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Comparative Developmental Toxicity of Nickel to *Gastrophryne carolinensis*, *Bufo terrestris*, and *Xenopus laevis*

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Abstract. The early embryo-larval developmental toxicity of nickel (Ni) to 3 amphibian species, *Xenopus laevis* (South African clawed frog), *Bufo terrestris* (southern toad), and *Gastrophryne carolinensis* (eastern narrow-mouthed toad), was evaluated using a modified FETAX model. Studies were initiated from late blastulae stage (Nieuwkoop and Faber [NF] stage 10 or Gosner stage 12) and completed at a common embryological-based test termination point, which represented the completion of the major stages of organogenesis (NF stage 46 for *Xenopus* or Gosner stage 26 for the toads). Results indicated that, in terms of lethality, *G. carolinensis* was the most sensitive and *X. laevis* was the least sensitive of the species tested. The 4-d LC50 in *X. laevis* value was approximately 7.2- and 2.8-fold greater than the *G. carolinensis* and *B. terrestris*, respectively. In terms of malformation, *X. laevis* was the most sensitive and *B. terrestris* was the least sensitive of the species tested. The 7-d EC50 (malformation) in *B. terrestris* was 10.6- and 7.0-fold greater than *X. laevis* or *G. carolinensis*, respectively. The chronic value (ChV) for growth in *X. laevis* was nearly 4.5-fold less than the ChV for growth determined for *B. terrestris.* As with the malformation endpoint, *X. laevis* was more sensitive than the other species, which were nearly equisensitive. Overall, the present study provides new data regarding the toxicity of Ni to larval amphibian species, which may be useful in the establishment of new aquatic life criteria for Ni.

The toxicity of nickel (Ni) to aquatic organisms has been the focus of relatively intense research in recent years. One impetus for this intensity has been the European Union's (EU) Existing Substances Risk Assessment process, within which Ni is a priority substance (EC 763/93). This comprehensive initiative has many regulatory consequences in the EU, including the setting of Environmental Quality Standards for water. The risk assessment has included a review of relevant ecotoxicological data on Ni. For the aquatic environment, amphibians were observed to be among the most sensitive species to Ni. Specifically, Birge and Black (1980) reported a chronic LC10 value of 0.0041 mg Ni/L for the eastern narrow mouthed toad, *Gastrophryne carolinensis.* Only the invertebrate *Ceriodaphnia dubia* has been reported to be more sensitive to Ni than *G. carolinensis*, with chronic No Observable Effects Concentration (NOEC) values for reproductive endpoints reported as low as 0.0034 mg Ni/L (Keithly *et al.* 2004). Considering that the mean ambient concentrations of Ni are approximately 15–20 μg Ni/L in the U.S. (Grandjean 1984) and 2.9 μg Ni/L in the EU (EURAS 2005), it is surprising that more widespread toxicological effects on developing amphibian species have not been observed.

However, the sensitivity of *G. carolinensis* to Ni found by Birge and Black (1980) contradicts results of other more recent studies involving the exposure of amphibian early life stages to Ni. For example, Herkovits *et al.* (2000) reported a NOEC of 1.8 mg Ni/L for *Bufo arenarum*, a South American toad. Additional studies suggest that amphibians in general, and toads in particular, are not exceptionally sensitive to Ni (Hopfer *et al.* 1991; Luo *et al.* 1993; Plowman *et al.* 1994; Sunderman *et al.* 1995). In Birge and Black (1980), details concerning experimental design that are more commonly provided in the scientific literature today were not presented, making the application of results for risk assessment purposes somewhat more complicated. For example, confirmation of the Ni concentrations tested was not provided, nor was the concentration of Ni in control treatments. It is possible that the results reported by Birge and Black (1980) may have been influenced by the experimental approach and methods used at that time.

With the development (Dumont *et al.* 1983), standardization (ASTM 1998; Bantle *et al.* 1998), and validation (Bantle *et al.* 1994a, 1994b, 1996, 1999; Fort and Stover 1998; Fort *et al.* 2003) of early amphibian embryo-larval developmental toxicity models, such as the Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX), re-evaluation of developmental toxicity databases for amphibians is warranted. FETAX is a 96-h chronic embryo-larval developmental toxicity assay. The FETAX model measures both lethal and sublethal endpoints

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including embryo lethality, malformation, and growth. In most cases, early embryo-larval development represents a highly sensitive life stage (Fort *et al.* 2003). FETAX represents the only standardized early life phase amphibian toxicity test model and is described in ASTM E1439-98 (ASTM 2004).

In the present study, the general FETAX model was used to evaluate the developmental toxicity of Ni. A modified FETAX model was adapted for use with three amphibian species: *Xenopus laevis* (South African clawed frog), *Bufo terrestris* (southern toad), and *G. carolinensis*. The primary adaptation included the use of a common embryological-based test termination point, which represented the completion of the major stages of organogenesis [NF stage 46 for *Xenopus* or Gosner stage 26 for the toads (Gosner 1960]. Results of definitive concentration-response studies evaluating the early embryo-larval developmental toxicity of Ni are presented in this report.

Materials and Methods

Test Animals and Materials

Adult male and female *X. laevis* were acquired from *Xenopus* 1 (Dexter, MI) and maintained at Fort Environmental Laboratories (FEL). The *B. terrestris* and *G. carolinensis* test embryos were acquired from University of Georgia, Savannah River Ecology Laboratory (SREL), Aiken, SC. Chemicals and reagents used in the culture of test organisms in this study were purchased from either the Sigma-Aldrich Company (St. Louis, MO) or Fisher Scientific (Houston, TX). Ni concentrations tested were prepared from dilutions of anhydrous Ni (II) chloride ($NiCl_2$) (99.99% purity) (Aldrich Chemical Company, Milwaukee, WI).

