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Assessing the toxicity and risk of salt-impacted winter road runoff to the early life stages of freshwater mussels in the Canadian province of Ontario☆

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ABSTRACT

In temperate urbanized areas where road salting is used for winter road maintenance, the level of chloride in surface waters has been increasing. While a number of studies have shown that the early-life stages of freshwater mussels are particularly sensitive to salt; few studies have examined the toxicity of salt-impacted winter road runoff to the early-life stages of freshwater mussels to confirm that chloride is the driver of toxicity in this mixture. This study examines the acute toxicity of field-collected winter road runoff to the glochidia of wavy-rayed lampmussels (*Lampsilis fasciola*) (48 h exposure) and newly released juvenile fatmucket mussels (*Lampsilis siliquoidea*) (<1 week old; 96 h exposure) under different water hardness. The chronic toxicity (28 d) to older juvenile *L. siliquoidea* (7–12 months old) was also investigated. The 48-h EC50 and 96-h LC50 for *L. fasciola* glochidia and *L. siliquoidea* juveniles exposed to different dilutions of road run-off created with moderately hard synthetic water (~80 mg $CaCO_3$/L) were 1177 (95% confidence interval (CI): 1011–1344 mg Cl^-/L) and 2276 mg Cl^-/L (95% CI: 1698–2854 mg Cl^-/L), respectively. These effect concentrations correspond with the toxicity of chloride reported in other studies, indicating that chloride is likely the driver of toxicity in salt-impacted road-runoff, with other contaminants (e.g., metals, polycyclic aromatic hydrocarbons) playing a *de minimis* role. Toxicity data from the current study and literature and concentrations of chloride in the surface waters of Ontario were used to conduct a probabilistic risk assessment of chloride to early-life stage freshwater mussels. The assessment indicated that chronic exposure to elevated chloride levels could pose a risk to freshwater mussels; further investigation is warranted to ensure that the most sensitive organisms are protected.

1. Introduction

Over the last four decades, the increase of chloride concentration in North American surface waters, particularly in northern latitudes, has corresponded with the increased application of road salt for the purpose of ice removal (Jackson and Jobbagy, 2005; Kaushal et al., 2005; OMOECC, 2009). The road salt used in Canada is most commonly in the form of sodium chloride (97%), with calcium chloride (2.9%), magnesium chloride and potassium chloride (0.1% each) employed less often (CCME, 2011). In Canada, approximately 4.75×10^6 tonnes of road salt were applied to roads in the winter of 1997 and 1998 for the purpose of deicing, with the greatest proportion (1.15×10^6 tonnes) being applied in the province of Ontario (Morin and Perchanok, 2000). Recent data indicate the amount of road salt applied to Canadian roads rose above 6.0×10^6 tonnes in 2009 (ECCC, 2012). Simultaneously, data from the Ontario's Provincial Water Quality Monitoring Network indicates that the mean concentration of chloride in surface waters of the province increased 68% from 1964 to 2005 (OMOECC, 2009). This increase in chloride concentrations represents a potential risk to freshwater ecosystems (Elphick et al., 2011; Gillis, 2011; Bartlett et al., 2012; Todd and Kaltenecker, 2012) and in fact there have been reports of community level impacts in some salt-sensitive biota (Collins and Russell, 2009).

A group of organisms that are at particular risk to increasing

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road salt application due to their sensitivity to chloride is freshwater mussels (Gillis, 2011; Pandolfo et al., 2012; Blakelsee et al., 2013; Beggel and Geist, 2015). North America holds the greatest diversity of freshwater mussels in the world but they are also one of the most threatened groups of organisms (Lydeard et al., 2004; Strayer et al., 2004). Laboratory studies have shown that the early life stages of freshwater mussels are particularly sensitive to salt exposure (Gillis, 2011; Pandolfo et al., 2012; Nogueira et al., 2015; Roy et al., 2015). For example, 24-h chloride EC50s for the glochidia of *Lampsilis fasciola* and *Epioblasma torulosa rangiana* exposed to sodium chloride were 199 (mean of LC50s 113 and 285 mg/L) and 244 mg/L (Gillis, 2011), respectively, while the next most sensitive freshwater species was *Ceriodaphnia dubia* (48-h LC50: 447 mg/L) (Hoke et al., 1992; CCME, 2011). Negative effects of salt exposure have also been reported in natural environments; Patnode et al. (2015) observed significant mortality of juvenile *Epioblasma torulosa rangiana* caged directly downstream of high salinity wastewater discharge relative to the reference site.

While lab-based studies have documented the acute effect of salt and selected formulations of road salt on some freshwater mussel species (e.g., Pandolfo et al. (2012)), the response of this group to salt-laden road runoff has not been examined. Winter road runoff can contain not only the components of road salt (e.g., sodium chloride, calcium chloride, sodium ferrocyanide, ferric ferrocyanide) (Pandolfo et al., 2012) but also other contaminants (e.g., metals, polycyclic aromatic hydrocarbons (PAHs), petroleum products, particulate matter) (Marsalek et al., 2003; Grapentine et al., 2004, 2008) that could pose a threat to freshwater mussels. For instance, Archambault et al. (2017) observed an increase in the concentration of PAHs in mussels downstream of road crossings. Winter melt events can deliver an acute pulse of salt to the receiving environment which can be particularly dangerous for sensitive biota, but also concerning is the effect of longer-term exposures that result from multiple pulses as well as the increased salinization of freshwaters.

Consequently, the objective of the current study was to investigate the effect of acute and chronic exposure of winter road runoff to the early life stages of freshwater mussels using laboratory exposures. As a result of the presence of freshwater mussels in a range of habitats with varying water chemistry and the toxicity of chloride being affected by the ionic composition of the exposure water (Elphick et al., 2011; Gillis, 2011), this study also examined the effect of water hardness on the toxicity of salt-impacted road runoff to freshwater mussels. The toxicity data generated from this study and data from the literature was used along with monitoring data from the province of Ontario to conduct a probabilistic risk assessment to investigate the potential risk that chloride may pose to freshwater mussel species in southern Ontario. The assessment was focused on southern Ontario because this region contains 42 of the 53 freshwater mussel species found in Canada (Metcalfe-Smith et al., 1998) and 13 of these species are classified as threatened or endangered in Ontario (OMNR, 2016). This region also receives the greatest magnitude of winter road salt in Canada (Morin and Perchanok, 2000).

