Evaluation Of Sublethal Toxicity Of Nitrite To A Suite Of Aquatic Organisms In Support Of The Derivation Of A Chronic Environmental Water Quality Benchmark

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Abstract

Nitrite is a naturally-occurring inorganic compound that occurs in aquatic environments as an intermediary between nitrate and ammonia in the nitrogen cycle. It is a contaminant of potential concern resulting from anthropogenic activities in some cases. While the acute toxicity of nitrite has been characterized in previous studies, its sublethal toxicity is less understood. To determine the sublethal toxicity of nitrite on freshwater organisms, a suite of organisms was tested including: two salmonids (*Oncorhynchus mykiss* and *O. kisutch*), an alga (*Pseudokirchneriella subcapitata*), an aquatic macrophyte (*Lemna minor*), and three invertebrates (*Ceriodaphnia dubia, Chironomus dilutus,* and *Neocloeon triangulifer*). Test organisms were exposed to nitrite concentrations ranging between 0.02 and 1.28 mg/L nitrite (NO_2-N). The toxicity tests were conducted according to procedures specified in standardized methods, allowing for the estimation of multiple endpoints for each test species. Species sensitivity distributions (SSDs) were generated using endpoints from the toxicity testing results, as well as data from previous studies, from which water chemistry approximated that used in the tests (*i.e.,* < 5 mg/L chloride, an important toxicity-modifying factor for nitrite). The mayfly, *N. triangulifer*, was the most sensitive species, followed by the two salmonids (which represented the second and third most sensitive species), although they were not as sensitive to nitrite exposure as reported in previous studies. The fifth percentile hazard concentration (HC5) generated from the SSD could be used for derivation of regulatory benchmarks and threshold values for site-specific aquatic risk assessments.

Highlights

- Sublethal effects of nitrite are tested with seven freshwater aquatic species.
- Mayfly larvae most sensitive to sublethal nitrite concentrations.
- Salmonids acutely sensitive to nitrite although low acute-to-chronic ratio.
- Toxicity data used in SSDs to generate environmental benchmark.

1. Introduction

Nitrite (NO_2) is an inorganic nitrogen compound which is an intermediary in the nitrification-denitrification processes of the nitrogen cycle, occurring between ammonia and nitrate. It can occur naturally as a result of the incomplete nitrification of ammonia by nitrifying aerobic bacteria or denitrification of nitrate by anaerobic bacteria, while anthropogenic inputs of nitrogen have led to increased concentrations in some surface waters. Examples of anthropogenic activities which have led to conditions of elevated nitrite include biological sewage treatment (Alleman 1985; Zhou et al. 2011), aquacultural wastewaters (John et al. 2020), blasting agents at mine operations (Bailey et al. 2013), and fertilizers or wastes at agricultural operations (Burns et al. 1995).

It has been demonstrated that nitrite is more acutely toxic to aquatic organisms than ammonia and nitrate under most conditions (Adelman et al. 2009; Soucek and Dickinson 2011; US EPA 2010); however, the sublethal effects on aquatic organisms has been relatively understudied. Nitrite can reduce the oxygen-carrying capacity of aquatic animals through oxidation of hemoglobin and hemocyanin (Kroupova et al. 2018), although it has not been well documented to which aquatic animals this toxic action may be of the most importance for long-term survival and growth; other modes of toxic action may exist (Soucek and Dickinson 2011). The effects of sublethal exposure to nitrite on freshwater fish, particularly salmonids, has been studied and results based on alterations of hemoglobin status following exposure to nitrite of < 0.1 mg/L (Brown and McLeay 1975; Russo and Thurston 1977; Smith and Russo 1975; Wedemeyer and Yasutake 1978). Although reduced growth has only been observed at nitrite concentrations orders of magnitude higher than the studies found to alter hemoglobin status (Gutierrez et al. 2011; Kroupova et al. 2008). Thus, there is some uncertainty regarding concentrations of nitrite at which reduced salmonid growth and survival occurs. In addition, it has been demonstrated that cladocerans (US EPA 2010) and mayflies (Kelso et al. 1999) are particularly sensitive to acute nitrite exposure, yet information is lacking on their sensitivity to longer term exposures. A recent review on toxicity of nitrite highlighted the lack of sublethal toxicity studies conducted with sensitive species, like aquatic insects, as a major data gap (Kroupova et al. 2018).

This study applied a suite of sublethal toxicity tests with fish (Order Salmoniformes), benthic aquatic insects (Orders Ephemeroptera and Diptera), a crustacean (Order Cladocera), an aquatic plant (Order Alismatales), and a green alga (Order Sphaeropleales).

Water chemistry can have a significant influence on the acute toxicity of nitrite. Increases in chloride, pH, alkalinity, dissolved oxygen, bromide, hardness, and humic substances have all been shown to reduce nitrite toxicity (Kroupova et al. 2005; Meinelt et al. 2010). Chloride is a particularly strong modifying factor of nitrite toxicity; this has led to the introduction of chloride-dependent water quality guidelines (BC MoE 1986; Russo et al. 1981). As less is known on the influence of these factors on sublethal toxicity, conducting sublethal tests with nitrite in water quality conditions representative of the site being assessed enhances the site-specificity of the results.

