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# Effects of Pond Water, Sediment and Sediment Extract Samples From New Hampshire, USA on Early *Xenopus* Development and Metamorphosis: Comparison to Native Species‡

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**In an effort to assess potential ecological hazards to amphibian species in selected regions within New Hampshire, the traditional Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX), a 14-/21 day tail resorption thyroid disruption assay and >30 day limb development tests were conducted with representative surface water and sediment samples. Two separate sets of samples collected from five sites were evaluated. The primary objectives of the study were to determine if samples were capable of inducing early embryo-larval maldevelopment, to determine if maldevelopment included limb defects, to determine if thyroxine co-administration altered the rates of limb malformation and to evaluate the impact of the samples on growth rates, developmental progress and metamorphic climax. Results from these studies suggested that pond water and sediment extract samples, but not whole sediment samples, from B2, FW, LP and W ponds were capable of inducing abnormal early embryo-larval development. In addition, water samples from B2 and W ponds induced significant abnormal hindlimb development. Some abnormal forelimb development was noted in the tail resorption studies, but not to the same extent as the hindlimbs. Each of the water samples induced appreciable developmental delay, including the paired reference site B1, which could be reversed by the addition of exogenous thyroxine.** 

## INTRODUCTION

The increase in the frequency of amphibian malformations reported over the last several years, combined with the more widespread occurrence of ectromelia, ectrodactyly, missing or misplaced eyes and internal abnormalities, now appears to be unique in recent environmental history.[1,2] Several factors with the potential to produce frog deformities have been proposed. Various groups have supported theories based on changes in predation, endoparasite infestation and disease, ultraviolet (UV) radiation, mineral depletion (e.g. calcium, magnesium) and natural or anthropogenic chemicals to cause malformation under certain conditions. These hypotheses have been reviewed recently by Burkhart *et al.*[3] The least understood of these potential causes for malformation is the role of natural or anthropogenic chemicals. Earlier studies[4] indicated that pond water and sediment samples collected from various sites in Minnesota were capable of inducing early embryo-larval maldevelopment, abnormal limb development and disruption of metamorphosis in *Xenopus laevis*. These results supported the hypothesis that the effects induced were due to exposure to either the pond water or sediment and appeared to be the result of either the presence of developmental toxicants or the absence of essential micronutrients. Previous studies[4–6] have ruled out the latter as a direct cause of the malformations induced in *X. laevis* in the laboratory. However, the role of ionic imbalances and nutritional deficiencies cannot be overlooked as potentially contributing to malformation in either the laboratory or

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field studies. Extensive focus in this work has been placed on identifying chemicals directly from field samples that may be responsible for the deformities induced in *X. laevis* in the laboratory.

Results from these studies suggested that a mixture of chemicals, including naturally occurring compounds, pesticides and anthropogenic organic compounds, were primarily responsible for the effects observed in *X. laevis*. Although these studies do not rule out the role of the previously mentioned factors such as parasites and UV radiation, they support the hypothesis that both naturally occurring and man-made chemicals may contribute to the incidence of malformed frogs found in the field in certain locales.

Increasing demands for routine developmental toxicity screening for ecological hazard assessment necessitates the evaluation and use of a battery of validated bioassays.[7–9] Alternative developmental toxicity tests, such as the Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX), provide a rapid, cost-effective method of evaluating developmental toxicants in the environment and a method of determining potential ecological hazards to amphibian populations. FETAX is a 96-h, static-renewal bioassay utilizing embryos of the South African clawed frog, *Xenopus laevis*, capable of detecting developmental toxicants in the environment.[10] FETAX has been used previously to evaluate the potential hazard of contaminated surface water,[5,11] sediments[5,12,13] and industrial well waters.[14]

In an effort to assess potential ecological hazards to amphibian species in selected regions within New England, FETAX,[15] a 14-/21 day tail resorption thyroid disruption assay[16,17] assay and >30 day limb development tests[18] were conducted with representative surface water and sediment samples. Two separate sets of samples collected from five sites were evaluated. The primary objectives of the study were to determine if samples were capable of inducing early embryo-larval mal-development; to determine if maldevelopment included limb defects, to determine if thyroxine co-administration altered the rates of limb malformation and to evaluate the impact of the samples on growth rates, developmental progress and metamorphic climax.