2. Methods

2.1. Runoff sample and exposure waters

Winter road runoff from the four lane Skyway Bridge, a section of the Queen Elizabeth Way (i.e., express highway) in Burlington, Ontario, Canada (43.298833, −79.797620) was collected on a number of days from the middle of January to early March 2012 (Supplementary Information, Table S1). Greater detail on the sample collection procedure is provided in Table S1. The runoff sample used in this study was collected January 31, 2012 and it had a chloride concentration of 14,400 mg/L, a level typical of salt-impacted winter runoff from this site in 2012 (124–25,500 mg Cl^-/L) (Table S1). However, compared to samples collected at other times of the year that are not influenced by salt-laden snow melt, the level of chloride in the runoff sample used in this study represents a worst-case exposure scenario for freshwater mussels, based on concentrations measured in runoff and surface water from around Ontario (Mayer et al., 1999; OMOECC, 2009). To simulate various exposure scenarios in the receiving environment, the different life stages of mussels were exposed to 1, 2, 5, 10, 20, 50, or 100% dilution of the runoff sample. Also, because water hardness can influence salt toxicity (Gillis, 2011; Elphick et al., 2011), exposures were conducted in runoff diluted with either moderately (MHW: ~100 mg $CaCO_3$/mL) or very hard (VHW: ~250 mg $CaCO_3$/L) reconstituted water (USEPA, 2002b). The physicochemical properties of dilutions used in each treatment for each set of experiments are presented in Tables S2–S5.

2.2. Glochidia

Gravid female wavy-rayed lampmussels (*L. fasciola*) were collected in July of 2012 from a site on the Grand River (43.494892, −80.470228) in Ontario, Canada. This site is located upstream of significant urban influences (Gillis, 2012) and has a stable population of *L. fasciola* (Gillis et al., 2017). Gravid female mussels were maintained at 14 ± 2 °C in a flow-through system at Environment and Climate Change Canada's (ECCC) aquatic life research facility (ALRF). Water supplied by the ALRF was City of Burlington tap water that has been sterilized and dechlorinated. The glochidia used in toxicity testing were isolated from these field-collected mussels. At the time of collection, *L. fasciola*, was listed as federally Endangered in Canada (COSEWIC, 1999), and therefore collections and this research was conducted under a Species at Risk permit (SARA C&A 12–020).

Toxicity testing with glochidia was performed in MHW or VHW using methods based on those outlined in ASTM (2006) and summarized by Gillis et al. (2010). For each test, glochidia with a viability >90% were isolated from a minimum of three female mussels and pooled in water composed of a mixture of MHW or VHW at 20 °C and ALRF water at 14 °C. Glochidia were transferred to this blended water to reduce any potential temperature or osmotic-related stress. The viability of pooled glochidia was confirmed to be >90% before the initiation of a test. Glochidia (~500–1000) were placed in four replicate test vessels (250-mL glass beakers with 100 mL of road runoff diluted with either MHW or VHW) for each treatment and incubated for 48 h at 20 ± 2 °C with a photoperiod of 16 h light: 8 h dark. Five control replicates containing MHW or VHW were included in each exposure. After 24 and 48 h of incubation, a subsample containing approximately 100 glochidia was removed from each replicate to assess viability. Viability was determined by counting the number of glochidia with open and closed valves before and after the addition of a concentrated sodium chloride solution (240 g/L). Equation (1) was used to calculate percent viability.

$$Percent\ viability = \left(\frac{\#closed\ after\ NaCl - \#closed\ before\ NaCl}{\#closed\ after\ NaCl + \#open\ after\ NaCl}\right) \times 100 \tag{1}$$

Water chemistry parameters (i.e., ammonia, pH, dissolved oxygen, conductivity) were measured in each dilution (i.e., treatment) at the start of each exposure (t = 0 h) and at the conclusion of each exposure (t = 48 h) (Table S6). An additional experiment using the method described above was also conducted by exposing *L. fasciola* glochidia collected from gravid females from the same field site to MHW that was spiked with a range (0–5 g/L) of concentrations of sodium chloride (NaCl) (≥99% purity; Fisher Scientific, Ottawa, ON, Canada). This experiment allowed a comparison of the toxicity of salt-impacted road runoff to the toxicity of sodium chloride alone.

2.3. Juvenile mussels

Two life stages of juvenile fatmucket mussel (*L. siliquoidea*) were obtained from Professor Chris Barnhart's laboratory at Missouri State University (MSU). Juvenile mussels were cultured in the laboratory from glochidia that were isolated from wild female mussels in the Silver Fork, Bourbeuse River, or Perche Creek in Missouri, USA (Barnhart, 2006). Largemouth bass (Chesapeake Fish Hatchery, Mount Vernon, MO, USA) were used as host for the glochidia in the MSU laboratory. *L. siliquoidea* are also relatively abundant in Ontario and found widely across the province. The use of cultured juvenile mussels in toxicity testing allows for appropriate replication and size consistency without placing pressure on wild populations. Mussels were shipped overnight to Burlington (ON) and given approximately two days to acclimate before being used in testing.

Juvenile mussels that had been newly released from fish gills (i.e., <1 week before test initiation) and juvenile mussels that had been released for 7–15 months (mean length: 1.23 cm standard deviation of length: 0.2 cm) were used in testing as outlined in ASTM (2006) and Roy et al. (2015). Older juvenile mussels were selected for testing based on mean length (e.g., ~1 cm) in order to standardize the size of juvenile mussels used in testing. Newly released juveniles were exposed to road runoff dilutions for 96 h in 50-mL glass beakers containing 30 mL of dilution solution at 20 ± 2 °C with a photoperiod of 12 h light: 12 h dark. Each dilution treatment had four replicates and five newly released juveniles were placed in each replicate beaker. Five control replicates containing MHW or VHW were incorporated in each test. As per ASTM (2006), 90% of the exposure solution in each replicate was renewed after 48 h. After 24, 48, 72, and 96 h of exposure, juvenile mussels were examined under a dissecting microscope (15 x magnification) for five minutes to observe movement of the foot inside or outside of the shell. If foot movement was observed, the mussel was considered alive. Water chemistry parameters were also measured every 24 h (Table S7).

