Bull. Environ. Contam. Toxicol. (2004) 72:1290–1296

DOI: 10.1007/s00128-004-0383-3

Bulletin of Environmental Contamination and Toxicology

Effects of Ammonia on the Early Life Stages of Northern Pike (*Esox lucius*)

E. A. Harrahy,[1] M. Barman,[2] S. Geis,[2] J. Hemming,[2] D. Karner,[2] A. Mager[2]

[1] Wisconsin Department of Natural Resources, Bureau of Watershed Management, 101 South Webster Street, Madison, WI 53702, USA
[2] Wisconsin State Laboratory of Hygiene, Biomonitoring Unit, 2601 Agriculture Drive, Madison, WI 53707, USA

Received: 14 November 2003/Accepted: 17 April 2004

The Wisconsin Department of Natural Resources (WDNR) is in the process of revising its acute and chronic surface water quality criteria for ammonia. Determination of effluent limitations for ammonia discharges based on chronic toxicity, or long-term impacts, will take into account temperature, pH, percent of stream flow used for dilution, and the presence of early life stages of fish. Because ammonia is generally less toxic to juvenile and adult fish at lower temperatures, chronic ammonia water quality criteria may be relaxed (to the extent that the relaxed water quality criteria would still be protective of them) when water temperatures are below 15°C for certain use-designated waters where either early life stages of fish are absent, or if present, have been shown to be tolerant of ammonia.

In revising its ammonia criteria, the WDNR is adapting the United States Environmental Protection Agency's (USEPA's) 1999 Update of Ambient Water Quality Criteria for Ammonia to reflect Wisconsin's fish species and surface water use designations. However, the USEPA document (1999) does not contain toxicity data for the northern pike (*Esox lucius*). Because the northern pike is a species that is widely distributed throughout Wisconsin, the WDNR asked the Wisconsin State Laboratory of Hygiene (WSLOH) to conduct toxicity tests with this species to generate data suitable for inclusion in Wisconsin's ammonia criteria database. Early life stages (pre-hatch embryonic, yolk-sac fry, and larval) of northern pike may be present in Wisconsin waters in late winter and early spring. Spawning typically occurs when the ice begins to melt, from late March to early April (Becker 1983). If the early life stages of northern pike (which may be present when water temperatures are below 15°C) are shown to be sensitive to ammonia, then lower chronic ammonia water quality criteria ("early life stages present" water quality criteria) may be necessary to protect them. The purpose of this research was to determine the effects of ammonia on hatching, larval mortality, final weight, and biomass of northern pike in laboratory chronic toxicity tests.

Correspondence to: E. A. Harrahy

MATERIALS AND METHODS

A chronic toxicity test was conducted following ASTM's Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes (ASTM 1998), with modifications to test temperature. ASTM recommends a test temperature of 15°C; however we conducted the toxicity test at a final temperature of 9°C. Fifteen degrees Celsius is not a temperature that is representative of conditions encountered by northern pike during their early life stages in Wisconsin. A temperature of 9°C falls close to the middle of the range of temperatures that early life stages of northern pike are exposed to in nature.