## MATERIALS

Two sets of five water and sediment samples from five separate sites were collected and shipped overnight via commercial carrier to the laboratory at 4°C. Four sites labeled B2, FW, W and LP were designated test sites in the present study. The fifth site labeled B1 was designated as a reference site. These designations were based on previous field observations in which abnormal indigenous frog species were identified in the test sites, but not at the reference site. Each of the sites contained comparable and adequate amphibian habitat and site characteristics. All samples were stored at 4°C upon receipt and throughout the holding period. Samples were thoroughly mixed before testing. The identity of the test sites was excluded from this report to protect the integrity of the sites and the research.

## METHODS

### Adult *Xenopus* care and breeding

*Xenopus* adult care, breeding and embryo collection were performed as described in American Society for Testing and Materials (ASTM) E1439–98[15] for conducting FETAX.

### 4 day development toxicity studies

**Aqueous samples with FETAX tests.** FETAX testing was conducted as described in ASTM E1439–98.[15] Groups of 20 normally developing blastula-stage embryos were placed in 60 mm covered plastic Petri dishes containing various control or treatment solutions. For each test, selected concentrations of each sample were tested in duplicate. Four separate dishes of 20 embryos were exposed to FETAX solution alone and designated FETAX solution controls. The FETAX solution consisted of 625 mg of NaCl, 96 mg of $NaHCO_3$, 75 mg of $MgSO_4$, 60 mg of $CaSO_4$. $H_2O$, 30 mg of KCl and 15 mg of $CaCl_2$ per liter of solution.[1] In addition, four dishes of 20 embryos were exposed to 6-aminonicotinamide, two sets at 5.5 mg $l^{-1}$ (ca. 4 day $EC_{50}$, malformation) and two sets at 2500 mg $l^{-1}$ (ca. 4 day d $LC_{50}$) as specified in ASTM E1439–98.[15] These treatments served as positive controls. Each treatment contained 8 ml of solution. Embryos were cultured at 23.0 ± 1.0°C. Samples for paired reference sites were also tested concurrently. All solutions were changed every 24 h during the 4 day test, dead embryos were removed and fresh solutions were added. Following 4 days of exposure (stage 46 embryos), embryos were fixed in 3% formalin (pH 7.0) and the number of malformed embryos that were living prior to fixation was determined using a dissecting microscope.[19] Two forms of test were performed. Screening tests consisted of experiments with undiluted samples and the appropriate controls indicated above. Definitive tests represented multi-concentration experiments in which six concentrations of the samples were examined in addition to controls. Screening tests determined the rates of mortality and malformation in undiluted samples, whereas definitive tests sought to determine specific point estimates to predict the magnitude of developmental toxicity.

**FETAX tests with aqueous sediment extracts.** Aqueous sediment extracts were prepared as described previously.[5,12] A weight of FETAX solution at 4:1 (v/w) was mixed together with each separate sediment sample in a 1-l plastic cubitainer. Specifically, 200 g of sediment (weight) was mixed with 800 g of FETAX solution in such a way as to minimize headspace and reduce volatilization. Aqueous soil mixtures then were tumbled in a rotary extractor for 48 h at 30 ± 2 rpm and 22 ± 2°C in the dark. The tumbled samples were allowed to settle overnight at 4°C. The samples then were centrifuged for ca. 20 min at 8000 rpm until the supernatant was completely clear. The extract was decanted and the pH and dissolved oxygen were measured. None of the samples deviated from the acceptable pH range of 6.5–9.0. Extracts were stored in a vented refrigerator at <5°C until use. Prior to testing, dis-

solved oxygen, pH, conductivity, hardness, alkalinity and ammonia-nitrogen were measured in each extract. Dissolved oxygen and pH also were measured prior to and after test renewal (waste). One aqueous sediment extract was prepared and tested for each site.

**FETAX tests with whole bulk sediment samples.** FETAX studies were performed in accordance with the methods cited in ASTM E1439–98,[15] with the following modifications for whole soil testing.[13] Testing was performed in 9-oz. specimen bottles equipped with a glass tube/100 μm Teflonmesh insert as the exposure chamber. For the screening tests, 35 g of sediment (wet weight) was placed in the bottom of the specimen jar and the exposure insert was added and filled with 140 ml of FETAX solution (dilution water). This represented a 1:4 dilution of sediment to dilution water. Blastula-stage embryos were placed on the Teflonmesh insert that rested over the top of the sediment in the sediment/water interface region. The test consisted of 20 embryos exposed to FETAX solution (standard negative control) alone placed in each of four replicates, 20 embryos exposed to either 5.5 or 2500 mg $l^{-1}$ 6-aminonicotinamide (6-AN, standard positive control) in each of two replicates in 60-mm Petri dishes, 20 embryos exposed to blasting sand (artificial sediment) in each of three replicates, 20 embryos exposed to paired reference site sediments in each of three replicates and 20 embryos exposed to each sediment sample in each of three replicates. Fresh solutions and sediment were provided every 24 h of the 4 day test. Dissolved oxygen and pH were measured prior to renewal and in the waste solutions from each successive day.