Chronic toxicity testing with 1-cm juvenile mussels was modeled after the methods of Jorge et al. (2013) performed in aerated 1-L glass beakers with 700 mL of dilution water at 20 ± 2 °C with a photoperiod of 16 h light: 8 h dark. Approximately 2 cm of well rinsed freshwater aquarium sand (CaribSea Super Naturals Premium Aquarium Substrate, Moonlight Sand, Fort Pierce, FL, USA) was placed on the bottom of each test vessel. Four control replicates containing MHW were included in each experiment. The larger juveniles were exposed to dilutions of runoff water using only MHW and not VHW. Ten juvenile mussels were placed in each replicate. Each replicate was fed 200 μL of food solution twice daily [food solution: 0.7% (v/v) Nanno 3600™ (*Nannochloropsis* sp.) (Reed Mariculture Inc., San Jose, CA, USA) and 2.7% (v/v) Shellfish Diet 1800™ (*Isochrysis*, *Pavlova*, *Thalassiosira*, and *Tetraselmis* sp.) (Reed Mariculture Inc. San Jose, CA, USA); 4.5×10^8 cells/mL Nanno 3600™ and 5.5×10^7 cells/mL Shellfish Diet 1800™]. Mussels were transferred to new test vessels every seven days in order to minimize fouling-induced changes in water chemistry over the course of the experiment. Mortality and water chemistry parameters (i.e., dissolved oxygen, pH, conductivity, ammonia; Thermo Scientific™ Orion™ VersaStar Pro™ multiparameter benchtop meter, Fisher Scientific, Ottawa, ON) were assessed in each test vessel at day 7, 14, 21, and 28 (Table S8). Mortality was determined by observing whether a mussel had attempted to bury in the sand and/or whether it was visibly filtering.

2.4. Chemical analysis

The chemical analyses of winter road runoff samples and dilutions used in tests were conducted by ECCC's National Laboratory for Environmental Testing (NLET) in Burlington, ON, Canada. Cyanide analysis was conducted by ALS Laboratories (Waterloo, ON). Standard methods were used to analyze samples at NLET and ALS and these methods are accredited to the standard ISO/IEC 17025 by the Canadian Association for Laboratory Accreditation (CALA) (EC, 2008).

2.5. Statistical analysis

Dilutions of the winter road runoff sample and measured concentrations of chloride causing 10, 25, or 50% reduction in glochidia viability (i.e., ECx) or mortality in juvenile mussels (i.e., LCx) were estimated by non-linear regression. A 4-parameter log-logistic model was used to determine standard errors and 95% confidence intervals for estimated effect concentrations. The *drc* package in R was used to perform non-linear regression and estimate effect concentrations (Ritz and Streibig, 2005).

2.6. Probabilistic risk assessment of chloride toxicity in mussel habitats

2.6.1. Problem formulation

The protection goal of this assessment exercise was to theoretically ensure there is no decline in freshwater mussel populations in Ontario due to acute or chronic exposure to chloride. The endpoint for this assessment is the probability that the early-life stages of 95% of freshwater mussel species will be protected from adverse effects due to exposure to chloride 95% of the time. This assessment endpoint has been used by a number of regulatory bodies (e.g., RIVM, USEPA) as a protection goal and in water quality criteria determination (Stephan et al., 1985; USEPA, 1995; Janssen et al., 2004). It is important to note that the species sensitivity distributions (SSDs) used in this study do include species that are considered vulnerable, threatened, or endangered in North America. Many regulatory bodies employ a different risk assessment approach when assessing the risk to vulnerable, threatened, or endangered species (United States Congress, 1973; Government of Canada, 2002). For example, the level of organization that is the focus of protection goals often shifts from the population, community, or ecosystem to the individual, as the loss of a single endangered individual is considered unacceptable.

2.6.2. Effect characterization

Toxicity data (e.g., ECx, LCx) from the current study and data found in the literature were used to construct SSDs for freshwater mussels due to acute or chronic exposure to chloride. Acute SSDs were constructed using LC50s and EC50s from acute toxicity tests. Chronic SSDs were constructed using LC10s and EC10s from acute and chronic toxicity tests. Effect concentrations (LC10s and EC10s) from acute tests were included in the chronic SSDs due the current study being the only study to investigate laboratory–based chronic exposure of freshwater mussels to road run-off and/or chloride. SSDs were constructed by giving each LCx or ECx a percent rank using the Weibull equation percent rank = (rank number/(total number of data points +1)). Distributions were visualized by graphing percent rank against concentration of chloride in water causing the effect. Percent rank can also be expressed as percent of SSD affected.

2.6.3. Exposure characterization

Concentrations of chloride measured in the surface waters of Ontario and specifically the Grand, Thames, Maitland, and Sydenham Rivers by the Ontario Ministry of the Environment and Climate Change's (OMOECC) Provincial (Stream) Water Quality Monitoring Network (PWQMN) in 2012, 2013, and 2014 were used to construct exposure distributions for freshwater mussel populations in Ontario. These rivers were chosen because they are known to contain populations of threatened and endangered freshwater mussel species: Grand (25 freshwater mussel species; 10 Species at Risk (SAR)), Thames (27 species; 5 SAR), Maitland (9 species; 2 SAR), and Sydenham (30 species; 11 SAR) Rivers. The Weibull equation described above was used to generate a chloride exposure distribution for surface waters in Ontario and each river.