Due to the paucity of sublethal toxicity information, chronic water quality guidelines for nitrite in many jurisdictions have not been recently updated; those guidelines that have been established have used derivation approaches constrained by the lack of complete datasets (*e.g.,* significant uncertainty factors). This study represented an opportunity to combine our data with other sublethal toxicity data to generate a statistically-robust Species Sensitivity Distribution (SSD), which could be used to derive a chronic environmental benchmark for this compound under the tested conditions.

2. Methods

Test water, nitrite concentrations and statistical analysis

Dilution Water. The formulated dilution water used in the toxicity tests was characterized by low chloride (*i.e.*, <2 mg/L) and high alkalinity and hardness (*i.e.,* each 150 mg/L as $CaCO_3$), and was formulated using reagent-grade salt additions to de-ionized water. A subsample from the dilution water used for each test was collected and measured for metals, alkalinity, sulfate, and chloride. The laboratory control water typically used for each of the toxicity tests was also tested concurrently with the formulated dilution water control.

Nitrite Concentrations. Nitrite concentrations were established in test treatments by addition of sodium nitrite ($NaNO_2$). Seven nitrite exposure concentrations were used in each toxicity test including: 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and 1.28 mg/L NO_2-N. The nitrite concentrations included the short-term acute (0.06 mg/L) and long-term chronic (0.02 mg/L) British Columbia provincial water quality guidelines for nitrite (BC MoE 1986). Subsamples from all test concentrations were collected at test initiation, at a mid-point in new *(i.e.,* freshly prepared solution used for renewal) and old (*i.e.,* test solutions prior to renewal) solutions, and at test termination. Subsamples for acute tests with salmonids were collected at test initiation in the low, middle and high test concentrations. Subsamples were analyzed for nitrite using Ion Chromatography with UV detection (EPA 300.1) to ensure that target/nominal concentrations were achieved.

Test concentration and endpoint determination. Test concentrations from the tests were calculated using analyzed nitrite concentrations; an average of the measured values was used for the exposure concentrations.

Effect concentrations were reported in concentrations of NO_2-N (*i.e.*, nitrite as nitrogen) and were determined using the software program, CETIS (Version 1.9.4; Tidepool Scientific Software, McKinleyville, California, USA). Confidence limits for the point estimate endpoints can be found in Supplementary Material- Part B.

The Lowest Effect Concentrations (or LECx) were calculated based on the method described in deBruyn and Elphick (2013), with “x” representing the percent minimum significant difference (PMSD) from each test (based on a Dunnett’s Comparison test). Briefly, the non-linear regression or linear interpolation model of the sublethal data was used to calculate the nitrite concentration at which a PMSD level of effect occurred. For example, if an 11% effect on biomass was the effect level that was statistically significant from the control organism response, the nitrite concentration necessary to result in this level of inhibition is calculated (*i.e.,* LEC11 or IC11).

Toxicity testing

Toxicity test methods were selected with aquatic organisms which were regionally relevant to British Columbia. Sublethal toxicity tests were included with aquatic organisms that have been reported to have particular sensitivity to nitrite in acute exposures. The battery of toxicity tests included broad trophic level representation and employed standardized methods that have been demonstrated to be reproducible and relevant, and therefore, as a whole, would be usable for assessment of water quality according to classifications outlined in Keddy et al. (1994).

Salmonid survival and growth test

Larval rainbow trout (*i.e.,* 2-6 days post swim-up) were received from Aqua Farms (Langley, BC) for testing using methods adapted from Lazorchak and Smith (2007) and Washington Department of Ecology (WDOE, 2016). The method was adapted from a 7-d duration to a 14-d duration. The test volume was increased from 500 mL to 2000 mL at Day 7; an 80% solution renewal was conducted daily with freshly prepared test concentrations. Fry were fed freshly hatched *Artemia* twice daily. Endpoints from the test were dry weight (IC10, IC20 and IC50), biomass (IC10, IC20 and IC50) and survival (LC50). The tests were considered acceptable if control survival was ≥90%. The 7-d test method also has an acceptability criterion of 1.5X growth increase from initiation to termination for the control fry; this was squared to 2.25X growth increase due to the doubling of the exposure duration. Additional details on the test method are provided in Supplementary Material- Part A; Table A1.