### 30 day limb development and 14-/21 day tail resorption studies

For the limb development studies, twenty blastulae were placed in 5-l test vessels containing varying concentrations of the samples. Dilutions were prepared in FETAX solution. Two separate aquaria containing 20 embryos each were exposed to FETAX solution alone. Treatment and control dishes contained a total of 4 l of solution. The pH of the test solutions was maintained at 7.8–8.0 mg $l^{-1}$. Organisms were cultured at 24 ± 0.5°C. Two separate experiments were performed with each of two pond water samples. Hindlimb development experiments were conducted for ca. 30 days from stage 8 through to stage.[16,18] In the second study, thyroxine was co-administered at a concentration of 1 μg $l^{-1}$ to determine if supplementation reduced the incidence of limb maldevelopment.

For the tail resorption studies, embryos were cultured in FETAX solution until stage 60 (ca. day 50), at which time exposure to the various samples was initiated. Larvae were fed Salmon Starterfish food for live bearers (Tetra Werke, Melle, Germany) starting at day 4 at a rate of ca. 250 mg of larvae per day. Dissolved oxygen and pH were monitored daily. Tail resorption exposure studies were conducted in general accordance with the methods of Fort and Stover[16] and Fort *et al.*[17] The rate of tail resorption was monitored for ca. 14–21 days, from stage 60 through to 66.

All solutions were renewed every 3 days during the test period, and dead embryos and larvae were removed and numbers recorded. Developmental stage was determined during the renewal process. Tests were terminated once the larvae reached stage 54 and stage 66 for the evaluation of hindlimb development and tail resorption assessment, respectively. At the completion of exposure, larvae were fixed in 3% (w/v) formalin (pH 7.0) and the gross effects on limb development and tail resorption were noted. To determine if effects induced on the rate of tail resorption were reversible by introducing thyroxine, additional studies were conducted in which 1 μg $l^{-1}$ of $T_4$ was co-cultured with the site waters tested and the rate of tail resorption was monitored.

### Data analysis

Mortality and malformation rates were determined for each test. Head–tail length of the surviving larvae was measured as an index of growth using an IBM-compatible computer and Sigma Scan (SPSS, Corte Madera, CA) digitizing software. For definitive tests, 4 day $LC_{50}$ and $EC_{50}$ (malformation) values were determined using trimmed Spearman–Karber analysis.[20] Significant impact on embryo-larval mortality, malformation and growth was determined using ANOVA (Bonferroni *t*-test, $P < 0.05$).

For limb development studies, the type and rate of hindlimb malformations among the control and exposed larvae were determined. Statistical comparisons of limb malformation rates of the control larvae, reference site larvae, and test site larvae were performed using ANOVA (Bonferroni *t*-test, $P > 0.05$).

Video images were captured using a Sony CCD-iris high-resolution color digital video camera. A Pentium 233-MHz computer with image processing software and a FlashPoint 128 (Integral Technologies, Inc., Indianapolis, IN) video frame grabber were used to digitize the tail length at developmental stages 63–66. A ruler videotaped with the larvae was used to monitor image distortion and calibrate the length measuring program to ensure accurate measurements of the larvae. Tail lengths were measured using Sigma Scan (SPSS, Corte Madera, CA). Statistical comparisons of the control and exposure treatments were performed using ANOVA (Dunnett's test, $P < 0.05$).

## RESULTS

### FETAX controls

FETAX solution, blasting sand controls, mortality and malformation met the criteria established in ASTM E1439–98[15] for the test acceptance (Table 1). In addition, mortality and malformation rates for 5.5 and 2500 mg $l^{-1}$ 6-AN positive control treatments were within the limits expressed in ASTM E1439–98[15] (Table 1).