2.6.4. Risk characterization

SSDs and exposure distributions enabled the determination of the probability that a certain percentage of effect concentrations or species within the SSD will be exceeded under each risk scenario, e.g., acute and chronic exposure in surface waters of Ontario and the four rivers described above. SSDs and exposure distributions were also used to construct a joint probability curve for each risk scenario. The area under the joint probability curve was determined for each risk scenario, which is representative of mean risk (Aldenberg et al., 2002). The coordinates for a joint probability curve (i.e., x: centile (y) of SSD or percent of species affected; y: percent of exposure distribution exceeding toxicity value) and area under the curve were calculated using the equations below in Excel.

Concentration at centile (y) of SSD = 10^((NORMSINV(centile (y) of species affected) − (y-intercept of SSD))/(slope of SSD))

Percent of exposure distribution exceeding toxicity value = 100*(1 − NORMSDIST(slope of exposure distribution * log (concentration at centile (y) of SSD)) + y-intercept of exposure distribution)

Area under joint probability curve (centile 1 to 100) = sum (1 − NORMSDIST(slope of exposure distribution * log (concentration at centile (y) of SSD)) + (y-intercept of exposure distribution))

3. Results and discussion

3.1. Glochidia

The 48-h EC50s for viability of *L. fasciola* glochidia, based on dilutions of winter road runoff, were 7.8% and 7.0% in MHW (80 mg $CaCO_3$/L) and VHW (237 mg $CaCO_3$/L), respectively (Table 1). The corresponding 48-h EC50s for road runoff based on chloride concentrations in the runoff sample were 1177 and 1032 mg/L, respectively (Table 1). The 48-h EC50 based on MHW spiked with NaCl was 942 mg Cl^-/L (Table 1). The similarity of the EC50s based on road runoff and MHW spiked with NaCl (Table 1) would indicate that chloride is likely responsible for much of the observed toxicity relative to other contaminants typically present in winter road runoff (e.g., metals, cyanide, PAHs, petroleum products). These findings support the work of Pandolfo et al. (2012), who observed that the acute toxicity of sodium chloride to rainbow mussel (*Villosa iris*) glochidia did not differ from the toxicity of three types of cyanide-containing road salts. The individual toxicity of three forms of cyanide found in road salt (sodium cyanide, sodium ferrocyanide, and ferric ferrocyanide) was also observed to be significantly less toxic to *V. iris* glochidia relative to chloride (Pandolfo et al., 2012). The lowest no observed effect concentration (NOEC) for *V. iris* exposed to cyanide was 0.5 mg/L (Pandolfo et al., 2012) and the total concentration of cyanide in the winter road runoff sample used in this study was 0.08 mg/L (Table S1). These findings support that the concentration of chloride in salt-impacted winter road runoff likely drives the toxicity to early-life stages of freshwater mussels.

The EC50s for the glochidia of *L. fasciola* observed in this study correspond with a similar level of acute toxicity that has been observed by others. Previous studies have reported 24-h EC50s for viability of *L. fasciola* glochidia that range from 113 to 1868 mg Cl^-/L (Bringolf et al., 2007; Valenti et al., 2007; Gillis, 2011). An increase in water hardness did not have a significant ameliorating effect on the toxicity of diluted road runoff to glochidia in this study. In contrast, Gillis (2011) observed a significant two-fold increase in the EC50 of *L. siliquoidea* glochidia exposed to chloride in soft water (47 mg $CaCO_3$/L) relative to moderately hard water (99 mg $CaCO_3$/L). However, the chloride EC50s for glochidia in moderately hard, hard (172 mg $CaCO_3$/L), and very hard water (322 mg $CaCO_3$/L) were not significantly different (Gillis, 2011), indicating that the protective effect of increased water hardness on chloride toxicity is only evident when comparing toxicity in soft water ($\ll$ 100 mg $CaCO_3$/L) to that in harder waters (i.e., >100 mg $CaCO_3$/L). The two levels of water hardness employed in the current study were chosen to simulate environmental conditions of winter road runoff entering two broad types of mussel habitat in road-dense southern Ontario, specifically water hardness in the lower Great Lakes (e.g., Lake Ontario, ~100 mg $CaCO_3$/L) and a number of riverine mussel habitats (e.g., Grand and Thames Rivers, ~300 mg $CaCO_3$/L). The results indicate that mussels inhabiting the ion-complex waters of the Grand, Thames and other mussel-rich (including Species at Risk), hard-water rivers are not afforded any further protection from salt exposure than those experiencing moderately hard water conditions.

3.2. Juveniles

Newly released (<1week old) juvenile *L. siliquoidea* were less sensitive to salt-impacted road runoff compared to *L. fasciola* glochidia (Tables 1 and 2). The 48-h LC50s for newly released juveniles based on dilutions of the road runoff sample were 16.0 and 21.9% in MHW and VHW, respectively, and were 2653 and 3442 mg/L based on the concentration of chloride, respectively (Table 2), which are two-fold greater than 48-h glochidia EC50s (*L. fasciola*). LC50s were consistently greater in VHW as opposed to MHW but they were not significantly greater (i.e., 95% confidence intervals overlapped) (Table 2). As mentioned above in relation to glochidia, it appears that the elevated hardness of VHW does not provide significant protection from salt toxicity as observed in earlier studies in softer waters (Gillis, 2011).

A number of studies have investigated the effect of acute exposure to NaCl or different road salt products on juvenile freshwater mussels (Table S9). Pandolfo et al. (2012) reported 48-h Cl^- LC50s for newly released (1-d old) *V. iris* exposed to NaCl and two types of road salt ranging from ~1086 to 1249 mg/L. Bringolf et al. (2007) reported 96-h LC50s for juvenile (i.e., ≤12-months old) *L. fasciola, L. siliquoidea* and *Villosa delumbis* of 2428, 2782, and 3190 mg/L of chloride, respectively. The acute LC50s for *V. iris*, a Species at Risk in Canada, reported by (Pandolfo et al., 2012) are both lower than those of the other species mentioned above, indicating that 1-d old juvenile *V. iris* appear to be more sensitive than the mussels used in this study (<1 week and 6–8 month old

Table 1
Concentrations causing 10, 25, and 50% inhibition of glochidia viability (ECx) of the wavy-rayed lampmussel (*Lampsilis fasciola*) exposed to winter runoff from the Skyway Bridge in Burlington, Ontario Canada diluted with reconstituted water of different hardness (80 and 237 mg/L) for 48 h and exposed to moderately hard water spiked with NaCl for 48 h. The 95% confidence interval (CI) has been determined for each ECx. The number in brackets is standard error. LCs and ECs have been calculated based on the percent dilution of the runoff sample and concentration of chloride (mg/L) in each dilution of the runoff sample.