Acute survival test with salmonids

Acute lethality (96-h LC50) tests using rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) were conducted following the Environment Canada test method (*i.e.,* EPS 1/RM/09; Environment Canada 1990) adapted as necessary for coho salmon. Tests with rainbow trout were conducted using smaller fry (*i.e.*, ≈0.1 g) and larger fry (*i.e.,* ≈1.0 g) with 0.5X concentration series from 0.64 to 10.24 mg/L mg/L NO_2-N and 0.16 to 2.56 mg/L NO_2-N, respectively. Fry were received from Aqua Farms (Langley, BC). The acute lethality test with coho salmon used 3.5 g juveniles obtained from Northern Divine Aquafarms (Sechelt, BC). In order to accommodate the loading density requirements from the test method (*i.e.,* 0.5 g wet weight per litre of test volume), two replicates of 25 L were employed per test concentration in the coho salmon test with five fish. Despite the increased test volume and the addition of a second replicate, the resulting loading density of this test was 0.8 g/L, which is above the Environment Canada (1990) biological test method, however, below loading densities of other acute survival methods with fish (OECD Test 203 2019). Salmonids used in the testing program were not acclimated for 14 days at the laboratory prior to testing as required by the Environment Canada (1990) biological test method. Rather, testing was conducted within a week of arrival and a concurrent reference toxicant test was conducted for the rainbow trout to ensure that the organisms were of appropriate sensitivity; no reference toxicant test was conducted with the coho salmon due to the lack of a historical reference toxicant database for this species in the laboratory. Additional details on these test methods are provided in Supplementary Material- Part A; Tables A2 and A3.

Chironomid survival and growth test

A 10-d survival and growth toxicity test using the chironomid, *Chironomus dilutus*, as the test species was conducted following methods that were adapted from the Environment Canada biological test method (*i.e.*, EPS 1/RM/32; Environment Canada 1997). A small amount of clean beach sand (1/2 teaspoon) was placed in each test container, followed by the addition of test solutions. Water renewals were conducted on a Monday-Wednesday-Friday schedule. Endpoints from the test were survival (LC50) and dry weight/growth (IC10, IC20 and IC50); tests were considered acceptable if ≥80% survival was observed in control exposures. Additional details on the test method are provided in Supplementary Material- Part A; Table A4.

Duckweed growth inhibition test

A 7-d survival and growth toxicity test with the duckweed, *Lemna minor*, was conducted following the Environment Canada test method (*i.e.,* EPS 1/RM/37; Environment Canada 2007a). The test was conducted as a static-renewal test with solution renewals conducted on Days 3 and 5 of the test exposure period. Endpoints from the test were the number of fronds (IC10, IC20 and IC50) and dry weight/growth (IC10, IC20 and IC50); the tests were considered acceptable if there was an ≥ 8-fold increase in the number of fronds from test initiation to termination in the control treatment. Additional details on the test method are provided in Supplementary Material- Part A; Table A5.

Nutrients (*i.e.,* sodium nitrate and potassium phosphate) were added to the formulated dilution water at concentrations according to the APHA formulation recommended in the Environment Canada (2007a) test method. Ferric chloride additions were replaced by ferric sulfate to minimize chloride alteration, which is a major toxicity modifying factor for nitrite. Similarly, manganese chloride ($MnCl_2$) additions were reduced to minimize chloride addition. Other trace elements (*i.e.*, B, Mo, Zn, Co, and Cu) were added to the water formulation as described in the Environment Canada (2007a) test method.

Algal growth inhibition test

A 72-h algal growth inhibition test with the green alga, *Pseudokirchneriella subcapitata* (recently renamed *Raphidocelis subcapitata)*, was conducted following the Environment Canada test method (*i.e.,* EPS 1/RM/25; Environment Canada 2007b). Endpoints from the test were inhibition of cell growth (IC10, IC20 and IC50). The tests were considered acceptable if the final density of algal cells in the control treatment was at least 16 times higher than the initial density. Additional details on the test method are provided in Supplementary Material- Part A; Table A6.

Nutrients (*i.e.,* sodium nitrate and potassium phosphate) were added to the formulated dilution water at concentrations recommended in the Environment Canada (2007b) method. Similar modifications made in the duckweed test solution (*i.e.,* ferric chloride substituted with ferric sulfate and reduced $MnCl_2$ concentration) were made in the algal growth test solution to minimize chloride alterations to the dilution water.

Mayfly survival and growth test

A 14-d survival and growth toxicity test with the mayfly, *Neocloeon triangulifer*, was conducted following methods adapted from Struewing et al. (2015). The test was conducted in 1-L glass jars with four replicates of each test concentration and ten mayflies per replicate. No water replacements were conducted on the first three days of the test to avoid disturbance of the mayflies, which are very sensitive at this stage. Following this static period, 80% of the water was renewed thereafter on a Monday-Wednesday-Friday schedule. Endpoints of the test were: survival (LC50) and dry weight/growth (IC10, IC20 and IC50); the tests were considered acceptable if ≥80% survival is observed in control exposures. Additional details on the test method are provided in Supplementary Material- Part A; Table A7.

Cladoceran survival and reproduction test

A 6 to 8-d three brood survival and reproduction test with the cladoceran, *Ceriodaphnia dubia*, was conducted following the Environment Canada test method (*i.e.,* EPS 1/RM/21, Environment Canada 2007c). Endpoints from the test were the inhibition of neonate reproduction (IC10, IC20 and IC50) and survival (LC50); the tests were considered acceptable if the control treatment had at least 80% survival and produced ≥15 neonates per organism. Additional details on the test method are provided in Supplementary Material- Part A; Table A8.