### FETAX tests

The results of FETAX tests with surface water, whole sediments and sediment extracts are presented in Table 2. None of the samples evaluated during the

**Table 1. Negative and positive FETAX control results**

| Control | % Mortality (*n*) | % Malformation (*n*) |
|---|---|---|
| Negative controls | | |
| FETAX solution (aqueous matrix) | 8.5 (400) | 9.9 (366) |
| Sand (solid matrix) | 7.9 (120) | 6.6 (111) |
| Positive controls | | |
| 5.5 mg $l^{-1}$ 6-Aminonicotinamide | 42.0 (200) | 82.0 (116) |
| 2500 mg $l^{-1}$ 6-Aminonicotinamide | 96.0 (200) | 100.0 (8) |

present study were substantially embryo-lethal. Sediment extracts of ponds B2, LP, FW and W from sample set 1 each induced elevated rates of abnormal development. Sediment extracts from B1 pond, however, did not induce developmental toxicity in the FETAX model. Interestingly, tests of whole sediment in the second sample set found each of the samples to be reasonably less toxic in terms of embryo-lethal and malforming potential than water samples of sediment extracts, with the exception of the B2 samples. Similar to the sediment extract tests, tests of water samples from set 2 produced similar low rates of mortality. However, definitive tests with water samples from each site determined the relative order of developmentally toxic potency to be: B2 $\geqslant$ W $>$ FW $>$ LP $>$ B1. Characteristic malformation induced in the affected sites included eye defects (ruptured pigmented retinas and misshaped lens), craniofacial defects and lower jaw maldevelopment, visceral hemorrhage and microencephaly. More specifically, the primary defects induced by B2 samples were mouth (100%), eye (72.4%), brain (27.6%), craniofacial (27.6%) and hemorrhage (37.9%). The primary defects induced by pond W included eye (100%), mouth (100%), brain (57.1%), face (57.1%) and hemorrhage (25%). The LP pond samples induced eye malformations (95.5%), mouth (86.4%), hemorrhage (63.6%), craniofacial and brain (93.3% for both) and gut deformities (22.7%). The FW samples caused eye (100%), mouth (100%), craniofacial and brain (93.3% for both) and visceral hemorrhage (16.7%). Further histopathological examination will be required to characterize more specifically these abnormalities. No characteristic malformations were found in the B1 reference pond samples. Only marginal effects on early embryo-larval growth were detected in samples from ponds B2, FW and W. Characteristic embryo-larval malformations are illustrated in Fig. 1.

## Long-term studies

**Limb development.** The impact of long-term exposure of *X. laevis* to both sets of water samples on growth stage attainment is presented in Table 3. Interestingly, each sample, including the paired reference pond B1, appreciably delayed development as measured by developmental stage. This trend was consistent in both sample sets.

**Table 2. Effects of selected samples on early embryo-larval development using FETAX**

| Site | Sample type/test format | Test no. | Mortality (%) | Malformed (%) | Growth (% of control) | $LC_{50}$ (%) | $EC_{50}$ (%) | MCIG (%) |
|---|---|---|---|---|---|---|---|---|
| B1 | Sediment extract/screen | 1 | 7.5 | 8.1 | 96.6 | NA | NA | NA |
| B2 | Sediment extract/screen | 1 | 27.5 | 100.0[a] | 90.7 | NA | NA | NA |
| LP | Sediment extract/screen | 1 | 12.5 | 62.9[a] | 93.5 | NA | NA | NA |
| FW | Sediment extract/screen | 1 | 22.5 | 96.8[a] | 93.9 | NA | NA | NA |
| W | Sediment extract/screen | 1 | 10.0 | 77.8[a] | 95.6 | NA | NA | NA |
| B1 | Sediment/screen | 1 | 17.5 | 36.4 | 96.8 | NA | NA | NA |
| | | 2 | 5.0 | 28.9 | 94.7 | NA | NA | NA |
| B2 | Sediment extract/screen | 1 | 20.0 | 100.0[a] | 87.4 | NA | NA | NA |
| LP | Sediment extract/screen | 1 | 27.5 | 34.5 | 101.0 | NA | NA | NA |
| | | 2 | 20.0 | 37.5 | 96.2 | NA | NA | NA |
| FW | Sediment extract/screen | 1 | 22.5 | 38.7 | 100.5 | NA | NA | NA |
| | | 2 | 27.5 | 41.4 | 96.8 | NA | NA | NA |
| W | Sediment extract/screen | 1 | 10.0 | 38.9 | 99.3 | NA | NA | NA |
| | | 2 | 2.5 | 35.9 | 95.3 | NA | NA | NA |
| B1 | Water/screen | 1 | 5.0 | 13.2 | 99.5 | NA | NA | NA |
| | | 2 | 7.5 | 21.6 | 98.6 | NA | NA | NA |
| B2 | Water/definitive | 1 | 22.5 | 100.0[a] | 84.1 | $>$100.0 | 79.5 | 100.0 |
| | | 2 | 12.5 | 100.0[a] | 80.6[b] | $>$100.0 | 73.2 | 90.0 |
| LP | Water/definitive | 1 | 12.5 | 40.0[a] | 101.0 | $>$100.0 | $>$100.0 | $>$100.0 |
| | | 2 | 12.5 | 65.7[a] | 93.1[b] | $>$100.0 | 81.7 | 100.0 |
| FW | Water/definitive | 1 | 17.5 | 54.5[a] | 98.7 | $>$100.0 | 98.2 | $>$100.0 |
| | | 2 | 15.0 | 97.1[a] | 95.2 | $>$100.0 | 59.6 | 80.0 |
| W | Water/definitive | 1 | 22.5 | 100.0[a] | 80.0[b] | $>$100.0 | 86.7 | 100.0 |
| | | 2 | 12.5 | 100.0[a] | 73.0[b] | $>$100.0 | 82.2 | 90.0 |