Type of exposure water	Time of exposure	EC10	95% CI	EC25	95% CI	EC50	95% CI
Based on percent dilution							
Moderately hard water	24 h	1.1 (0.2)	0.7–1.5	2.6 (0.3)	2.0–3.2	6.0 (0.4)	5.2–6.9
	48 h	2.2 (0.4)	1.3–2.8	4.1 (0.5)	3.1–5.0	7.8 (0.5)	6.7–9.0
Very hard water	24 h	0.7 (0.2)	0.3–1.2	1.9 (0.3)	1.2–2.6	4.9 (0.5)	3.8–5.9
	48 h	0.8 (0.3)	0.2–1.4	2.4 (0.6)	1.3–3.6	7.0 (0.9)	5.1–8.9
Based on concentration of Cl^-							
Moderately hard water	24 h	163 (30)	103–224	384 (45)	293–476	903 (64)	773–1034
	48 h	319 (57)	203–435	613 (69)	471–755	1177 (81)	1011–1344
Very hard water	24 h	135 (31)	72–199	319 (47)	224–415	753 (67)	616–891
	48 h	113 (43)	25–200	341 (83)	172–511	1032 (142)	739–1324
Moderately hard water spiked with NaCl							
	24 h	213 (20)	171–255	436 (26)	382–490	892 (37)	816–969
	48 h	330 (31)	266–393	557 (33)	490–625	942 (39)	862–1023

Table 2
Concentrations causing 10, 25, and 50% mortality (LCx) in juvenile *Lampsilis siliquoidea* newly released from fish gills (<2 mm in length; < 1 week old at test initiation) exposed to winter runoff from the Skyway Bridge in Burlington, Ontario Canada diluted with reconstituted water of different hardness (80 and 237 mg/L) for 96 h. The 95% confidence interval (CI) has been determined for each LCx. The number in brackets is standard error. LCs and ECs have been calculated based on the percent dilution of the runoff sample and concentration of chloride (mg/L) in each dilution of the runoff sample.

Type of exposure water	Time of exposure	LC10	95% CI	LC25	95% CI	LC50	95% CI
Based on percent dilution							
Moderately hard water	24 h	8.8 (1.4)	6.1–11.5	11.8 (1.4)	9.2–14.5	16.0 (1.6)	12.8–19.0
	48 h	8.8 (1.4)	6.1–11.5	11.8 (1.4)	9.2–14.5	16.0 (1.6)	12.8–19.0
	96 h	7.0 (1.2)	4.7–9.3	9.8 (1.2)	7.4–12.2	13.8 (1.5)	10.9–16.7
Very hard water	24 h	18.4 (11.8)	−4.6–41.5	19.7 (2.4)	15.0–24.4	21.1 (8.5)	4.5–37.7
	48 h	13.8 (1.9)	10.1–17.5	17.4 (1.7)	14.1–20.7	21.9 (2.0)	17.9–25.8
	96 h	6.1 (1.5)	3.1–9.0	11.0 (1.9)	7.3–14.7	20.0 (3.1)	13.8–26.1
Based on concentration of Cl^-							
Moderately hard water	24 h	1552 (223)	1114–1990	2029 (212)	1614–2444	2653 (239)	2184–3121
	48 h	1552 (223)	1114–1990	2029 (212)	1614–2444	2653 (239)	2184–3121
	96 h	814 (179)	463–1166	1361 (213)	943–1779	2276 (295)	1698–2854
Very hard water	24 h	2880 (1189)	550–5210	3097 (257)	2594–3602	3332 (868)	1631–5034
	48 h	2196 (291)	1626–2766	2749 (259)	2242–3256	3442 (310)	2834–4049
	96 h	984 (241)	512–1456	1763 (297)	1181–2345	3159 (486)	2206–4112

L. siliquoidea) and by Bringolf et al. (2007). These studies, which were conducted in water of similar hardness (Pandolfo et al. (2012) 160–170 mg $CaCO_3$/L; Bringolf et al. (2007) 170–190 mg $CaCO_3$/L, current study 100–250 mg $CaCO_3$/L), illustrate that sensitivity to chloride is influenced by mussel species and potentially also the age of the juvenile mussel employed, though comparisons across juveniles of different ages within a species are rare.

To our knowledge, this was the first study to examine the effect of chronic exposure to chloride, road salts, and/or winter road runoff on juvenile freshwater mussels under controlled laboratory conditions. Lower LC50s were observed for larger juvenile *L. siliquoidea* compared to newly released juveniles, as would be expected based on the longer duration of the older mussel exposure, i.e., 28 d (Tables 2 and 3). The 96-h LC50 of newly released juveniles (2276 mg Cl^-/L) and the 7-d LC50 for older juveniles (2366 mg Cl^-/L) were similar (Tables 2 and 3), though as mentioned, because test duration can influence toxic response, these LC50s (96-h and 168-h exposures) may not be directly comparable. Bringolf et al. (2007), also reported acute salt toxicity in the range of 2428–3190 for 96-h LC50s for juveniles (≤12-months old) of the three mussel species (*L. fasciola, L. siliquoidea, V. delumbis*).

Table 3
Concentrations causing 10, 25, and 50% mortality (LCx) in juvenile *Lampsilis siliquoidea* (1–2 cm in length) exposed to winter runoff from the Skyway Bridge in Burlington, Ontario Canada diluted with moderately hard water for 28 d. The 95% confidence interval (CI) has been determined for each LCx. The number in brackets is standard error. LCs and ECs have been calculated based on the percent dilution of the runoff sample and concentration of chloride (mg/L) in each dilution of the runoff sample.