Species Sensitivity Distribution.

Species Sensitivity Distributions (SSDs) represent the variation in sensitivity of species to a chemical parameter by a statistical or empirical distribution function of responses for a sample of species (Posthuma et al. 2002). The statistical distribution is

then used to calculate a concentration expected to be safe to most species of interest. The Median Hazardous Concentration Affecting 5% of the species (*i.e.,* the HC5) from the SSD was used to establish water quality guidelines and objectives, the approach suggested by the Canadian Council of Ministers of the Environment (CCME 2007).

The *ssdtools shiny web* application was used to estimate HC5 values (BC MOECCS 2021). This application uses the R package (ssdtools version 0.2.0) to plot the toxicity data using maximum likelihood estimation (MLE). Six distributions (*i.e.*, Gompertz, Weibull, Gaama, log-Gumbel, log-logistic and log-normal) were fitted to the toxicity data and a model averaging approach was applied with the best models to estimate an HC5 value. The best models were selected based on goodness-of-fit tests (*i.e.,* Anderson-Darling and Akaike information criterion), as well as a visual assessment of the lower tails of the distribution, to ensure that only statistically-appropriate models were included in the derivation of the HC5.

An SSD was calculated that included the data reported here for low chloride conditions of the formulated water and literature values from studies that also used low chloride concentrations (*i.e.*, < 5.0 mg/L Cl). Values for the fathead minnow and Topeka shiner were available from Adelman et al. (2009) and for a *C. dubia* test from US EPA (2010). Because *C. dubia* was one of the test species conducted in this study, the geometric mean of chronic values was calculated and included in the SSD.

Separate SSDs were calculated using IC10 and LECx endpoint values, respectively. The provincial and federal guidance for the derivation of Water Quality Guidelines for Aquatic Life (BC MOECCS 2019; CCME, 2007) states that an EC/IC representing a no-effects threshold is the most preferred endpoint. As the LECx represents the point at which test produced a statistically distinguishable effect (deBruyn and Elphick 2013), it was considered an appropriate estimate of the most preferred endpoint. The LECx method also allows for use of regression-based statistical data evaluation, which is preferred (BC MOECCS 2019; CCME 2007).

3. Results

Test water, nitrite concentrations and statistical analysis

Dilution Water. The water chemistry for the dilution water used in the toxicity tests is provided in Table 1. The concentrations of major ions were approximate (*i.e.*, within 20%) to targeted concentrations with the following exceptions: sodium and potassium concentrations were elevated in the green algae and duckweed tests due to addition of nutrients (*i.e.,* sodium nitrate and potassium phosphate), chloride concentrations were lower in the mayfly test media in comparison to the other test waters.

Table 1. Water chemistry results for dilution water used in sublethal toxicity tests.

Parameter	Algae	Duckweed	Rainbow trout/ Cladoceran	Chironomid/ Amphipod	Mayfly
Nutrients (mg/L)					
Ammonia (as N)	<0.005	<0.005	NA	NA	NA
Nitrate (as N)	4.29	44.2	NA	NA	NA
Nitrite (as N)	0.002	0.002	<0.001	0.0014	<0.001
Phosphate [a]	0.168	1.78	NA	NA	NA
Anions (mg/L)					
Alkalinity [b]	100	105	106	100	99.4
Chloride	0.74	0.60	0.77	0.57	0.22
Sulfate	100	101	101	99.2	94.2
Total metals (mg/L)					
Calcium	30.9	32.5	29.9	29.6	33.1
Magnesium	6.42	6.10	6.66	6.28	6.69
Potassium	2.79	7.22	2.29	2.02	2.16
Sodium	50.3	109	48.9	46.7	49.4

NA: Not analyzed [a]- Phosphate, ortho-, dissolved (as P); [b]-Total, as $CaCO_3$

Test concentrations. Analyzed nitrite concentrations (reported in Supplementary Material- Part B) were within 15% of nominal concentrations in new/fresh test solutions and were stable in test solutions in most of the toxicity tests conducted and therefore average analyzed concentrations were used in effect endpoint determination. Analyzed nitrite concentrations deviated from nominal in test solutions of the duckweed, chironomid, cladoceran and mayfly tests and these deviations are described and discussed below.

Concentrations of nitrite analyzed in subsamples collected from test containers prior to solution renewal (*i.e.*, old solutions) of the *Lemna minor* (duckweed) test on Days 5 and 7, were slightly increased in comparison to nominal concentrations (Supplementary Material- Part B; Table B5). A concentration of nitrite ranging from between 0.030 and 0.052 mg/L was observed in the old solutions of the control treatments (both dilution water and laboratory control water) indicating that the duckweed naturally produced a small amount of nitrite. Upon correction for background, the test concentrations in Day 5 and 7 old test solutions were within 20% of nominal concentrations. Thus, low concentrations of nitrite observed in *Lemna minor* test solutions with no nitrite added is likely associated with the combination of the presence of excess nitrate in the test media (*i.e.,* 44.2 mg/L as N) and the reported ability of this plant to reduce nitrate to nitrite (Joy, 1969).