Fertilized northern pike eggs were transported to the WSLOH in 2°C water on April 1, 2003 by WDNR Southern Region fisheries staff. The test began that day when forty eggs were randomly allocated to each of 18 flow-through egg hatching chambers (three replicates for each of six treatments). Hatching chambers consisted of 500 mL plastic beakers connected to an inlet tube that allowed water to enter at the bottom of the beaker and an outlet tube that allowed water to exit near the top of the beaker. Flow rate (through gravity) was maintained at about 50 L/day using pipette tips. Eggs were suspended in the water column on Nitex® mesh silicone-glued to a plastic ring that rested on a collar about 5 cm above the bottom of the beaker. Hatching chambers were randomly placed in a walk-in incubator set at 3°C. Incubator temperature was increased by 0.5°C per day until it reached 9°C, and then was maintained at 9°C for the remainder of the test. Based on the results of two previous range-finding chronic toxicity tests (*unpublished results*), treatments in the present test consisted of 0, 5, 10, 20, 40, and 80 mg N/L (nominal concentrations; total ammonia nitrogen) and were prepared in 50-L plastic carboys by mixing an appropriate amount of stock solution (1,000 mg N/L stock solution) with dechlorinated tap water. The stock solution was prepared by mixing dried ammonium chloride (NH_4Cl; certified ACS, assay 99.9%, Fisher Chemical, Fairlawn, NJ) with Milli-Q® water. Solutions in each carboy were renewed daily. Ammonia concentrations were measured as total ammonia nitrogen (mg N/L) in each hatching chamber weekly using the automated phenate method (USEPA 1993). Temperature, dissolved oxygen and pH were measured every day in one replicate of each treatment such that each replicate was measured every three days. Hardness and alkalinity were measured in the dechlorinated dilution water weekly. The photoperiod was set to 24 hours dark during the egg stage. Once hatching began, the photoperiod was set to 14 hours light, 10 hours dark to mimic the environmental photoperiod for that time of year. Egg viability was recorded and unviable eggs (those that appeared cloudy or had fungus) were removed daily. After hatching ceased, surviving larvae were counted and thinned. Ten surviving larvae were randomly selected from each hatching chamber (with the exception of replicate C of the 5 mg/L nominal treatment) and transferred to a larger, 3 L plastic flow-through beaker to prevent crowding effects on growth. Only five larvae were transferred from replicate C of the 5 mg/L nominal treatment because only five eggs hatched in that chamber. Results of previously conducted range-finding chronic toxicity tests (*unpublished results*) indicated that there was a significant positive relationship between number of larvae in a chamber and the final weight of those

larvae. Each beaker contained a thin strip of Nitex® mesh. Larvae were fed live brine shrimp three times per day at a rate of 0.1 mL concentrated brine shrimp, beginning when yolk sacs appeared to be nearly absorbed on some individuals. Five days later, the feeding rate was increased to 0.2 mL concentrated brine shrimp per chamber (fed three times per day). Mortality was recorded and dead larvae were removed daily. The test was terminated at 52 days, when larvae entered the juvenile stage, or when they began to resemble adults. Final survival was recorded and total dry weight of survivors was measured for each chamber.

Statistical analyses were conducted using a PC-version of SAS® (SAS Institute, Cary, NC). Results with $p < 0.05$ were considered significant. Actual measured ammonia concentrations were used in all statistical analyses. One-way analysis of variance (ANOVA) was used to determine if there were differences among treatments in hatching success, larval mortality, and weight of survivors. Hatching success and larval mortality data were arc-sin square-root transformed prior to analysis because results for each of these effects were analyzed as percentages. Average weight of a surviving individual was calculated by dividing the total weight of fish in a replicate chamber at the end of the test, by the number of fish weighed (number of survivors). Because there were significant reductions in both survival and growth (as weight of surviving individuals), ANOVA was also used to determine if there were differences among treatments in biomass (the product of survival and growth). Biomass was calculated by dividing the total weight of fish in a replicate chamber at the end of the test, by the number of larvae in the chamber at the start of the larval phase of the test. All weight and biomass data were log-transformed prior to analysis to give the best fit, and to address any heterogeneity of variances. Multiple comparison (least significant difference (LSD) and Ryan-Einot-Gabriel-Welsch multiple range (REGWQ)) tests were conducted to compare hatching success, mortality, weight of survivors, and biomass among treatments, where there were significant F-tests (ANOVAs). Analyses of sublethal measures (weight of survivors and biomass) were conducted without those treatments that had mortality significantly different from the control. No observed effect concentrations (NOECs) and lowest observed effect concentrations (LOECs) were estimated where possible. Maximum acceptable toxicant concentrations (MATCs) were also calculated (as the geometric mean of the NOEC and the LOEC) where possible.

EC20 values (effective concentration for 20% of the population, or the concentration that reduces the measured parameter of interest 20% relative to the control) were estimated for hatching success, larval mortality, weight of surviving individuals, and biomass using Proc Probit (SAS Release 8.01, SAS Institute 2001). Estimations of EC20 values for sublethal measures were conducted without those treatments with mortality significantly different from the control. Values estimated for weight of surviving individuals and for biomass were converted to equivalent EC20 values at a standard pH of 8.0 (USEPA 1999) to allow comparison to EC20 values that have been published for other species.