Type of exposure water	Time of exposure	LC10	95% CI	LC25	95% CI	LC50	95% CI
Based on percent dilution							
Moderately hard water	7 d	8.8 (0.7)	7.4–10.1	10.6 (0.7)	9.3–11.9	12.8 (0.7)	11.4–14.3
	14 d	8.8 (0.7)	7.4–10.1	10.6 (0.7)	9.3–11.9	12.8 (0.7)	11.4–14.3
	21 d	4.9 (0.7)	3.6–6.2	7.5 (0.8)	6.0–9.0	11.6 (1.0)	9.6–13.5
	28 d	3.1 (0.5)	2.0–4.2	5.7 (0.7)	4.3–7.1	10.6 (1.1)	8.4–12.7
Based on concentration of Cl^-							
Moderately hard water	7 d	1550 (139)	1278–1822	1915 (130)	1660–2170	2366 (144)	2085–2648
	14 d	1550 (139)	1278–1822	1915 (130)	1660–2170	2366 (144)	2085–2648
	21 d	857 (120)	621–1093	1326 (138)	1055–1596	2049 (178)	1700–2400
	28 d	488 (87)	318–659	940 (121)	702–1178	1810 (194)	1429–2190

This study focused on the effects of winter road runoff to larval and juvenile mussels. Adult mussels were not examined as previous studies have shown that the early life stages are more sensitive than adults (Ingersoll et al., 2007; Keller et al., 2007). Hartmann et al. (2016) observed that adult *Anodonta anatina* displayed an avoidance behaviour to pulse exposures of a de-icing salt solution by tightening the closure of their valves and suggested that adult mussels may be able to avoid the adverse effects of such pulse exposures, depending on the length of the pulse. While juvenile mussels have also displayed this behaviour (Wang et al., 2007), it is not clear for what period of time they could avoid exposure before the accumulation of waste and lack of oxygen begin to cause adverse effects.

3.3. Probabilistic risk assessment of chloride toxicity in mussel habitats

The toxicity data used to construct the acute and chronic SSD for the early-life stages of freshwater mussels is presented in Tables S9 and S10. The chloride exposure distributions for the entire province of Ontario and for four rivers in Ontario that are known to contain threatened or endangered mussel species (i.e., Grand, Maitland, Sydenham, and Thames Rivers) and acute and chronic SSDs are presented in Fig. 1. A relatively low percentage of the acute SSD overlaps with the different exposure distributions using chloride levels recorded between April and early November (Fig. 1). The probability of exceeding the 5th centile of the acute SSD ranges from 9.1×10^{-14}% to 1.5%, which means that 5% of species represented in the acute SSD will be affected ≤1.5% of the time (Fig. 1, Table 4). The frequency of exceedance of the 5th centile of the acute SSD is below the stated assessment endpoint for this study, i.e., <95% of the time, which means that acute exposure to chloride likely represents a *de minimis* risk to the early-life stages freshwater mussel populations in Ontario between April and early November. The joint probability curves constructed with the exposure distributions and acute SSD for each risk scenario also indicated that acute exposure to chloride likely does not represent a considerable risk in these months, as the curves are below the proposed *de minimis* – low risk boundary (Fig. 2) (USEPA, 2002a, 2004). It is important to note that the monitoring data on concentrations of chloride in the surface waters of Ontario is based on a relatively low number of grab samples throughout the year. Consequently, we cannot discount the possibility that peaks in chloride concentration could occur within the monitoring period that could result in acute mortality. Also, the timing of the monitoring program (i.e., April to early November) would not include data on runoff that may occur in the winter months, which could contain many significant loading events.

The probability of exceeding the 5th centile of the chronic SSD ranges from 67.8 to 96.7%, which is considerably greater than the assessment endpoint for this risk assessment (Fig. 1, Table 4). The joint probability curves for each risk scenario exceed the low – intermediate risk boundary but not the intermediate – high risk boundary (Fig. 2). The frequent exceedance of the 5th centile of the chronic SSD would indicate that chronic exposure to chloride poses a level of risk to the early-life stages of freshwater mussels that warrants further investigation. It is also important to consider the threatened or endangered species' position in the SSDs. The 95th