Nitrite concentrations analyzed in new test solutions of the *Chironomus dilutus* test averaged 96% and 102% of nominal on Days 0 and 5, respectively (Supplementary Material- Part B; Table B3). However, nitrite concentrations in old test solutions from Days 5 and 10, were substantially reduced from nominal concentrations. Additional investigations were undertaken to assess nitrogen compounds in the chironomid test. Firstly, ammonia and nitrate were measured in Day 5 old test solutions. This analysis determined that total nitrogen concentrations increased with an increase in nominal nitrite concentrations (Supplementary Material- Part B; Table B3), despite the non-monotonic increase in nitrite concentration with the concentration series in Day 5 old test solutions. Also, a solution from the laboratory control treatment was sampled at a mid-point of the test and analyzed for nitrite. This solution was found to contain 0.472 mg/L of nitrite (as N), despite no addition of nitrite to this treatment. These changes in nitrite concentrations in test solutions may have been related to the reported role of chironomid species in nitrogen cycling; *Chironomus* spp. host bacteria in their gut involved in nitrogen redox reactions (Poulsen et al. 2014; Samuiloviene et al. 2019; Stief et al. 2010). Collectively, these analytical results indicate that the chironomids were likely

actively involved in nitrogen cycling under the test conditions. Based on these findings, only analyzed nitrite concentrations from new solutions were used in the statistical analyses of the test results.

Nitrite concentrations analyzed in new test solutions for the mayfly and *C. dubia* tests averaged within 10% of nominal but both had reduced nitrite concentrations on one of the two sets of old test solutions. Old test solutions sampled on Day 7 from the mayfly and *C. dubia* tests had nitrite concentrations of 62% and 50% of nominal, respectively; nitrite concentrations were within 15% of nominal in the other set of old test solutions analyzed for the two tests (Supplementary Material- Part B; Table B7 and B8). Average analyzed concentrations of all test solutions, both new and old, were within 20% of nominal concentrations for both tests, despite the slight reduction in nitrite in one set of old test solutions and therefore the average analyzed concentrations were used in the effect concentration determination.

Toxicity test results

Results of the reference toxicant tests conducted during the testing program fell within the range for organism performance of the mean ± two standard deviations, based on historical results obtained by the laboratory with these tests. Thus, the sensitivity of the organisms used in these tests was appropriate. The control performance met the requirements of the test methods in all cases.

Results from the of toxicity tests conducted as part of this study are summarized in Table 2 and described below.

Table 2. Summary of sublethal toxicity endpoints calculated for nitrite to freshwater organisms.

Taxon	Species	Life stage	Duration	Endpoint	IC10	IC20	IC50	NOEC	LECx
					mg/L NO_2-N				
Sublethal									
fish	*Oncorhynchus mykiss*	fry (0.1g)	14-d	biomass	0.71	1.03	>1.25	-	0.72[a]
invertebrate	*Chironomus dilutus*	3rd instar	10-d	growth	>1.28	>1.28	>1.28	1.28	-
invertebrate	*Ceriodaphnia dubia*	<24h	6-d	reproduction	0.10	0.15	0.32	-	0.136[a]
invertebrate	*Neocloeon triangulifer*	<24h	14-d	biomass	0.039	0.046	0.069	-	0.059[a]
aquatic plant	*Lemna minor*	Three frond	7-d	growth	>1.29	>1.29	>1.29	1.29	-
aquatic plant	*Pseudokirchneriella subcapitata*	Exponential growth	72-h	growth	>1.37	>1.37	>1.37	1.37	-
Acute									
fish	*Oncorhynchus kisutch*	fry (3.5g)	96-h	survival	-	-	1.14	-	-
fish	*Oncorhynchus mykiss*	fry (0.1g)	96-h	survival	-	-	2.75	-	-
		juvenile (1.0g)	96-h	survival	-	-	1.16	-	-

EC: Effect concentration; NOEC: No Observed Effect Concentration; LECx (deBruyn and Elphick, 2013): Lowest Effect Concentration.

In the *O. mykiss*, *C. dubia* and *N. triangulifer* LECx endpoints presented for these three tests represent IC16, IC37 and IC10, respectively.

Survival and growth test with rainbow trout

No reductions in growth or survival of rainbow trout were observed, except for the highest test concentration of 1.25 mg/L (as N), which resulted in a 20% reduction in survival after 14 days in comparison to the control treatment groups. Biomass (which incorporates both growth and survival) resulted in the most sensitive effect concentrations. An IC10 for biomass was estimated at 0.71 mg/L NO_2-N. A 10.4% reduction in biomass was determined to be the PMSD and, therefore, an IC10.4 estimated at 0.72 mg/L NO_2-N was determined to be the LECx for this test. Detailed results of the toxicity test are presented in Supplementary Material- Part B; Table B1.