Methods

Experimental Animals. *Xenopus* adult care, breeding, and embryo collection were performed by FEL, as described in ASTM E1439-98 (ASTM 1998). Dechlorinated tap water was used as the in-laboratory site water for maintaining adult *X. laevis.* FETAX solution (ASTM 1998; Dawson and Bantle 1987) was used in the laboratory as the culture and dilution water, and also served as the negative control for the bioassays. FETAX solution is an ionically balanced reconstituted water medium that provides satisfactory survival and growth of developing amphibian embryos. FETAX solution consisted of 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4{\cdot}2H_2O$, 30 mg KCl, and 15 mg $CaCl_2$ in ASTM type I grade deionized water, brought to a final volume of 1 L. Adult *X. laevis* were fed a Salmon Chow diet *(Xenopus* 1, Dexter, MI) daily *ad libitum. Bufo* and *Gastrophryne* adult collection, care, breeding, and embryo collection were performed by SREL. Adult male and female *B. terrestris* and *G. carolinensis* were collected from uncontaminated wetlands in South Carolina, including Ginger's Bay and ephemeral wetlands near Risher Pond and the SREL Conference Center. The field-collected adult specimens were then held in outdoor mesocosms at SREL until breeding was completed. Well water was used for maintaining the adult specimens in the mesocosms and for the short-term culturing of the resulting egg masses. The adult males and females were injected with 600 IU and 800 IU, respectively, of human chorionic gonadotropin (hCG) [1,000 IU/ml 0.9% (v/v) saline solution] to induce breeding. The egg masses were then collected and shipped overnight in cold well water to FEL via commercial carrier.

Prior to sorting and staging, the gelatinous coat was removed from *X. laevis* embryos by swirling the eggs in a 2% (w/v) solution of L-cysteine (pH 8.1) for 2 min followed by thorough rinsing in FETAX solution. Gelatinous coats from *B. terrestris* and *G. carolinensis* embryos were not removed prior to testing. Previous studies in our laboratory have indicated that removal of the gelatinous coat from *Xenopus* embryos does not appreciably alter sensitivity to a variety of developmental toxicants including metals (Bantle *et al.* 1998). Nieuwkoop and Faber (1994) and Gosner (1960) were used as guides for staging the *X. laevis*, *B. terrestris*, and *G. carolinensis*, respectively.

Analytical Testing

Culture and Site Water Characterization. The embryo-larval culture waters (FETAX solution and SREL well water) and the adult specimen source waters (dechlorinated tap water, SREL Conference Center Wetland, Risher Wetlands and Ginger's Bay) were physicochemically characterized. Samples were filtered using 0.45-µm membrane filters prior to sample preservation, and all analyses were reported as dissolved analytes. General water chemistry, which included pH, dissolved oxygen (DO), conductivity, hardness, and ammonia-nitrogen were analyzed in water samples from each respective site, in accordance with EPA methods (USEPA 1980). In addition, chloride, sulfate, alkalinity (bicarbonate and carbonate), orthophosphate-phosphorus, organic carbon, calcium, magnesium, potassium, sodium, and Ni concentrations were also determined, based on EPA methods.

Nickel Measurement of Test Concentrations. Soluble Ni analysis was performed on 0.45-µm filtered aliquots of each working (test) concentration collected at test initiation and termination. Ni analyses were performed using inductively coupled plasma-mass spectrometry (ICP-MS) in accordance with US EPA Method 6020; method detection limit (MDL) was 0.5 µg Ni/L.

Embryo-Larval Developmental Assay. FETAX, as described in ASTM E143 9-98 (ASTM 2004), was used as the model for the bioassays of Ni, using *X. laevis, B. terrestris*, and *G. carolinensis* as test species. Standard FETAX uses blastula stage *Xenopus* embryos raised from Nieuwkoop and Faber (NF) stage 8–10 to stage 46 (Nieuwkoop and Faber 1994), exposing whole embryos to the test material for approximately 4 d with daily renewals of the test solutions. At the end of the test period, the surviving larvae are preserved, scored for malformations using a dissecting scope, photographed and measured to determine growth inhibition, and archived for future reference. Two definitive assays testing 12–14 aqueous concentrations of Ni per test were performed with each test species. Each test concentration was set up in quadruplicate, with 20 organisms per replicate (80 organisms per concentration). The incubation temperature was 23° ± 1°C for *X. laevis* and 21° ± 1°C for *B. terrestris* and *G. carolinensis*, which were optimal for each species. Previous studies have indicated that a 2°C difference between tests only slightly alters developmental rates but does not confer change in organism sensitivity to toxicants (Fort *et al.* 2004). FETAX solution alone was used as the negative control. Two concentrations of 6-aminonicotinamide (6-AN) (2,500 mg/L and 5.5 mg/L) were concurrently tested (4 replicates of 20 organisms each) and served as positive controls for embryo-lethal and malformation-inducing effects in amphibian larvae (ASTM 2004). Assays were initiated at late blastula/early gastrula (NF stage 10 for *Xenopus* and the comparable Gosner stage 12 for *Bufo* and *Gastrophryne)* and terminated at NF stage 46 (4 d for *Xenopus)* or comparable Gosner stage 26 (7 d for *Bufo* and *Gastrophryne)*, which marked the completion of the major stages of organogenesis. All testing was performed in 500-ml polyethylene containers containing a total volume of 100 ml of test solution. Each

Table 1. General culture and source water physicochemical characteristics[a]