RESULTS AND DISCUSSION

Ammonia concentrations measured in the definitive chronic toxicity test were consistently within only about 24% of the desired nominal concentrations (Table 1) because the stock solution had been calculated as ammonium (NH_4) instead of as nitrogen (N). All statistical analyses were conducted using average measured (not nominal) concentrations. Summary statistics for dissolved oxygen, pH and temperature are also presented in Table 1. While the majority of pH readings were close to the overall mean pH of 7.6, five of the 308 readings ranged from 8.48 to 9.83, and may be considered to be "spikes". The reason for the occurrence of these spikes, which were observed in three of the six treatments, is unknown. It took seven days for the temperature to reach 9°C. Alkalinity of the dechlorinated dilution water ranged from 283 to 311 mg/L (with a mean of 293 mg/L and standard deviation of 10.49 mg/L; n=6). Hardness of the dechlorinated dilution water ranged from 136 to 172 mg/L (with a mean of 152 mg/L and standard deviation of 17.71 mg/L; n=6).

Larvae began emerging from the eggs on April 16, 2003, or on day 15 of the test. Hatching continued for five days. The number of larvae in a chamber after thinning was equal to 10 at the start of the second phase of the test for all replicates of all treatments except for replicate C of the 3.83 mg N/L treatment which contained only five larvae. Larvae were fed live brine shrimp beginning on May 2, or day 31 of the test. The test was terminated on May 23, or day 52, when larvae appeared to have entered the juvenile phase of development and resembled adults.

Mean hatching success ranged from 34.16% in the 3.83 mg N/L treatment to 65.83% in the 15.10 mg N/L treatment, but there were no significant differences in hatching among treatments (Table 2). The estimated EC20 value for hatching success was equal to 43.20 mg N/L; however, it was not possible to calculate 95% confidence limits for this value. Two embryos died in a control replicate on a day when pH in that replicate spiked to 8.48; however, no other pH spikes occurred during the embryo stage and so it was not possible to determine if there was a relationship between pH and embryo mortality.

Mean mortality ranged from 0% in the control treatment to 100% in the 62.67 mg N/L treatment. ANOVA on arc-sin square-root transformed data indicated there were significant differences in mortality among treatments, and the results of the REGWQ multiple comparison test are shown in Table 2. The estimated NOEC, LOEC, and MATC values were equal to 15.10, 30.38, and 21.42 mg N/L, respectively. The estimated EC20 value for mortality was equal to 20.56 mg N/L, with 95% confidence limits of 14.19 and 46.17 mg N/L. Examination of mortality data on days of observed pH spikes, and on days following pH spikes, did not indicate increasing larval mortality with increasing pH.

Only five sac fry hatched in one replicate of the 3.83 mg/L treatment. Because the range-finding tests indicated that final weight was dependent on number of larvae in a chamber, this replicate was not included in any weight of survivors or

Table 1. Ammonia (Mean Conc) in mg N/L, dissolved oxygen (Mean DO) in mg/L, pH (Mean pH), and temperature (Mean Temp) in °C, measured during the early life stage toxicity test conducted with northern pike. Standard deviation (Std. Dev.) and sample size (*N*) are also presented for each parameter.

	Nominal Concentration (mg N/L)					
	0	**5**	**10**	**20**	**40**	**80**
Mean Conc	0.05	3.83	7.71	15.10	30.38	62.67
Std. Dev.	0.04	0.20	0.42	5.35	1.48	2.21
N	8	8	8	8	8	7
Mean DO	8.7	8.7	8.9	9.0	9.1	8.7
Std. Dev.	0.8	0.8	0.7	0.6	0.6	0.8
N	52	52	52	52	52	48
Mean pH	7.6	7.6	7.6	7.6	7.6	7.7
Std. Dev.	0.4	0.1	0.2	0.3	0.1	0.4
N	52	52	52	52	52	48
Mean Temp	8.5	8.6	8.7	8.7	8.7	8.5
Std. Dev.	1.3	1.2	1.3	1.2	1.2	1.4
N	52	52	52	52	52	48

*Limit of detection = 0.013 mg N/L

biomass analyses. In addition, because mortality was significantly lower in the 30.38 mg N/L and 62.67 mg N/L treatments than in the control treatment, these treatments were not included in any weight of survivors or biomass analyses. Mean weight of survivors ranged from 4.13 mg in the 15.10 mg N/L treatment to 5.48 mg in the control treatment. ANOVA on log-transformed data indicated there were significant differences in weight of survivors among treatments (Table 2). The LSD multiple comparison test showed the 15.10 mg N/L treatment to be significantly different from all lower treatments, resulting in estimated NOEC, LOEC, and MATC values equal to 7.71 mg N/L, 15.10 mg N/L, and 10.79 mg N/L, respectively. However, the more conservative REGWQ test indicated no significant differences among treatments, and prohibited estimation of NOEC, LOEC, and MATC values. The estimated EC20 value for weight of survivors was equal to 13.21 mg N/L, with 95% confidence limits of 2.94 and 53.03 mg N/L. This EC20 value was converted to an equivalent EC20 value of 8.25 mg N/L at a standard pH of 8.0 (USEPA 1999).