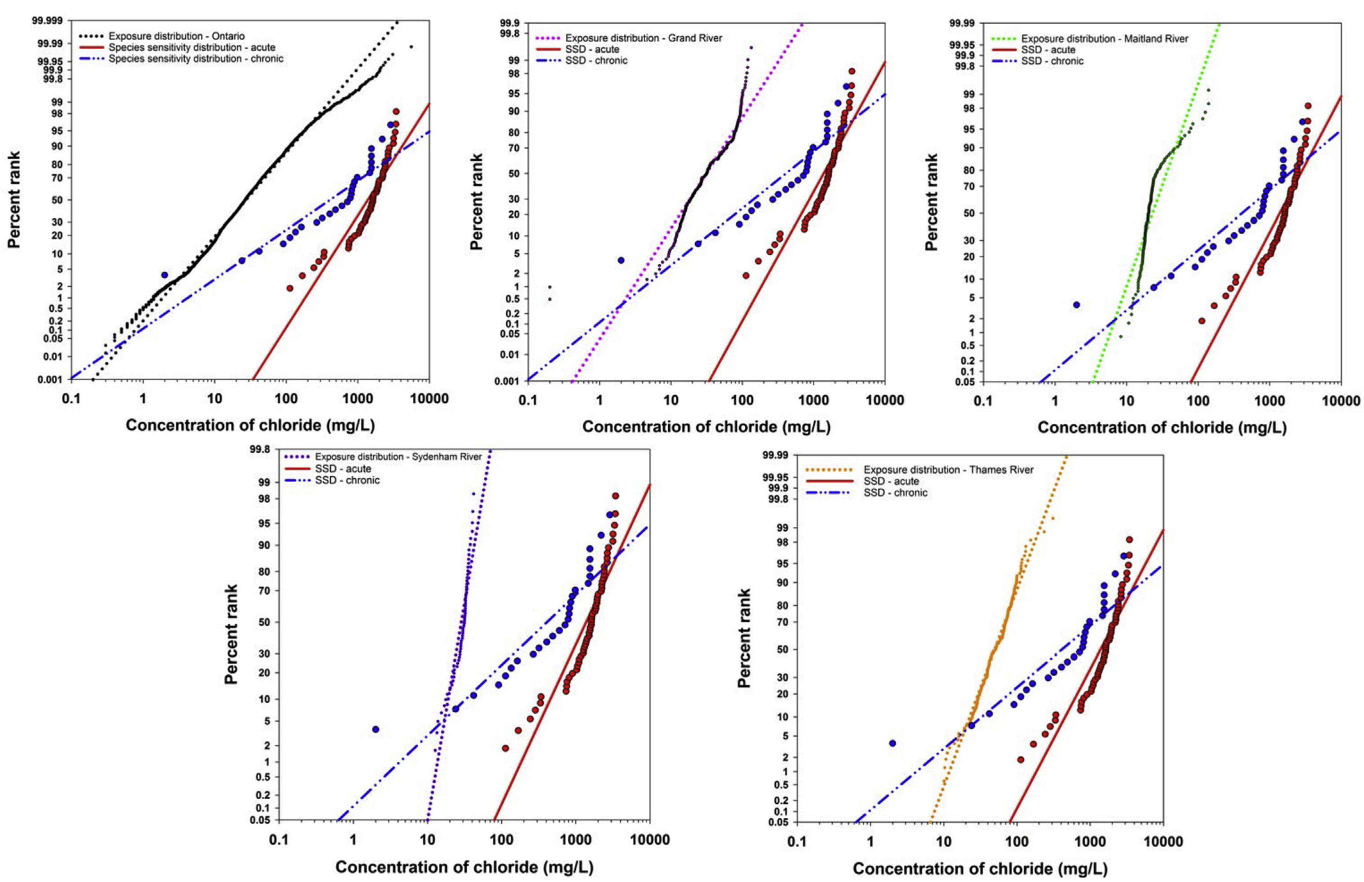

Fig. 1. Species sensitivity distributions for early-life stages of freshwater mussels acutely and chronically exposed to chloride. Exposure distributions constructed using data from Ontario Ministry of the Environment and Climate Change's Provincial Stream Water Quality Monitoring Network collected in 2012, 2013, and 2014. (Top left-hand corner; clockwise) Exposure distributions were created from data collected through the entire province of Ontario, Grand River, Maitland River, Thames River, and Sydenham River.

Table 4
Parameters of species sensitivity distributions constructed for acute and chronic exposure of the early-life stages of freshwater mussels exposed to chloride. Parameters of exposure distributions constructed using data from Ontario Ministry of the Environment and Climate Change's Provincial Stream Water Quality Monitoring Network collected between April and November in 2012, 2013, and 2014. The concentration that represents the 5th centile of species sensitivity distributions, i.e., concentration at which 5% of species are affected, and the concentrations that represent the 95th centile of exposure distributions are reported. The probability of exceeding the 5th centile of the acute and chronic species sensitivity distribution based on each exposure distribution are reported. The area under the joint probability curves (JPC) (i.e., mean risk) for each risk scenario are presented.

Distribution	n	r^2	slope	y − intercept	5th centile	95th centile	Probability of exceedance		Area under JPC	
							Acute	Chronic	Acute	Chronic
Species sensitivity distributions										
Acute	55	0.85	2.66	−8.35	331 mg/L					
Chronic	26	0.84	1.17	−3.06	16 mg/L					
Exposure distributions										
Ontario	7306	0.99	2.00	−2.87		182 mg/L	1.5%	67.8%	0.3%	11.3%
Grand River	204	0.87	2.28	−3.41		166 mg/L	1.0%	74.6%	0.2%	11.8%
Maitland River	123	0.81	3.95	−5.38		60 mg/L	2.3×10^{-4}%	72.9%	0.0002%	7.5%
Sydenham River	60	0.88	7.24	−10.58		49 mg/L	9.1×10^{-14}%	96.8%	3.0×10^{-9}%	8.7%
Thames River	163	0.98	3.75	−6.36		136 mg/L	0.1%	96.7%	0.03%	14.8%

n: number of data points.
r^2: coefficient of determination.