	Embrvo-Larval Culture Water		Adult Source Water			
			X. laevis	*B. terrestris*		*G. carolinensis*
Analytical Parameter (unit)	100% FETAX Solution	SREL Well Water	Dechlorinated Tap Water	Conference Center Wetlands	Risher Wetlands	Ginger's Bay
pH (su)	7.7 (0.1)	6.7 (0.3)	7.2 (0.2)	5.6 (0.3)	5.6 (0.1)	5.6 (0.1)
Dissolved Oxygen (mg/L)	7.6 (0.2)	10.2 (0.4)	8.7 (0.3)	9.8 (0.2)	7.2 (0.2)	6.5 (0.1)
Conductivity (µS/cm)	1,482.0 (5.8)	44.3 (1.7)	370.0 (8.5)	8.2 (0.5)	29.7 (1.1)	46.3 (1.3)
Hardness (mg/L)	100.0 (0.9)	6.0 (0.3)	78.0 (2.3)	2.0 (0.2)	14.0 (0.8)	4.0 (0.9)
Ammonia-Nitrogen (mg/L)	0.06 (—)	0.09 (0.01)	0.13 (0.02)	0.19 (0.03)	1.21 (0.05)	4.16 (0.07)
Chloride (mg/L)	371.0 (2.3)	2.2 (0.3)	61.2 (3.3)	2.1 (0.02)	5.0 (0.3)	4.9 (0.07)
Sulfate (mg/L)	110.0 (1.1)	1.5 (0.2)	75.4 (1.4)	2.3 (0.01)	1.3 (0.1)	1.9 (0.03)
Bicarbonate Alkalinity (mg/L)	57.4 (1.3)	27.6 (0.4)	25.1 (0.6)	<20.0 (—)	<20.0 (—)	<20.0 (—)
Carbonate Alkalinity (mg/L)	<20.0 (—)	<20.0 (—)	<20.0 (—)	<20.0 (—)	<20.0 (—)	<20.0 (—)
Orthophosphate-P (mg/L)	<0.1 (—)	<0.1 (—)	<0.1 (—)	<0.1 (—)	<0.1 (—)	<0.1 (—)
Organic Carbon (mg/L)	<1.0 (—)	<1.0 (—)	1.0 (0.03)	1.6 (0.08)	27.0 (1.2)	22.0 (1.2)
Calcium (mg/L)	17.9 (0.3)	0.6 (0.1)	30.3 (1.5)	0.4 (0.05)	3.3 (0.2)	1.1 (0.3)
Magnesium (mg/L)	13.6 (0.2)	0.2 (0.03)	3.5 (0.5)	0.3 (0.02)	0.6 (0.03)	0.3 (0.04)
Potassium (mg/L)	15.9 (0.5)	<0.5 (—)	3.8 (0.4)	<0.5 (—)	1.5 (0.02)	1.3 (0.06)
Sodium (mg/L)	240.0 (1.1)	11.9 (0.4)	37.0 (2.5)	1.2 (0.07)	2.9 (0.04)	2.3 (0.05)
Nickel (µg/L)	1.1 (0.03)	<0.5 (—)	1.7 (0.1)	0.5 (0.02)	1.0 (0.02)	2.7 (0.03)

[a] All results reported as dissolved (soluble) with standard error of the mean reported in parentheses; $N = 4$.
— Not determined.

test replicate was renewed daily by removing 75% of the test solution, along with any dead larvae, and replacing with fresh test solution. Mortality counts and developmental stages were recorded daily. At test termination, surviving specimens were anesthetized using 3-aminobenzoic acid ethyl ester (MS-222), preserved in 3% formalin, scored for malformations (Bantle *et al.* 1998), and archived for future reference.

Data Analysis. The percent mortality and malformation were determined for each Ni concentration tested. Head to tail length of the surviving larvae were measured as an index of growth (sample growth over control growth, expressed as a %) using a digital camera and SigmaScanPro® 5.0 image analysis software (SPSS, Inc., Chicago, IL). The primary endpoints were the lethal concentration at which 10% and 20% mortality occurred (LC10 and LC20) and the median lethal concentration (LC50), lowest observed adverse effect concentration (LOAEC), no observed adverse effect concentration (NOAEC), and chronic value (ChV) for survival; effect concentration at which 10% and 20% malformation occurred (EC10 and EC20) and the median teratogenic concentration (EC50), LOAEC, NOAEC, and ChV for malformation; and LOAEC, NOAEC, and ChV for growth. Initial range-finding tests, based broadly on the results of Birge (1978) and Birge and Black (1980) using an augmented log scale, were performed using *Xenopus* and *Bufo* species. The two definitive concentration-response tests were then performed, based on the range-finding results, using a focused series of 12–14 different measured concentrations of Ni. Significant differences of each treatment from the FETAX solution control (LOAEC and NOAEC values) were determined by ANOVA (Bonferroni *t*-test, $p < 0.001$ or Dunnett's Method, $p < 0.05$ [parametric data sets]) or Kruskal Wallis (KW)-ANOVA (Dunn's Method, $p < 0.05$ [non-parametric data sets]) using SigmaStat® 2.03 statistical software. The LC10 and EC10 (malformation), LC20 and EC20 (malformation), and the LC50 and EC50 (malformation) values were calculated using Probit 1.5 and Trimmed Spearman-Karber 1.5 (US EPA, Cincinnati, OH), respectively. Chronic values (ChVs) were calculated as the geometric mean of the LOAEC and NOAEC values.