Mean biomass also ranged from 4.13 mg in the 15.10 mg N/L treatment to 5.48 mg in the control treatment. ANOVA on log-transformed data indicated there were no significant differences in biomass among treatments (Table 2). The estimated EC20 value for biomass was equal to 13.44 mg N/L, with 95% confidence limits of 4.38 and 26.64 mg N/L. This EC20 value was converted to an equivalent EC20 value of 8.40 mg N/L at a standard pH of 8.0 (USEPA 1999).

Results of this study indicate that northern pike are more tolerant of ammonia than seven of the eleven species for which chronic data are presented in the

Table 2. Mean, standard deviation (Std. Dev.), and sample size (*N*) for hatching success, larval mortality, weight of survivors, and biomass measured in the early life stage toxicity test conducted with northern pike. Results of analysis of variance (ANOVA) and multiple comparison tests (LSD and REGWQ; where there were significant ANOVAs) are also presented. Means with the same letter are not significantly different from each other.

Effect Parameter	ANOVA F (*p*-value)	Multiple Compar. Test	Treatment					
			0.05	**3.83**	**7.71**	**15.10**	**30.38**	**62.67**
Hatching								
Mean (%)			63.33	34.17	55.83	65.83	43.33	49.16
Std. Dev.			3.82	18.93	10.10	16.64	16.27	9.46
N			3	3	3	3	3	3
	2.31 (0.1093)							
Larval Mortality								
Mean (%)			0	0	6.67	0	66.67	100
Std. Dev.			0	0	5.77	0	35.12	0
N			3	3	3	3	3	3
	28.97 (<0.0001)	LSD	A	A	A	A	B	C
		REGWQ	A	A	A	A	B	C
Wt. of Survivors								
Mean (mg)			5.48	5.20	5.43	4.13	1.84	0
Std. Dev.			0.70	0.18	0.42	0.55	0.74	0
N			3	2	3	3	2	0
	4.54 (0.0454)	LSD	A	A	A	B	--	--
		REGWQ	A	A	A	A	--	--
Biomass								
Mean (mg)	--	--	5.48	5.20	5.06	4.13	1.02	0
Std. Dev.	--	--	0.70	0.18	0.26	0.55	0.88	0
N	--	--	3	2	3	3	2	0
	4.20 (0.0539)	--	--	--	--	--	--	--

USEPA's 1999 ammonia criteria document. Comparison of standardized EC20 values [EC20 values converted to equivalent EC20 values at a standard pH of 8.0 (fish) or to equivalent EC20 values at a standard pH of 8.0 and temperature of 25°C (invertebrates)] indicated that, of the species listed in USEPA's 1999 ammonia criteria document, only *Ceriodaphnia dubia*, *Ceriodaphnia acanthina*, *Daphnia magna*, and *Ictalurus punctatus* (channel catfish) are more tolerant of chronic exposure to ammonia than northern pike.

Because juvenile and adult fish species are generally more tolerant of ammonia when water temperatures are low, the WDNR has proposed relaxing water quality criteria for ammonia when water temperatures are low and early life stages of fish are absent. Three species were chosen by the WDNR to represent those species that may have early life stages present for a month or more when water temperatures are less than 15°C (and that may impact the length of an early life stage-absent criteria period): white sucker (*Catostomus commersoni*), burbot (*Lota lota*), and northern pike. Before the present study was conducted, chronic toxicity data were not available for the northern pike. Chronic toxicity values (standardized EC20 values) determined in the present study are greater than the WDNR's proposed early life stage-absent criteria across a range of possible pH and temperature values. Therefore, the early life stage-absent water quality criteria for ammonia proposed by the WDNR can be expected to be protective of early life stage northern pike.

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