2008). Newly hatched tadpoles in these contaminated ponds are not only exposed to the highest concentrations of chloride within a season, but this study demonstrates that they are also at their most sensitive. Increasingly warm winters and earlier springs only serve to exacerbate this issue, with spotted salamanders and wood frogs laying eggs as early as mid-March (Gilhen, 1984).

Spotted salamander larvae and wood frog tadpoles are often absent from ponds and wetlands with excessively high chloride concentrations (Collins and Russell, 2009; Karraker et al., 2008). This begs the question of whether their absence is due to selective behaviour or if it is chemical exclusion. Behavioural choice seems less likely when considering that other amphibian species are indiscriminately found in ponds of varying chloride concentrations, despite their varied sensitivities to chloride (Helmreich et al., 2010; Turtle, 2000). Chloride is considered toxic to all Nova Scotian amphibians; it is lethal at elevated levels and at sublethal concentrations, it negatively affects fitness (Collins and Russell, 2009; Sanzo and Hecnar, 2006). Many amphibian species also tend to exhibit high site fidelity to their natal pond, regardless of changes in habitat quality, including chemical contamination (Turtle, 2000; Wagner and Lotters, 2013). Even when amphibians do not breed in their natal pond, they are typically restricted to a small range and are likely to only have contaminated ponds available to them within that range

(Wagner and Lotters, 2013). Therefore, it is more likely attributable to chemical exclusion, where elevated chloride concentrations are eliminating sensitive species at their embryonic or larval stages.

Spotted salamanders and wood frogs may exhibit higher sensitivities than other Nova Scotian species due to their terrestrial nature. These two species have a more terrestrial adult life than their highly aquatic relatives, such as green frogs (Gilhen, 1984; Vitt and Caldwell, 2013). While chloride has been shown to be detrimental to spotted salamander larvae and wood frog tadpoles, green frogs are much less affected by it (Collins and Russell, 2009; Karraker and Ruthig, 2009). Green frogs are primarily aquatic throughout their lifecycle and thus may be better adapted to aquatic contaminants and fluctuations in water chemistry (Gilhen, 1984; Vitt and Caldwell, 2013). Dougherty and Smith (2006) found that American toads exhibited the highest tolerance to chloride and were virtually unaffected, as was also demonstrated in this study. This could be attributed to their evolutionary history, having adapted to coastal habitats that receive an influx of salt from adjacent marine water (Gilhen, 1984). Toads are also highly terrestrial, with thick skin covering the majority of their body that is more impervious than most frog species (Gilhen, 1984). This suggests that morphology factors into chemical sensitivity. My results demonstrate that variations in chloride sensitivity exist not only among species, but among larval developmental stages as well.

Median lethal concentrations also decreased for all tested stages of wood frog tadpoles over the 96 hour testing period. This may indicate that wood frog tadpoles were more sensitive to the physiological and toxic stress associated with chloride throughout the duration of exposure time. Harless et al. (2011) demonstrated that their LC_{50} calculations for wood frog tadpoles were highest following 24 hours of exposure, and had decreased by at least 40% in each instance by the end of the 96 hour period. If exposure time was a critical factor, this suggests that the negative effects of exposure depend on multiple variables. The type of chemical applied and its ability to accumulate in the environment are vital factors, but also how long the chemical persists in aquatic systems as well as the fluctuations in its concentrations throughout spring and summer months (Harless et al., 2011; Mayer and Ellersieck, 1986). There may also have simply been a lag response in mortality, suggesting that even extreme short-term exposure to chloride would be lethal to amphibian larvae (Harless et al., 2011; Mayer and Ellersieck, 1986).