Results

Analytical Testing

The results of the culture and site water characterization are presented in Table 1. All results are presented as dissolved (soluble) analytes. Ni concentrations in the egg collection surface water ranged from <0.5 µg Ni/L (SREL well water) to 2.7 µg Ni/L (Ginger's Bay). Measured Ni concentrations were based on 2 samples collected at test initiation and termination and averaged to express a single representative concentration. Of the 31 test concentrations analyzed, the difference between initial and final test concentrations was <5% for 26 of the treatments, with all but 1 occurring at low concentrations (<0.5 mg Ni/L).

Embryo-Larval Development Assays

Controls. No mortality or malformation was observed in *X. laevis* exposed to FETAX solution. Both parameters were within the acceptable limits (≤ 10.0%) for mortality and malformation as outlined in ASTM E1439-98. The 2,500 mg/L 6-AN reference solution induced 62.5% and 31.3% mortality, respectively, in Tests 1 and 2, and 100% malformation in both tests. The 5.5-mg/L 6-AN reference toxicant induced 20.0% and 0.0% mortality and 53.1% and 56.3% malformation in Tests 1 and 2, respectively.

In the *B. terrestris* assays, mean mortality and malformation frequencies of 3.2% and 0.0%, respectively, were observed in the FETAX solution controls. The incidence of both mortality and malformation observed in the FETAX solution control were within the acceptable limits (≤ 10.0%) as outlined in ASTM E1439-98. The 2,500-mg/L 6-AN positive control

Table 2. Effect of nickel on *Xenopus laevis*, *Bufo terrestris,* and *Gastrophryne carolinensis* embryo-larval mortality

		Mortality					
Test Species	Test No.	LC10 (CI) (mg/L)[a]	LC20 (CI) (mg/L)[a]	LC50 (CI) (mg/L)[b]	NOAEC (mg/L)[c]	LOAEC (mg/L)[d]	ChV (mg/L)[e]
X. laevis	1	4.63 (ND)	5.36 (5.29–5.42)	7.88 (7.72–8.05)	6.90	9.39	8.15
	2	4.79 (ND)	5.51 (5.25–5.76)	8.02 (7.95–8.08)	6.90	9.39	8.15
B. terrestris	1	0.88 (0.54–1.23)	0.97 (0.72–1.12)	2.91 (2.67–3.16)	0.90	2.33	1.62
	2	1.36 (1.00–1.68)	1.45 (1.28–1.62)	3.06 (2.88–3.25)	0.90	2.33	1.62
G. carolinensis	1	0.19 (0.00–0.49)	0.21 (0.11–0.31)	1.19 (1.03–1.38)	0.45	0.91	0.68
	2	0.18 (0.00–0.44)	0.20 (0.16–0.24)	1.11 (0.96–1.28)	0.45	0.91	0.68

[a] LC10 and LC20 (lethal concentrations at which 10% and 20% mortality occurs, respectively) for *X. laevis* were determined using linear interpolation. N = 1,040–1,200 organisms for each test reported.
[b] LC50 (lethal concentration at which 50% mortality occurs) was calculated using Trimmed Spearman-Karber. CI represents a 95% confidence interval.
[c] NOAEC (no observable adverse effect [mortality] concentration) was calculated using ANOVA and Bonferroni t-test ($p < 0.001$).
[d] LOAEC (lowest observable adverse effect [mortality] concentration) was calculated using ANOVA and Bonferroni t-test ($p < 0.001$).
[e] ChV (chronic value) was determined by the geometric mean of the LOAEC and NOAEC.

Table 3. Effect of nickel on *Xenopus laevis, Bufo terrestris*, and *Gastrophryne carolinensis* embryo-larval development

		Malformation						Growth		
Test Species	Test No.	EC10 (CI) (mg/L)[a]	EC20 (CI) (mg/L)[a]	EC50 (CI) (mg/L)[b]	NOAEC (mg/L)[c]	LOAEC (mg/L)[d]	ChV (mg/L)[e]	NOAEC (mg/L)[f]	LOAEC (mg/L)[g]	ChV (mg/L)[e]
X. laevis	1	0.23 (0.09–0.30)	0.24 (0.22–0.26)	0.39 (0.37–0.41)	0.09	0.25	0.17	0.09	0.25	0.17
	2	0.26 (0.14–0.32)	0.27 (0.26–0.28)	0.44 (0.42–0.46)	0.09	0.25	0.17	0.09	0.25	0.17
B. lerrestris	1	0.97 (0.61–1.29)	1.07 (0.88–1.36)	3.74 (3.14–4.45)	0.64	0.90	0.77	0.64	0.90	0.77
	2	1.43 (0.87–1.91)	1.60 (1.31–1.89)	4.12 (3.58–4.73)	0.64	0.90	0.77	0.64	0.90	0.77
G. carolinensis	1	0.22 (0.16–0.27)	0.24 (0.15–0.33)	0.63 (0.57–0.70)	0.09	0.23	0.16	0.45	0.91	0.68
	2	0.18 (0.14–0.23)	0.20 (0.14–0.26)	0.59 (0.53–0.66)	0.07	0.09	0.08	0.45	0.91	0.68

[a] EC10 and EC20 (effect concentrations at which 10% and 20% malformations occur, respectively) were calculated using Probit analysis. CI represents a 95% confidence interval.
[b] EC50 (effect concentration at which 50% malformations occur) was calculated using Trimmed Spearman-Karber. CI represents a 95% confidence interval.
[c] NOAEC (no observable adverse effect [malformation] concentration) was calculated using ANOVA and Dunnett's Method ($p < 0.05$).
[d] LOAEC (lowest observable adverse effect [malformation] concentration) was calculated using ANOVA and Dunnett's Method ($p < 0.05$).
[e] ChV (chronic value) was determined by the geometric mean of the LOAEC and NOAEC.
[f] NOAEC (no observable adverse effect [growth inhibition] concentration) was calculated using KW-ANOVA and Dunn's Method ($p < 0.05$) or ANOVA and Dunnett's Method ($p < 0.05$).
[g] LOAEC (lowest observable adverse effect [growth inhibition] concentration) was calculated using KW-ANOVA and Dunn's Method ($p < 0.05$) or ANOVA and Dunnett's Method ($p < 0.05$).

induced 100% mortality in both tests. The 5.5-mg/L 6-AN positive control induce a mean mortality and malformation frequencies of 5.7% and 33.2%, respectively.