[a]Significantly greater than B1 (reference) site (Bonferroni *t*-test, $P < 0.05$).
[b]Significantly less than B1 (reference) site (Bonferroni *t*-test, $P > 0.05$).

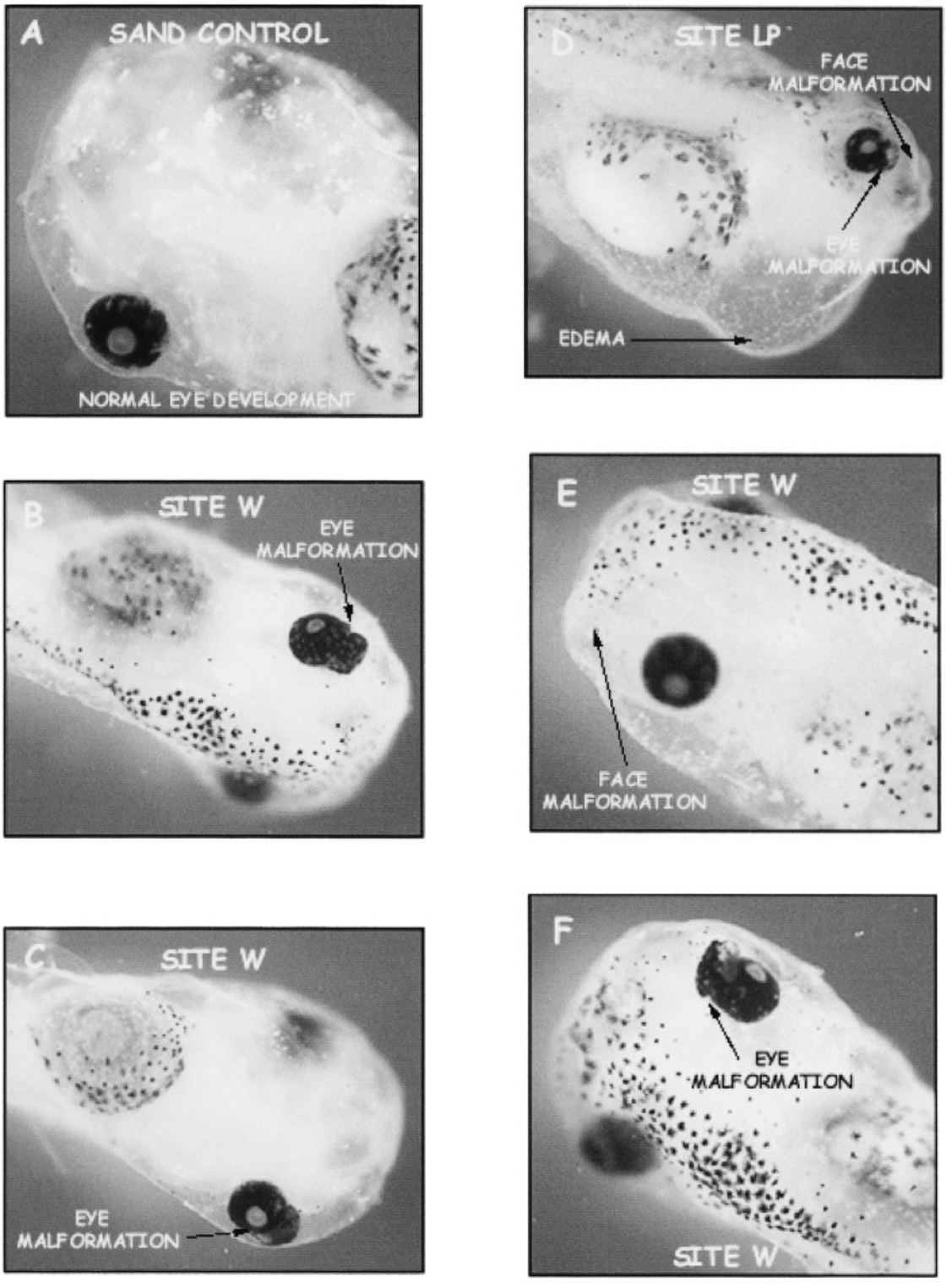


**Figure 1.** Representative characteristic malformations induced in 4 day larvae by site W and LP samples: (A) embryo exposed to blasting sand and FETAX solution culture media for 4 days, demonstrates normal eye and facial development; (B,C,F) eye malformation characterized by blebbing of pigmented retinal cells; (D,E) representative facial malformation.

Addition of exogenous thyroxine (1 $\mu g\, l^{-1}$) increased the rates of development in each sample, but most substantially in the LP and W samples (Table 4). These results suggested that the organisms are capable of responding to thyroxine and that developmental delay may be the result of decreased thyroxine/triodothyroxine production. Further studies are being performed to determine the validity of the hypothesis.

Only B2 and W samples induced appreciable abnormal limb development (Table 5) Malformation included reduction deficits, soft-tissue flexures and digit malformations in samples from both sites. Addition of 1 $\mu g\, l^{-1}$ thyroxine