Acute lethality tests with salmonids

Results from the acute lethality tests with rainbow trout and coho salmon conducted with nitrite resulted in 96-h LC50s ranging from 1.14 to 2.75 mg/L NO_2-N. Smaller rainbow trout had higher LC50s than larger rainbow trout. The 96-h LC50s were similar for juvenile coho salmon and rainbow trout. Detailed results of the toxicity test are presented in Supplementary Material- Part B; Table B2.

Chironomid survival and growth

No reduction in *C. dilutus* survival was observed after 10 days in any of the tested nitrite concentrations (Supplementary Material- Part B; Table B4). Growth of the chironomids (*i.e.*, dry weights of larvae) was also unimpaired with no statistically different growth in nitrite concentrations from between 0.018 and 1.28 mg/L NO_2-N in comparison to the control treatment.

Duckweed growth inhibition test

No significant inhibition of frond growth was observed in the 7-d exposure at any of the nitrite concentrations (Supplementary Material- Part B; Table B6). The dry weight of the fronds was also not reduced. Therefore, the IC10s and IC50s are greater than the highest nitrite concentration of the test (*i.e.*, 1.29 mg/L NO_2-N).

Algal growth inhibition test

No reduction in cell yield was observed in the nitrite concentrations tested; both the 72-h IC10 and IC50 endpoints were > 1.37 mg/L NO_2-N (Supplementary Material- Part B; Table B7).

Mayfly survival and growth test

Reduced mayfly survival was determined in test concentrations of 0.064 mg/L NO_2-N and above; the 14-d LC50 was estimated at 0.091 mg/L NO_2-N. Growth (dry weight) of the mayfly was not significantly reduced at concentrations lower than 0.139 mg/L NO_2-N, demonstrating that survival was a more sensitive endpoint than growth. Biomass was the most sensitive endpoint, with an IC10 estimated at 0.039 mg/L NO_2-N, although a 36.6% reduction in biomass was determined to be the PMSD and, therefore, an IC36.6 estimated at 0.059 mg/L NO_2-N was determined to be the LECx for this test.

Cladoceran survival and reproduction test

No survival of *C. dubia* was observed in the highest concentration tested (1.24 mg/L NO_2-N) and the 6-d LC50 was estimated at 0.76 mg/L NO_2-N. Reduced reproduction of *C. dubia* was also observed to occur in a dose-dependent manner with a 10% reduction (IC10) in reproduction estimated at 0.10 mg/L NO_2-N. As a 16.4% reduction in reproduction was determined to be the

PMSD (based on a Dunnett's Comparison test), an IC16.4 would represent the point at which significant reduction in reproduction occurred; this was estimated at 0.136 mg/L NO_2-N, representing the LECx for this test.

Species Sensitivity Distribution.

The battery of toxicity tests conducted allow the dataset, in conjunction with additional data from the literature, to meet the Canadian Council of Ministers of Environment (CCME) requirements to generate a water quality benchmark through the use of an SSD (*i.e.,* three fish species, three invertebrate species and an algae/plant) (CCME, 2007).

Two uncertainties associated with the salmonid toxicity data generated in the sublethal testing portion of the study needed to be addressed prior to the inclusion of these data into the SSD. First, the study determined that nitrite toxicity differed depending upon the life stage of salmonids used in the tests; significantly higher sensitivity to nitrite was observed with juvenile rainbow trout in comparison to swim up fry rainbow trout. Because the less-sensitive life stage (*i.e.,* fry) was used for the sublethal exposures with rainbow trout, the potential for higher sensitivity of larger rainbow trout was addressed prior to the inclusion of these data in the SSD analysis. Second, acute toxicity effect concentrations (*i.e.*, 96-h LC50s) were determined for coho salmon, while no sublethal toxicity data were generated, due to logistical limitations (*e.g.*, lack of available coho salmon).

To address these two uncertainties, an acute-to-chronic ratio (ACR) for salmonids was generated from the acute and chronic tests conducted with rainbow trout. The salmonid ACR was calculated by dividing the acute LC50 (96-h survival: 2.75 mg/L NO_2-N) by the sublethal IC10 (14-d biomass: 0.71 mg/L NO_2-N) from nitrite exposures with swim-up rainbow trout (0.1g). This ACR was then applied to the 96-h nitrite LC50s for the larger rainbow trout fry (1.0g) and coho salmon fry (3.5g) of 1.16 mg/L and 1.14 mg/L, respectively, to establish estimates of sublethal effect thresholds for the more sensitive larger fish. The results of this calculation (*i.e.*, 96-h LC50 ÷ salmonid ACR) for rainbow trout and coho salmon was then included in the sublethal SSD.