Mean frequencies of mortality and malformation of 7.5% and 0.0%, respectively, were observed in *G. carolinensis* exposed to FETAX solution. The frequency of both mortality and malformation observed in the FETAX solution control were within the acceptable limits ($\leq$ 10.0%) as outlined in ASTM E1439-98. The 2,500-mg/L 6-AN solution induced 100% mortality in both assays. The 5.5-mg/L 6-AN solution induced a mean mortality frequency of 21.9% and an average incidence of malformation of 59.2%.

Nickel. Endpoint data for tests with *X. laevis, B. terrestris*, and *G. carolinensis* are presented in Tables 2 and 3. The concentration-response relationships for mortality, malformation, and growth are illustrated in Figures 1a–c, respectively.

Lethality. X. laevis. The mean LC10, LC20, and LC50 values were 4.71 mg Ni/L, 5.44 mg Ni/L, and 7.95 mg Ni/L, respectively (Table 2). The mean NOAEC and LOAEC values for mortality were 6.90 mg Ni/L and 9.39 mg Ni/L, respectively resulting in a ChV (mortality) of 8.15 mg/L. *B. terrestris.* The mean LC10, LC20, and LC50 values were determined to be 1.12 mg Ni/L, 1.21 mg Ni/L, and 2.99 mg Ni/L, respectively (Table 2). The mean NOAEC, LOAEC, and ChV values for mortality were 0.90 mg Ni/L, 2.33 mg Ni/L, and 1.62 mg Ni/L, respectively. *G. carolinensis.* The mean LC10, LC20, and LC50 values were 0.19 mg Ni/L, 0.21 mg Ni/L, and 1.15 mg Ni/L, respectively (Table 2). The mean NOAEC, LOAEC, and ChV values for mortality were 0.45 mg Ni/L, 0.91 mg Ni/L, and 0.68 mg Ni/L, respectively.

Malformation and Growth. X. laevis. The mean EC10, EC20, and EC50 (malformation) values were 0.25 mg Ni/L, 0.26 mg Ni/L, and 0.42 mg Ni/L, respectively (Table 3). The