Chloride toxicity primarily stems from its ability to disrupt osmoregulation (Kaushal et al., 2005), a function that is especially vital to developing amphibian larvae which so readily absorb surrounding liquids and gases through their skin (Vitt and Caldwell, 2013). Sodium, chloride and urea contribute heavily to plasma osmotic pressure (Katz, 1973; Liggins and Grigg, 1985). Ionic concentrations of plasma increase with rising concentrations in the environment, resulting in blood hypertonicity and dehydrated cells (Kaushal et al., 2005). Amphibian larvae exposed to elevated salinity are then forced to allocate more energy to osmoregulation (Gomez-Mestre et al., 2004). Gomez-Mestre et al. (2004) speculated that if larval mortality due to chloride toxicity cannot be attributed to dehydration alone, the increased energy expense of osmoregulation results in lowered metabolism rates and highly stressed larvae. Immunoassays of thyroid hormone content also yielded results that suggest chloride may repress amphibian hormones and disrupt normal development (Gomez-Mestre et al., 2004). I found that the youngest amphibian larvae are the most sensitive to chloride toxicity. Although not tested, this would imply that the younger they are, the more vulnerable they are in stressful conditions. While this may be attributed in part to their size, it is also because their premature, developing systems are unequipped to deal with extreme chemical stressors (Vitt and Caldwell, 2013).

Wood frog tadpoles at Gosner stage 19 were highly sensitive to chloride at room temperature, while those acutely exposed at a cold temperature were less sensitive. The median lethal concentration over a 96 hour period at 22˚C was 97.01 mg/L. This closely corresponds with the 96 hour LC_{50} values found for wood frog tadpoles at Gosner stage 19 in the earlier portion of this study (85.77 mg/L from Devon, N.S. and 121.54 mg/L from Harrietsfield, N.S.). The earlier experiments to determine differences in sensitivity across developmental stages were also conducted at 22˚C, so those results are comparable and to be expected. However, wood frog tadpoles of the same Gosner stage and weight range yielded significantly different results when acutely exposed to chloride at 12˚C. The 96 hour LC_{50} result calculated was 972.27 mg/L. Temperature is an important factor that directly affects the rate of metabolism, which is especially evident in ectotherms (Cairns et al., 1975; Hayes et al., 2010; Mayer and Ellersieck, 1986). Surrounding environmental heat increases metabolic rate, including the rate at which toxic chemicals are absorbed and metabolized (Hayes et al., 2010; Mayer and Ellersieck, 1986). Though some exceptions exist, there is typically a positive correlation between temperature and toxicity in aquatic organisms (Giese, 1968; Cairns et al., 1975). Giese (1968) concluded that the temperature coefficient of respiration indicates a 10˚C temperature increase tends to double or even quadruple the rate of reaction in ectotherms.

My study demonstrates the important role that temperature plays in affecting acute toxicity. The 96 hour LC_{50} results for 12˚C are more applicable to the conditions faced by newly hatched wood frog tadpoles, such as those at Gosner stage 19 used for this experiment. The colder temperature of 12˚C is reflective of the environment in Nova Scotia during early spring. Warm temperatures such as 22˚C are unlikely to frequently occur until late May to June. While there would still be newly hatched wood frog tadpoles at this time, the majority are likely to have reached a later stage in development. The bulk of snow and ice would typically be melted by late May as well, meaning that those wood frogs hatching late would not be exposed to the extreme chloride concentrations present in fresh runoff during early spring. Although young wood frog tadpoles are less sensitive to chloride at 12˚C, their median lethal concentration still falls well within the range of chloride present in ponds and wetlands during early spring. Mortality due to chloride toxicity is therefore still likely to be occurring in their natural habitats at this time.

Acute Toxicity of Green Deicers

Calcium magnesium acetate (CMA) was considered a prime example for green deicing alternatives, with a low suspected toxicity and rising popularity throughout Canada. Spotted salamander, wood frog, and spring peeper larvae were chosen for these experiments due to their breeding season. All three species are considered early breeders, in that their eggs are typically laid and hatched before mid-June (Gilhen, 1984). Spotted salamanders and wood frogs may lay their eggs as early as late March, while most other amphibian species native to Nova Scotia are summer breeders, breeding between June-August (Gilhen, 1984). CMA also biodegrades quickly at temperatures exceeding 20˚C (Environment Canada, 2012; Fay and Shi, 2012). It will remain in road runoff during winter and early spring months, but is unlikely to be present following mid-June when temperatures rise and remain steady (Environment Canada, 2012; Fay and Shi, 2012). Early breeding species are therefore the most relevant species to test in regards to CMA because their larvae are the most heavily exposed (Gilhen, 1984; Fay and Shi, 2012).