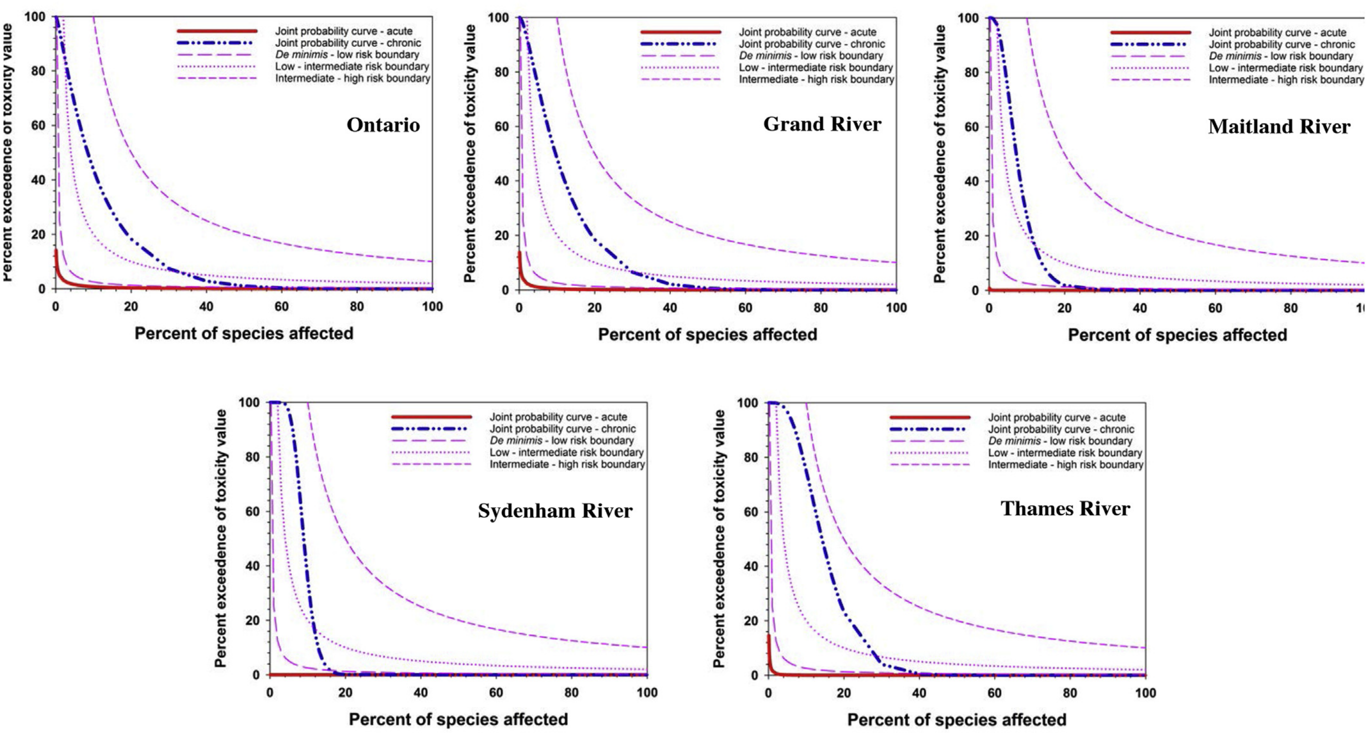

Fig. 2. Joint probability curves constructed using acute and chronic species sensitivity distributions for early-life stages of freshwater mussels and exposure distribution for surface waters in Ontario, Grand River, Maitland River, Sydenham River, and Thames River. Each graph contains *de minimis* − low risk (risk product: 0.25%), low − intermediate risk (risk product: 2%), and intermediate − high risk (risk product: 10%) boundaries.

centile of the exposure distributions range from 49 to 182 mg/L of chloride, which exceeds the lowest reported 24-h EC50 for glochidia viability of the provincially threatened wavy-rayed lampmussel (*L. fasciola*) (113 mg/L) in the acute SSD (Table 4 and Table S9). For the chronic SSD, the 95th centile of the exposure distributions exceeds EC10s for glochidia viability of *L. fasciola* and the federally endangered northern riffleshell (*E. torulosa rangiana*) (COSEWIC, 2010). This means that ≥5% of measured chloride concentrations in Ontario and in these four specific rivers could have an adverse effect on the early-life stages of multiple threatened or endangered freshwater mussel species. The mean risk (i.e., area under the joint probability curve) for each risk scenario indicates that chloride concentrations in the Maitland and Sydenham Rivers pose a lower risk to the early life stages of mussels than the Grand and Thames Rivers (Table 4). The watersheds for both the Grand and Thames Rivers encompass considerably more urban areas than the Maitland and Sydenham Rivers, and it appears that the difference in mean risk is likely due to the higher proportion of connected impermeable areas (Kaushal et al., 2005). This is also likely linked to the higher concentrations of chloride in the Grand and Thames Rivers than those observed in the Maitland and Sydenham Rivers.

The lack of data related to chronic effects of chloride on early-life stages of freshwater mussels generates a considerable amount of uncertainty around the chronic SSD in this assessment (Table S9). There is a clear need for studies examining the effects of chronic exposure (i.e., >4 d) on juvenile and adult freshwater mussels, at various life stages. Another source of uncertainty in this assessment relates to the temporal relationship of the exposure data. Water at each station is only sampled four to five times a year (April to early November) for analysis of chloride. While there are multiple sampling stations on each river (e.g., seven stations along Grand River), it is not possible to characterize peaks in chloride concentrations due to winter melting or precipitation events with so few samples spread over a relatively long period of time. Consequently, short-term pulses or even extended periods of considerably higher concentrations of chloride may be occurring but have not been incorporated into these exposure distributions due to the infrequency and timing of sampling especially during late winter and early spring when there is the greatest potential for peak concentrations (Perera et al., 2010), which would result in an underestimation of risk. Corsi et al. (2015) observed that the greatest increase in chloride concentration in urban streams in the northern United States was in the winter. The exposure distributions also do not capture the length of time that a particular concentration of chloride may persist at a site, so chloride levels may peak and fall quickly or remain elevated for extended periods of time. This type of data is important from the perspective of understanding the probability of chronic exposure, especially when exposure to elevated chloride is likely as a result of a pulse corresponding with a melt or rain event (Evans and Frick, 2001; Marsalek et al., 2003). Measuring the concentration of chloride with greater resolution (i.e., on an hourly basis) at particular sites along rivers that mussel populations are known to inhabit would allow for concentration peaks to be captured and provide information on the length of time that particular pulses of chloride are sustained.

4. Conclusions

This study found that the toxicity of salt-impacted winter road runoff to early-life stages of freshwater mussels closely matches the documented toxicity of chloride to mussels. Road runoff was acutely toxic to half of the exposed mussels (EC50) at dilutions as low as 6%. The concentration of chloride is likely the driver of toxicity in winter road runoff. The probabilistic risk assessment of chloride exposure to early-life stages of freshwater mussels in Ontario found that acute exposure likely represents a *de minimis* risk from April to early November, but chronic exposure to elevated chloride represents a risk that requires further investigation. Further research needs to be conducted on the effect of chronic exposure of all life stages of freshwater mussels to chloride, with particular consideration of threatened and endangered species.

Further research is also needed to characterize potential exposure with greater resolution, i.e., identification of the magnitude and duration of pulses of chloride both in the winter and non-winter months. The current monitoring regime provides little data on the concentration of chloride in surface water in Ontario throughout the winter and provides low resolution throughout the non-winter months. Consequently, the current monitoring program does not characterize peaks in chloride concentration associated with winter thaws and salt-laden runoff, which could result in acute mortality.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.07.001.

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