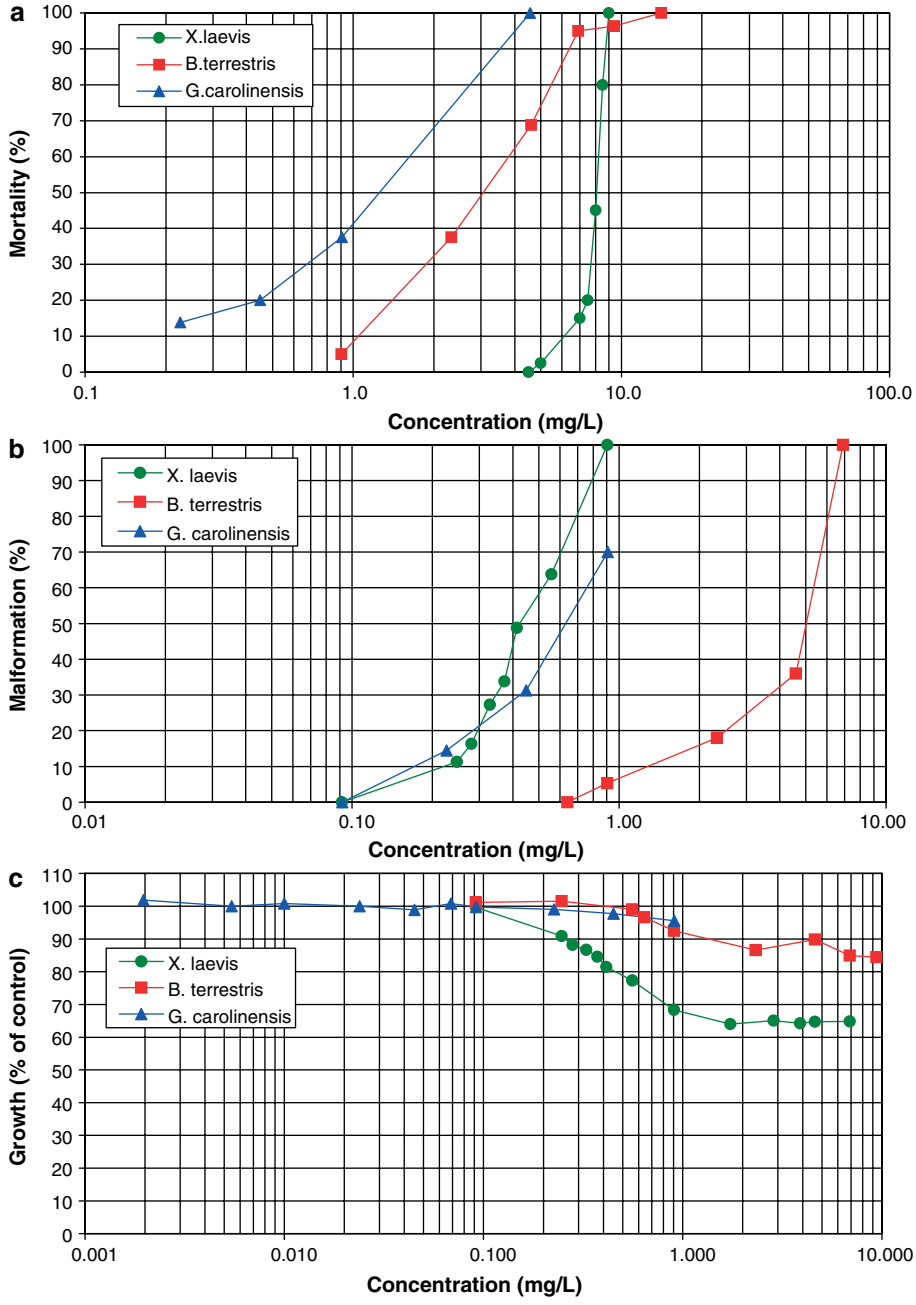

Fig. 1. **(a)** Embryo lethal effect of Ni on *X. laevis, B. terrestris*, and *G. carolinensis.* **(b)** Effect of Ni on the induction of abnormal development in *X. laevis, B. terrestris*, and *G. carolinensis.* **(c)** Effect of Ni exposure on larval growth in *X. laevis, B. terrestris*, and *G. carolinensis*

mean NOAEC, LOAEC, and ChV values for malformation were 0.09 mg Ni/L, 0.25 mg Ni/L, and 0.17 mg Ni/L, respectively. The major types of malformations induced in both Tests 1 and 2 were head and facial dysmorphogenesis, abnormal mouth development, rupture of the pigmented retina, miscoiling of the gut, visceral hemorrhaging, and abnormal myotome and notochord development. Edema, hemorrhage, and microcephaly were noted at concentrations ≥1.74 mg Ni/L. Photographs of representative malformations induced in *X. laevis* are presented in Figure 2A. Mean NOAEC, LOAEC, and ChV values of 0.09 mg Ni/L, 0.25 mg Ni/L, and 0.17 mg Ni/L for larval growth, respectively, were determined (Fig. 1c). *B. terrestris.* The mean EC10 (malformation) value was 1.20 mg Ni/L, respectively (Table 3). The mean EC20 (malformation) value was 1.33 mg Ni/L, respectively. The mean EC50 (malformation) value was 3.94 mg Ni/L. Mean NOAEC, LOAEC, and ChV values for malformation were 0.64 mg Ni/L, 0.90 mg Ni/L, and 0.77 mg Ni/L, respectively. The major types of malformations induced in both tests were visceral edema, head and facial dysmorphogenesis, abnormal mouth development, and rupture of the pigmented retina. Abnormal myotome development and microcephaly were observed at concentrations ≥6.9 mg Ni/L. Photographs of representative malformations found in *B. terrestris* are presented in Figure 2B. Mean NOAEC, LOAEC, and ChV values for growth were 0.64 mg Ni/L, 0.90 mg Ni/L, and 0.77 mg Ni/L, respectively (Fig. 1c). *G. carolinensis.* The mean EC10 (malformation) value was 0.20 mg Ni/L. The average EC20 (malformation) value was determined to be 0.22 mg Ni/L, respectively (Table 3). The mean EC50 (malformation) value