Spotted salamander, wood frog, and spring peeper larvae were acutely exposed to CMA in aerated and non-aerated conditions at 22˚C. Their 96 hour LC_{50} values for the aerated experiments were 2376.06 mg/L for spotted salamanders, 2588.45 mg/L for wood frogs, and 2831.0 mg/L for spring peepers. These values fall well beyond the typical 10-100 mg/L range of CMA found within roadside ponds and wetlands (Horner, 1988; Transportation Research Board, 1991). Direct sampling of fresh road runoff has even been known to yield concentrations as high as 5000 mg/L (Horner, 1988; Transportation Research Board, 1991). Although this is low when compared to chloride concentrations in runoff that may exceed 18,000 mg/L, it is still potentially significant in terms of acute and chronic toxicity (Environment Canada, 2001). CMA is less popular than salt-based deicers due to its significantly higher cost of production, and thus it is less prevalent in runoff and aquatic habitats (Environment Canada, 2012; Transportation Research Board, 1991; Vitaliano, 1992). CMA is also less effective and requires approximately 1.2-1.6

times more when applied alone to achieve the same results as sodium chloride (Manning and Crowder, 1989; Transportation Research Board, 1991). Its primary use currently is to supplement salt-based deicers (Fay and Shi, 2012).

Acute toxicity tests that did not use aeration yielded significantly lower median lethal concentrations than their aerated counterparts. The 96 hour LC_{50} result for spotted salamander larvae was 982.06 mg/L, wood frog tadpoles at 465.17 mg/L, and spring peeper tadpoles at 268.40 mg/L. As before, these experiments were conducted at 22˚C with larvae of similar weight and developmental stage. All three test species were significantly more sensitive to CMA toxicity under non-aerated conditions than aerated. This was to be expected when considering the known potential for CMA to deplete oxygen in water bodies (Brenner and Horner, 1992; Transportation Research Board, 1991). Brenner and Horner (1992) demonstrated that without aeration, full oxygen depletion occurred within 48 hours of CMA introduction at 20˚C. This poses a particular threat to amphibian larvae which primarily inhabit low oxygen environments with poor rates of water turnover, such as ephemeral ponds and wetlands (Gilhen, 1984; Vitt and Caldwell, 2013). It is expected that sensitivity to CMA without aeration is more reflective of natural occurrences. However, all CMA experiments on larvae were conducted at room temperature and it is expected that larvae would be less sensitive at lower temperatures (Harless et al., 2011). Unfortunately, there are very few studies examining acute CMA toxicity in regards to amphibian larvae, nor relating CMA and amphibians in general.

Spotted salamander eggs were also tested to determine hatching success over increasing concentrations of calcium magnesium acetate. The experiments were conducted at 12˚C with aeration, using a range of 250-9000 mg/L of CMA. Hatching success remained high (77%-84%) throughout the 250-6000 mg/L treatments. Only the 9000 mg/L treatment yielded a significantly decreased success of 23%. Further testing would be required to pin point the concentration between 6000-9000 mg/L at which hatching success begins to decline. It is possible that the favourable experimental conditions yielded higher hatching success than would be seen in the field. If aeration was removed, then a decline in success would be expected as oxygen is depleted over the 96 hour testing period. The same could be assumed for an increase in experimental temperature. There has been little research conducted on CMA concentrations in relation to amphibian hatching success.

Propylene glycol was also tested as a green deicing alternative. It is considered relatively harmless to humans and other animals even at relatively high concentrations (Ramakrishna and Viraraghavan, 2005), and the results of this study support that conclusion. The 96 hour LC_{50} values ranged from 18,068.47 mg/L for wood frog tadpoles to 53,168.26 mg/L for American toads. The acute toxicity is therefore negligible when compared to most other deicing agents, with a low potential for direct lethality (Ramakrishna and Viraraghavan, 2005). However, examining acute exposures likely underestimates the overall toxicity of this compound.