appreciably reduced the rate of these abnormalities. The most notable malformation included in the site water + thyroxine studies was asymmetrical limb emergence, which is common when development

 

**Table 3. Effect of selected aqueous samples on long-term larval growth**

| Sample set | Test day | FETAX solution | Growth stage[a] | | | | |
|---|---|---|---|---|---|---|---|
| | | | B1 | B2 | FW | W | LP |
| 1 | 0 | 13 | 13 | 13 | 13 | 13 | 13 |
| | 5 | 46 | 46 | 46 | 46 | 46 | 46 |
| | 20 | 52 | 46 | 46 | 46 | 46 | 46 |
| | 30 | 53–54 | 48 | 48 | 48 | 48 | 48 |
| | 35 | 55 | 48 | 48 | 48 | 48 | 48 |
| | 60 | 65–66 | 50 | 50 | 50 | 50 | 50 |
| | 65 | 67 | 51 | 51 | 51 | 51 | 51 |
| | 70 | 67+ | 52–53 | 52–53 | 52–53 | 52–53 | 52–53 |
| | 80 | 68 | NA[b] | NA | NA | NA | NA |
| | 87 | 69 | NA | NA | NA | NA | NA |
| 2 | 0 | 13 | 13 | 13 | 13 | 13 | 13 |
| | 5 | 46 | 46 | 46 | 46 | 46 | 46 |
| | 17 | 51 | 48 | 48 | 48 | 48 | 48 |
| | 35 | 54–55 | 48 | 49 | 49 | 49 | 49 |
| | 52 | 64 | 48 | 53–54 | 51 | 55 | NA |
| | 65 | 66–67 | 48 | 54 | 51 | 55 | NA |
| | 94 | 70 | 48 | 54 | 51 | 55 | NA |

[a]Stage based on that described by Nieuwkoop and Faber.[19]
[b]NA = no remaining larvae to assess developmental stage.