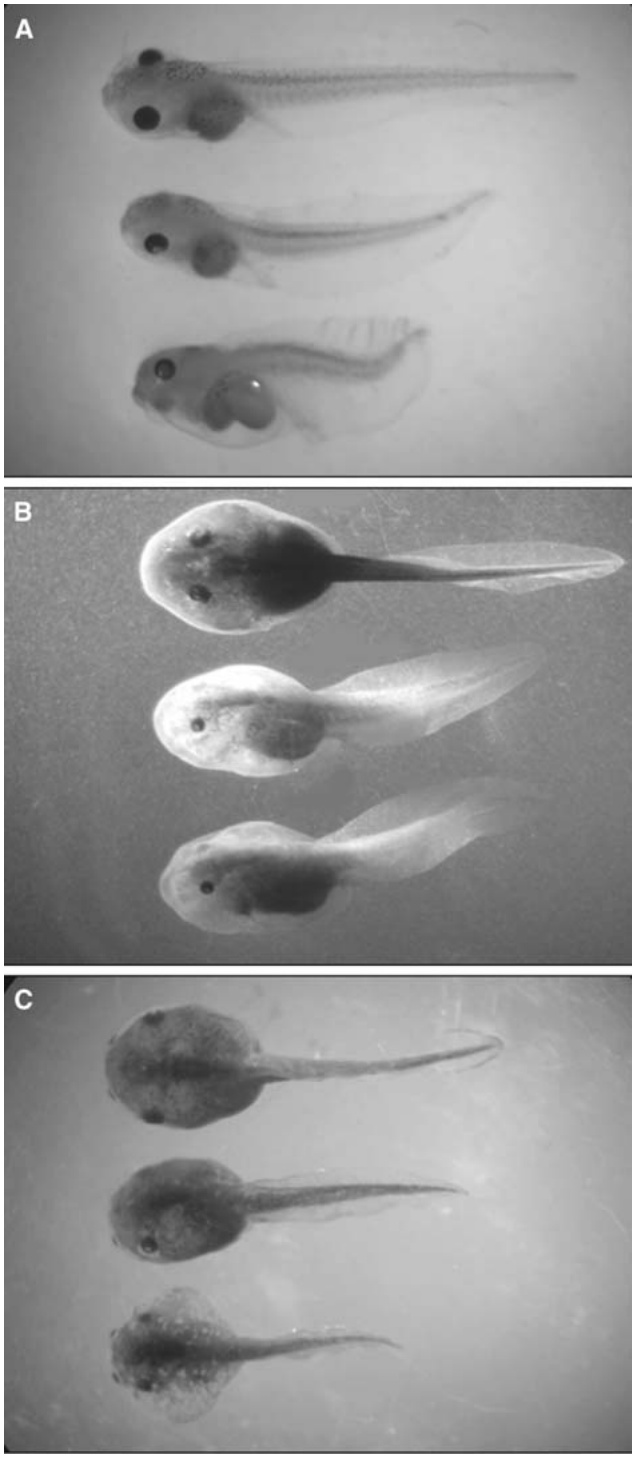

Fig. 2. **(A)** Effects of Ni on developing *X. laevis.* **Top**: 0.0 mg/L Ni (FETAX solution control). **Middle:** 0.37 mg/L Ni. Major types of malformation induced were head and facial dysmorphogenesis, abnormal mouth development, gut miscoiling, and abnormal myotome development. **Bottom:** 6.9 mg/L Ni. Major types of malformation induced were head and facial dysmorphogenesis, abnormal mouth development, gut miscoiling, abnormal myotome and notochrod development visceral hemorrhage, edema, and ruptured pigmented retina. **(B)** Effects of Ni on developing *B. terrestris.* **Top:** 0.0 mg/L Ni (FETAX solution control). **Middle:** 4.6 mg/L Ni. The major type of malformation induced was head and facial dysmorphogenesis. **Bottom:** 14.1 mg/L Ni. Major types of malformation induced were head and facial dysmorphogenesis, abnormal mouth development, abnormal myotome development visceral edema, ruptured pigmented retina, and microcephaly. **(C)** Effects of Ni on developing *G. carolinensis.* **Top:** 0.0 mg/L Ni (FETAX solution control). **Middle:** 0.45 mg/L Ni. Major types of malformation induced were head and facial dysmorphogenesis and abnormal mouth development. **Bottom:** 0.91 mg/L Ni. Major types of malformation induced were head and facial dysmorphogenesis, abnormal mouth development, visceral edema, ruptured pigmented retina, and microcephaly

was 0.61 mg/L Ni. The mean NOAEC, LOAEC, and ChV values for malformation were 0.08, 0.16, and 0.12 mg Ni/L, respectively. The major types of malformations induced in both tests were edema, head and facial dysmorphogenesis, and abnormal mouth development. Also, rupture of the pigmented retina and microcephaly were induced at Ni concentrations ≥0.45 mg Ni/L. Representative malformations observed in *G. carolinensis* exposed to Ni are presented in Figure 2C. Mean NOAEC, LOAEC, and ChV values for growth were 0.45, 0.91, and 0.68 mg Ni/L, respectively (Fig. 1c).

Discussion and Conclusions

Results from the present early embryo-larval developmental toxicity evaluation of Ni indicated that, in terms of embryo-lethality, *G. carolinensis* was the most sensitive and *X. laevis* was the least sensitive of the species tested. The 4-d LC50 in *X. laevis* value was approximately 7.2- and 2.8-fold greater than the *G. carolinensis* and *B. terrestris*, respectively. In terms of malformation, *X. laevis* was the most sensitive and *B. terrestris* was the least sensitive of the species tested. The 7-d EC50 (malformation) in *B. terrestris* was 10.6- and 7.0-fold greater than *X. laevis* or G. *carolinensis*, respectively. It should be noted that many of the test specimens that died in the tests with *B. terrestris* were malformed. However, since the standardized test only considers organisms living at the conclusion of the test, these malformed dead organisms were not reflected in the *B. terrestris* malformation data sets. *X. laevis* was more sensitive than the other species, which were nearly equisensitive. The ChV for growth value in *X. laevis* was nearly 4.5-fold less than the ChV for growth value found for *B. terrestris.* Overall, the results from each test conducted per species were consistent and reproducible. Further, the concentration-response curves and the types of malformations observed were consistent between tests for each species studied.

In terms of endpoint sensitivity, malformation and growth were consistently the most sensitive endpoints. In *X. laevis* and *B. terrestris*, the malformation and growth endpoints were equisensitive. In *G. carolinensis*, the malformation endpoint was the most sensitive, with the mortality and growth endpoints demonstrating equal sensitivity. Variation in species sensitivity and endpoint sensitivity between species is not uncommon and has been reported in historical toxicological studies in amphibians with Ni (Linder and Grillitsch 2000).

Of the species evaluated in the present study, only *G. carolinensis* was evaluated in both the present study and the

study performed by Birge and Black (1980) with Ni. The other amphibian species evaluated by Birge and Black (1980) included *B. fowleri* and *Ambystoma opacum.* These investigators reported LC10 and LC50 values of 0.41 mg Ni/L and 11.03 mg Ni/L, and 0.06 mg Ni/L and 0.41 mg Ni/L in *B. fowleri*, and *A. opacum*, respectively. Birge and Black (1980) reported LC10 and LC50 values of 0.004 mg Ni/L and 0.05 mg Ni/L in G. *carolinensis*, respectively. In the present study, the most conservative LC10 and LC50 values for *G. carolinensis* were 0.18 mg Ni/L and 1.11 mg Ni/L, respectively, which are orders of magnitude greater than values reported by Birge and Black (1980). The most sensitive endpoint measured in the present study with *G. carolinensis* was the NOAEC value for malformation (0.07 mg Ni/L). This NOAEC value was also greater than the LC50 value reported by Birge and Black (1980).