The primary concern lies with chronic toxicity that may impede amphibian larval development and otherwise decreases overall fitness (Marais and Waitz, 2009; Seeland et al., 2012). Propylene glycol is widely known as an endocrine disruptor (Seeland et al., 2012). Environmental stimuli influence the central nervous system, which has the potential to negatively affect hormone production and release from the hypothalamus and pituitary (Hayes, 2000). The ability for peripheral endocrine tissues to receive those hormones may also be disrupted (Hayes, 2000). It may also act as a penetration enhancer, allowing other more immediately harmful substances to be readily absorbed (Seeland et al., 2012). Although glycols are not acutely toxic, deicing mixtures may exhibit dangerous chronic toxicity due to harmful additives (Marais and Waitz, 2009; Ramakrishna and Viraraghavan, 2005). Between 10-20% of glycol mixtures used for deicing contain additives to inhibit metal corrosion and rust, as well as thickening agents and surfactants (Marais and Waitz, 2009; Ramakrishna and Viraraghavan, 2005). This enhances the potential toxicity to levels often unknown due to the interaction and dynamic between various additives and the glycol base (Marais and Waitz, 2009; Ramakrishna and Viraraghavan, 2005). There has been very little research on propylene glycol toxicity, whether acute or chronic, on amphibian larvae.

Acute Triclosan Toxicity

All six test species of amphibian larvae were sensitive to low concentrations of triclosan under all experimental conditions. While the median lethal concentration varied for acute toxicity, no species exhibited any remote tolerance. Mink frogs and green frogs were the least sensitive, with 96 hour LC_{50} results of 981.23 µg/L and 651.38 µg/L respectively. Spring peepers were the most sensitive at 180.76 µg/L. Spotted salamanders (400.58 µg/L), wood frogs (427.88 µg/L), and American toads (389.72 µg/L) all demonstrated comparable results. For African clawed frogs, Matsumura et al. (2005) calculated a 96 hour LC_{50} of 820 µg/L for Gosner stage 30, and Palenske et al. (2010) found a similar range throughout tadpole development. Blanchard's cricket frog (367 µg/L), Woodhouse's toad (152 µg/L), and the southern leopard frog (562 µg/L) were also sensitive to acute triclosan toxicity (Palenske et al., 2010). Other types of aquatic organisms such as minnows and rainbow trout have exhibited similar median lethal concentrations, at 260 µg/L and 350 µg/L respectively (Chiba, 1998; Orvos et al., 2002).

As with chloride, this study found an inverse relationship between Gosner stage and triclosan sensitivity. Younger American toad tadpoles were significantly more sensitive to chloride than their older counterparts. The 96 hour LC_{50} calculation for individuals at Gosner stage 19 was 361.02 µg/L, whereas the calculation for those at Gosner stage 37 was 592.18 µg/L. Small spotted salamanders were also more sensitive than large, with LC_{50} results of 417.0 µg/L and 608.41 µg/L respectively. African clawed fogs exhibited a similar trend in other studies, found to be more sensitive at younger stages. Palenske et al. (2010) calculated a range of 96 hour LC_{50} results between 259 µg/L and 664 µg/L. Ishibashi et al. (2004) demonstrated this trend in rainbow trout, with LC_{50} results from 399 µg/L as embryos to 602 µg/L as 24 hour old larvae.

Wood frog and American toad tadpoles were also acutely exposed to triclosan under varying experimental conditions, such as changes in temperature and lighting, as well as static versus static renewal. None of these tests yielded much difference. There was a slight increase in triclosan sensitivity for tadpoles of both species at 22˚C as opposed to 12˚C, but not to the significant extent as was found in the previous chloride experiments. There was also no change between light and dark conditions, which were tested because triclosan is known to degrade under prolonged exposure to light (Palenske et al., 2010). Static renewal tests were also done for that reason, in case degradation was occurring over the 96 hour testing period and influencing the results. However, these tests yielded no different results than those conducted statically.