**Table 4. Effect of exogenous thyroxine administration on larval growth rates[a]**

| Sample set | Test day | FETAX solution | Growth stage[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | B1 | B2 | FW | W | LP |
| 2 | 0 | 13 | 13 | 13 | 13 | 13 | 13 |
| | 5 | 46 | 46 | 46 | 46 | 46 | 46 |
| | 9 | 48 | 46 | 46 | 46 | 46 | 46 |
| | 15 | 50 | 48 | 48 | 48 | 48 | 48 |
| | 21 | 52 | 48 | 48 | 48 | 48 | 48 |
| | 27 | 54 | 50 | NA[c] | 48 | 50 | 50 |
| | 31 | 55 | 50 | NA | 50 | 56 | 56 |
| | 38 | 56 | 53 | NA | 53 | 56 | 56 |
| | 40 | 57 | 53 | NA | 53 | 56 | 59 |

[a]Thyroxine (1 $\mu$g l$^{-1}$) administered exogenously via test solution.
[b]Stage based on that described by Nieuwkoop and Faber.[19]
[c]NA = no remaining larvae to assess developmental stage.

is significantly accelerated. Characteristic limb malformations are illustrated in Fig. 2.

**Tail resorption.** Results of tail resorption thyroid disruption studies conducted on set 1 water samples collected from each site are provided in Figs 3 and 4. Pond water samples from B2 and LP both decreased the rate of tail resorption. The FW and W samples increased the rate of tail resorption. The reference site (B1) pond water samples had no appreciable effect on the rate of tail resorption.

**Physical/chemical water quality.** Results of water quality analyses are reported in Table 6. Water quality characteristics were fairly consistent with those collected previously from this region. No trends between the water quality characteristics of the samples or extracts and the toxicological effects induced were detected. Thus, although the matrix of the samples may have modulated the toxicity observed in the present study, it was not the primary cause of the effects observed.

**Table 5. Effects of surface water samples from New England sites on hindlimb development**

| Sample set | Site/sample | % Malformation (SEM) | Type of malformation |
|---|---|---|---|
| 1 | FETAX solution | 0.0 (–) | – |
| | B1 | 0.0 (–) | – |
| | B2 | 37.5 (5.2) | Reduction deficits, axial flexure, fused digits |
| | FW | 5.0 (1.3) | Reduction deficits |
| | W | 42.5[a] (3.8) | Reduction deficits, soft tissue, atresia, fused digits |
| | LP | 3.8 (0.9) | Fused digits |
| 2 | FETAX solution | 0.0 (–) | – |
| | B1 | 0.0 (–) | – |
| | B2 | 42.3[a] (5.8) | Reduction deficits, axial flexure, fused digits |
| | FW | 0.0 (–) | – |
| | W | 32.3 (8.3) | Reduction deficits, soft tissue, atresia, fused digits |
| | LP | 1.3 (0.5) | Fused digits |
| 2 | FETAX solution | 0.0 (–) | – |
| | FETAX solution + thyroxine[b] | 0.0 (–) | – |
| | B1 + thyroxine | 0.0 (–) | – |
| | B2 + thyroxine | 1.3 (1.3) | Asymmetric emergence |
| | FW + thyroxine | 0.0 (–) | – |
| | W + thyroxine | 2.5 (0.9) | Asymmetric emergence |
| | LP + thyroxine | 0.0 (–) | – |

[a]Significantly greater than B1 (reference) site sample (Bonferroni *t*-test, $P > 0.05$).
[b]Thyroxine concentration = 1 $\mu$g l$^{-1}$.

## DISCUSSION

Results from these studies suggested that pond water and sediment extract samples, but not whole sediment