The SSD models generated with IC10 and LECx values for all species tested are presented in Figure 1 and the HC5 estimates are presented in Table 3. SSD models generated with this dataset determined that three models (*i.e.,* gamma, log-normal and Weibull) fit the data well (*e.g.,* delta AICs < 1; Supplemental Information Part B Table B9), and therefore all three were used in a model average to derive the HC5s. The HC5s for IC10 and LECx values were 0.052 and 0.064 mg/L NO_2-N, respectively. The lower HC5 estimate resulting from the inclusion of IC10 rather than the LECx would provide a higher level of conservatism, which is of particular importance for the mayfly, whose effect concentration was close to the HC5 estimates.

Table 3. Hazard Concentrations (HC5) for nitrite derived using Species Sensitivity Distributions with IC10 and LECx values from sublethal toxicity tests with low chloride concentrations (*i.e.,* <5.0 mg/L).

Endpoints used	Model	HC5 (mg/L NO_2-N)	SE	LCL	UCL
IC10	Model average of gamma, log-normal and Weibull	**0.052**	0.103	0.0096	0.381
LECx	Model average of gamma, log-normal and Weibull	**0.064**	0.107	0.013	0.406

SE: Standard Error, LCL: Lower Confidence Limit, UCL: Upper Confidence Limit

4. Discussion

Organism performance in the formulated dilution water was similar to that observed in the standard laboratory control water in the majority of tests conducted, with the exception of mayfly growth, which was lower in the formulated dilution water in comparison to the laboratory control water. However, the 14-d dry weight of the mayflies from the dilution water was within a range of dry weights observed in control water historically (*i.e.,* based on internal laboratory data) and higher than that observed in a 14-d test with the same species conducted by Soucek et al. (2020). Overall, the use of the data produced using formulated water in this study provides a significant increase of measured sensitivity of the aquatic organisms to nitrite under low chloride concentrations.

Information on the sublethal sensitivity of aquatic organisms to exposure to nitrite derived from these toxicity tests was in the same range as the reported nitrite sensitivities from the primary literature, as follows.

Ephemeroptera species, such as the mayfly, *N. triangulifer*, are considered to be pollution-intolerant, and mayflies have been among the most sensitive species to acute nitrite exposure (Kelso *et al.*, 1999). The results from this study indicate that they are the most sensitive species to nitrite exposure that have been tested. As this species was found to be 2 to 3 times more sensitive to nitrite than any of the other species tested, additional work addressing the effects of nitrite to this species may be warranted. For example, very low chloride conditions, as in the formulated dilution water of the mayfly test *(i.e.,* 0.22 mg/L), may exacerbate the effects of nitrite.

The 6-d IC50 for *C. dubia* reproduction determined in this study fell within the range of IC50s reported in Dave and Nilsson (2005) and US EPA (2010) (*i.e.*, 0.22 to 2.4 mg/L as N). In US EPA (2010), *C. dubia* was the second most sensitive species tested in acute and chronic exposures to nitrite, consistent with relative sensitivities observed in the suite of tests conducted in this study. The acute LC50 for *C. dilutus* was estimated to be 15.6 mg/L NO_2-N by US EPA (2010). The concentrations employed in this study with *C. dilutus* were ten-fold below this acute threshold and no sublethal effect was observed. No information was previously available in the literature with respect to the sensitivity of plants and algae to nitrite; in comparison to the other aquatic organisms tested in this study, the results from the plant and algae tests characterize them as relatively insensitive to nitrite exposure, which is not surprising considering the important role of nitrogenous species as nutrients for primary producers.

Salmonids have been observed to be one of the more sensitive aquatic organisms to nitrite; results from studies with salmonids were the basis for the current provincial guideline (BC MOE 1986; 0.02 mg/L NO_2-N). The nitrite concentration which caused a reduction in rainbow trout survival and growth determined in this study (*i.e.,* a 14-d IC20 of 1.03 mg/L NO_2-N) was similar to the nitrite concentration which caused adverse growth effects (*i.e.*, LOEC of 1.0 mg/L NO_2-N) from a 28-d test with rainbow trout (Kroupova et al. 2008). The acute tests conducted in this study indicate that coho salmon were comparable to rainbow trout in their sensitivity to nitrite. In contrast, the BC Aquatic Life Guideline for nitrite was derived from studies which identified nitrite concentrations associated with an increase in blood methemoglobin in salmonids (MetHb; the form of hemoglobin not able to carry oxygen) at nitrite concentrations that were much lower (*i.e.*, <0.1 mg/L as N) than the sublethal growth effect concentrations reported in this study (*i.e.*, >0.5 mg/L as N). In the literature, a clear connection between the observed increase of MetHb at nitrite concentrations <0.1 mg/L (as N) and an associated reduction in the growth of salmonids is lacking. For example, no significant reduction in salmonid growth was described in the studies that originally reported increased MetHb at <0.1 mg/L (as N) (Brown and McLeay 1975; Wedemeyer and Yasutake 1978). The lack of effects on growth at these concentrations (*i.e.,* <0.1 mg/L (as N) in the 14-d sublethal test conducted in the water used here further suggests there is no growth impairment to salmonids under these low nitrite concentrations.