It is evident that acute triclosan toxicity poses a threat when many aquatic organisms are sensitive to such low concentrations. However, it is unlikely that Nova Scotian amphibian larvae are exposed to lethal levels of triclosan in their natural habitats (Palenske et al., 2010). The highest concentration found within surface waters of North America is 2.3 µg/L (Kolpin et al., 2002), though this excludes systems that act as deposit sites for waste water effluent or are situated nearby said facilities (Dann and Hontela, 2011). Although natural levels of triclosan are not typically high enough to cause direct mortality, sub-lethal levels may still negatively impact amphibian populations (Palenske et al., 2010). Smith and Burgett (2005) found that American toad tadpoles chronically exposed to 2.3 µg/L over a 14 day period exhibited a significant negative effect on survivorship. Their activity was reduced and mortality rate increased, as found in previous studies (Fraker and Smith, 2004; Smith and Burgett, 2005).

Thyroid hormones control the complex process by which tissues and organs are modified from larval structures to recreate adult features (Shi, 2000). At metamorphosis, amphibian larvae reach their peak of circulating thyroid hormones (Gutleb et al., 2000; Shi, 2000). Environmental contaminants can disrupt this system, especially those that inhibit the proper function of thyroid hormones (Hayes, 2000). Inhibition of thyroid hormone synthesis has been shown to prolong or even to prevent metamorphosis (Gutleb et al., 2000; Shi, 2000). The potential for triclosan to act as an endocrine disruptor is a primary concern for prolonged exposure throughout larval development.

Ecological Importance

Availability of viable habitats and breeding sites is essential for amphibian persistence. Many function in metapopulation structures involving separate, interacting subpopulations, making their situation even more sensitive (Hanski et al., 1996; Sinsch, 1992). Subpopulations are in constant balance between increasing and decreasing numbers within any given network of ponds or wetlands (Hanski et al., 1996; Levins, 1969). High adult and juvenile mortality often tips that balance when exacerbated by external factors, such as increased chemical contamination (Cushman, 2006; Hanski et al., 1996; Levins, 1969). Contamination reduces the number of viable habitats and breeding sites among subpopulations that are largely dependent on juvenile survival and dispersal (Cushman, 2006). High levels of contamination may act as a stressor for the whole metapopulation, leading to population sinks and decreasing both community structure and diversity (Cushman, 1996; Levins, 1996). Contamination of key source ponds is likely to disrupt the balance of the entire system (Levins, 1996; Sinsch, 1992). Not only would amphibian community numbers decline, but diversity would as well.

This study demonstrates that amphibian species exhibit varying tolerances to most chemical contaminants. Some ponds contain high enough levels of certain contaminants to eliminate sensitive species, perhaps removing them entirely from the community. Decreased diversity is a poor result in itself, and can interfere with the complex relationships held by amphibians with one another and their community (Wilbur, 1997). Contamination often alters intraspecies and interspecies competition due to the elimination of sensitive species (Collins and Russell, 2009; Wilbur, 1997). Amphibians are also considered key members of their ecosystems, in part due to their involvement in multiple trophic levels of the food web (Blaustein, 2001; Tanara, 1975). As tadpoles, they are a major component of biomass and will metamorphose to become predators as well as prey (Blaustein, 2001; Tanara, 1975). Disrupting the diversity and abundance of amphibian communities can therefore have large scale, detrimental effects (Wilbur, 1997).

It is also vital to consider temporal scales and varying environmental conditions when examining the effects of chemical contamination. Amphibian habitats receive the highest influx of contaminated road runoff in spring months (Collins and Russell, 2009; Helmreich et al., 2010). Small, ephemeral ponds located near roadsides are often saturated or even created solely from runoff (Forman et al., 2003; Williams, 2006). This poses a particular concern not only for early breeders, but for young life stages of tadpoles in general. The results of this study demonstrate the importance of examining multiple developmental stages to understand the full scope of contaminant interaction. Contaminant concentrations vary in natural habitats over the spring and summer seasons based on temperature and chemical longevity, as well as a number of other factors shown in this study. The complex interaction among variables cannot be overlooked in research of amphibian ecotoxicology.

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