Several possible differences in experimental factors could be attributed to the differences in responses observed with *G. carolinensis* between Birge and Black (1980) and the present study. These factors include genetic variability, differences in test conditions, physicochemistry of the test media, test duration, and analysis of the data (Burkhart *et al.* 2003). Genetic variability in the organisms used in the various studies may have contributed to differences. The source of the field-collected indigenous organisms used in toxicological studies of this type could potentially alter the results obtained. The source waters, from which the adult *G. carolinensis* were captured, contained trace levels of Ni (2.7 μg Ni/L) and were extremely soft (4.0 mg/L as $CaCO_3$). The water in which these adults were bred contained <0.5 μg Ni/L and was also soft (6.0 mg/L as $CaCO_3$). Thus, since these physicochemical water quality characteristics are not unique to regions in which *G. carolinensis* reside, the source water most likely did not contribute directly to the differences in sensitivity in the form of adaptation to Ni. Herkovits *et al.* (2000) reported a 7-d LC50 value in *B. arenarum* of 1.80 mg Ni/L. Khangarot and Ray (1987) reported a 4-d static LC50 value in *B. melanostictns* of 25.32 mg Ni/L. We reported LC10 and LC50 values of 0.88 mg Ni/L and 3.06 mg Ni/L for *B. terrestris*, which is in a similar order as the results in *B. fowleri* found by Birge and Black (1980) and Herkovits *et al.* (2000), considering species differences (same genus).

Differing test conditions could have a significant impact on differences in results between different studies. The general laboratory conditions used in the present study were nearly identical to those used for FETAX, with the primary exception of the use of greater test volumes for each species (100 ml) to accommodate testing with the toad species. Many details on experimental conditions used by Birge and Black (1980) were not presented. Although it is difficult to infer the specific test conditions used by Birge and Black (1980), the physical conditions appear to be similar to those in the present study. In the present study, the Ni concentrations in test solutions were specifically quantified, and, therefore, provided more confidence in estimates of toxicity compared to the use of nominal values. A MDL of 0.5 μg Ni/L was sufficient to quantify Ni concentrations in all test exposures. The FETAX solution media used as a diluent of the Ni stock solution and in culturing the organisms in the present study was reasonably similar to the culture media used by Birge and Black (1980) in terms of pH [7.7 (range 7.3–7.9) in FETAX solution vs. 7.4 reported in Birge and Black (1980)] and hardness [100 mg/L (as $CaCO_3$) in FETAX solution vs. 95–103 mg/L (as $CaCO_3$) reported in Birge and Black (1980)]. The media hardness in the Khangarot and Ray (1987) study was 185 mg/L (as $CaCO_3$), which was greater than that reported for the Birge and Black (1980) study (95–103 mg/L) and may have resulted in the greater LC50 values obtained. Thus, it is unlikely that the basic physicochemical characteristics of the culture/dilution water significantly contributed to the difference in results between the two studies. However, more specific differences in the water chemistry of exposure media could explain the differences between the present study and Birge and Black (1980). For example, recent studies on fish and aquatic invertebrates indicate that Ni toxicity decreases with increasing hardness and dissolved organic carbon (DOC) concentrations (Hoang *et al.* 2004; Keithly *et al.* 2004). Additionally, chronic Ni toxicity to *Oncorhynchus mykiss, Daphnia magna*, and *Pseudokirchneriella subcapitata* generally increases with increasing pH. Since the pH and hardness of the culture media used in both studies were similar, and nondetectable levels of organic carbon were present, it would appear that differences in the factors that influence Ni toxicity did not appreciably exist between the present studies and the work of Birge and Black (1980).

Exposure duration is another possible source of variation. The duration of the tests used in the present study was based on the attainment of a specific embryological stage (Gosner stage 26), which is consistent with the standardized FETAX model. Thus, differences in species sensitivity could be partially attributed to differences in the actual duration of exposure. However, test termination at a consistent embryo/larval stage for each species evaluated facilitates a direct means of comparison in response at a developmental level. Birge and Black (1980) exposed specimens for 8 d, which may have increased sensitivity somewhat since the effects of Ni appear to be manifested in larval development (post-Gosner stage 20) rather than embryo development (pre-Gosner stage 14).

In terms of *X. laevis*, several investigators have evaluated the toxicity of Ni. Linder *et al.* (1991) reported a 4-d LC50 value of 1.80 mg Ni/L using a 4-d FETAX protocol, whereas Hopfer *et al.* (1991) reported a 4-d LC50 value of 21.40 mg Ni/L using a similar protocol. The only apparent difference in the studies is the pH of the test media, 6.0 for the Linder *et al.* (1991) study and 6.8 for the Hopfer *et al*. (1991) study. In the present study, the 4-d LC50 value for *X. laevis* was approximately 8.0 mg Ni/L. The malformation syndromes induced by Ni in *X. laevis* in both studies (Linder *et al*. 1991; Hopfer *et al.* 1991) were similar to those obtained in the present study.

The advent and use of standardized methodologies for evaluating toxicity in early life stage amphibians continues to provide the scientific and regulatory communities with information needed for sound decision-making. Overall, the present study provides reliable and relevant data regarding the developmental toxicity of Ni to amphibian species, particularly those that have been historically reported to be highly sensitive. Our findings were generally more similar to those of Linder *et al.* (1991), Herkovits *et al.* (2000), and Khangarot and Ray (1987), but did not support the conclusions of Birge and Black (1980). The data generated should be useful while new aquatic life criteria for Ni are being considered.

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