Findings from the current study provide additional evidence supporting the possible adaptation of salmonids to nitrite exposure. The rainbow trout tests conducted during this study determined acute LC50s that were only slightly higher than the sublethal growth IC20 determined from the 14-d exposure; 96-h LC50s were as low as 1.14 mg/L NO_2-N in comparison to a 14-d IC20 of 1.03 mg/L NO_2-N. Therefore, the data indicate a low acute-to-chronic ratio for salmonids and nitrite exposures. This is consistent with other data that show salmonids activate coping mechanisms which can reverse initial increases in MetHb due to nitrite exposure (Almendras 1987; Gutierrez et al. 2011) by reducing MetHb back to functional hemoglobin (Ha et al. 2019), leading to decreasing MetHb over time, during continued nitrite exposure (Doblander and Lackner 1997; Zusková et al. 2013). This suggests that initial increases in %MetHb in salmonids at lower nitrite concentrations (*i.e.,* <0.1 mg/L as N) may be temporary, and not associated with long-term reduction in the performance of salmonids. In summary, the data generated in this study indicate a threshold for effects on long-term growth for salmonids is of a higher magnitude (*i.e.,* >0.1 mg/L) than the MetHb thresholds used in the derivation of the provincial guideline (*i.e.*, <0.1 mg/L).

The HC5 values derived from the SSD models with the data from this study, together with data for water with low chloride reported in the literature, provide the basis for an environmental benchmark for long-term nitrite exposure under these conditions. As the sublethal toxicity tests were conducted with low ambient chloride, the results of the toxicity tests likely

represent a conservative assessment of the effects of nitrite. Thus, the HC5s determined represent a baseline environmental benchmark which could be adjusted for use on a site-specific basis with conditions of higher chloride. Additional research may be required to assess the modification of nitrite toxicity by chloride in long-term exposures, as has been done previously for short-term exposures.

Statements And Declarations

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Author contributions: Josh A. Baker: Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Roles/Writing – original draft. Graham Matheson: Investigation; Visualization; Roles/Writing – original draft. Guy Gilron: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing – review & editing. David K. DeForest: Conceptualization; Project administration; Methodology; Supervision; Writing – review & editing

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Figures

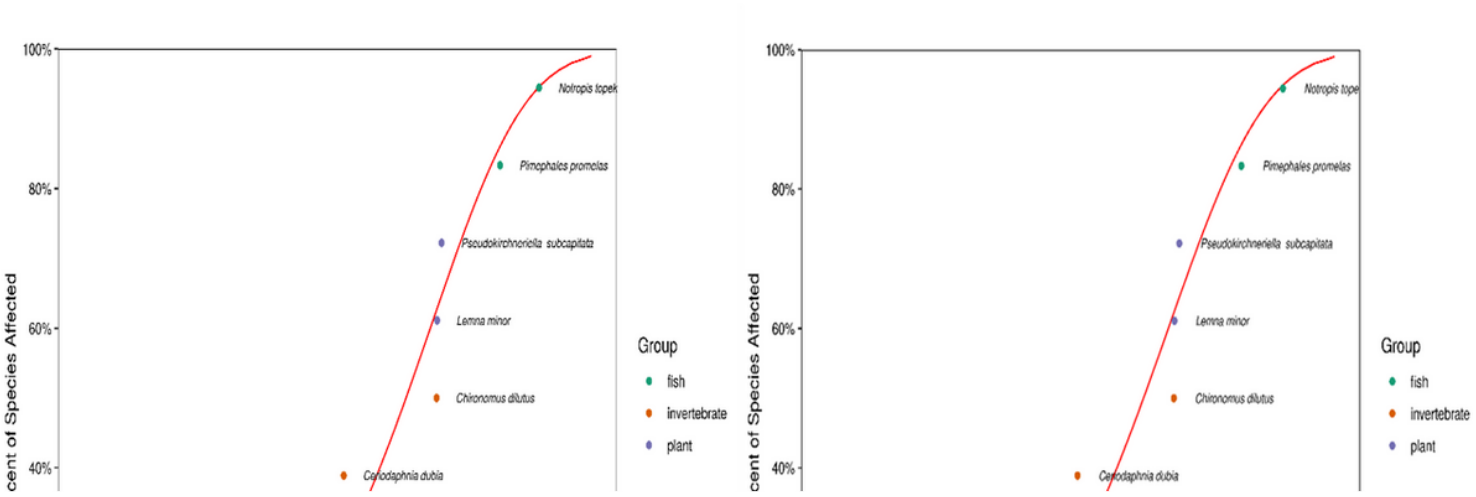

Figure 1

Species Sensitivity Distributions using IC10s (left) and LECx (right) for nitrite from sublethal toxicity testing with chloride of <5.0 mg/L [HC5s: 0.052 and 0.064 mg/L NO_2-N for IC10s and LECxs, respectively].

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