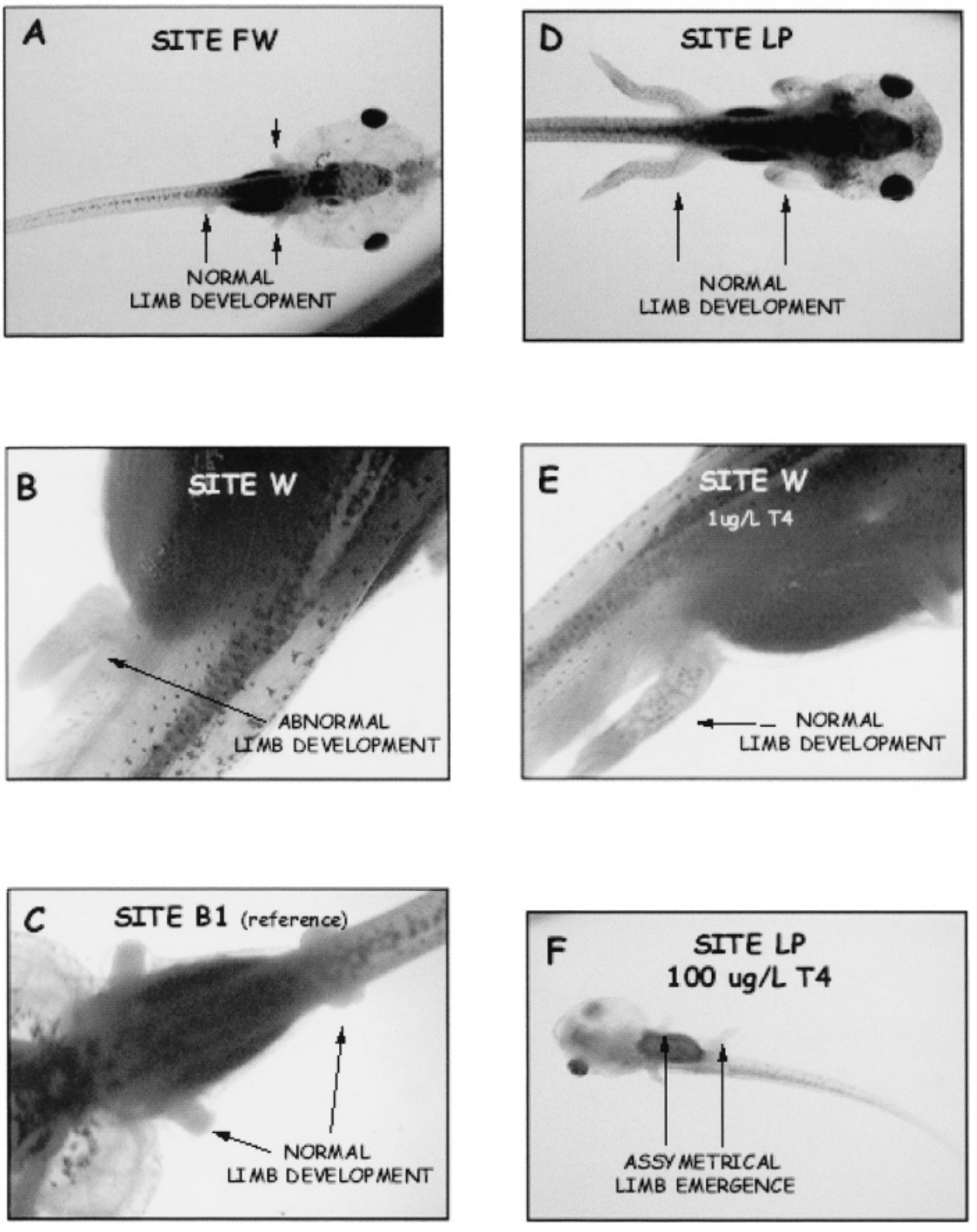


**Figure 2.** Representative characteristic limb malformation induced in premetamorphic *X. laevis* by site FW, W and LP samples: (A) normal limb emergence and development; (B) abnormal limb development characterized by abnormal soft-tissue development, including webbing and reduction deficits distal to femur; (C,D) normal limb development at various stages of limb elongation; (E) normal limb development in larvae exposed to site W surface water with 1 $\mu g\, l^{-1}$ thyroxine; (F) asymmetrical limb emergence induced by exposure to 100 $\mu g\, l^{-1}$ thyroxine; (G–I) fused digits and axial rotation of soft tissue in distal region of hindlimb induced by site B2 surface water.

samples, from ponds B2, FW, LP and W were capable of inducing abnormal early embryo-larval development. In addition, water samples from ponds B2 and W induced significant abnormal hindlimb development. Some abnormal forelimb development was noted in the tail resorption studies, but not to the same extent as the hindlimbs.

Each of the water samples induced appreciable developmental delay, including the paired reference site B1, which could be reversed by the addition of exogenous thyroxine. We have observed developmental delay of varying magnitudes in several uncontaminated reference sites in previous studies using both *Xenopus* and several *Rana* species (Fort *et al.*, unpublished data). Thus, although the magnitude of the developmental delay is somewhat surprising, the effect itself is not. We observed the same response in Washington,[12] Minnesota[5,6] and to a lesser extent in Vermont.[5] Because the FETAX solution control developed a normal rate, the effect appeared to be the result of some unidentified factors in the water/sediment extracts.

 

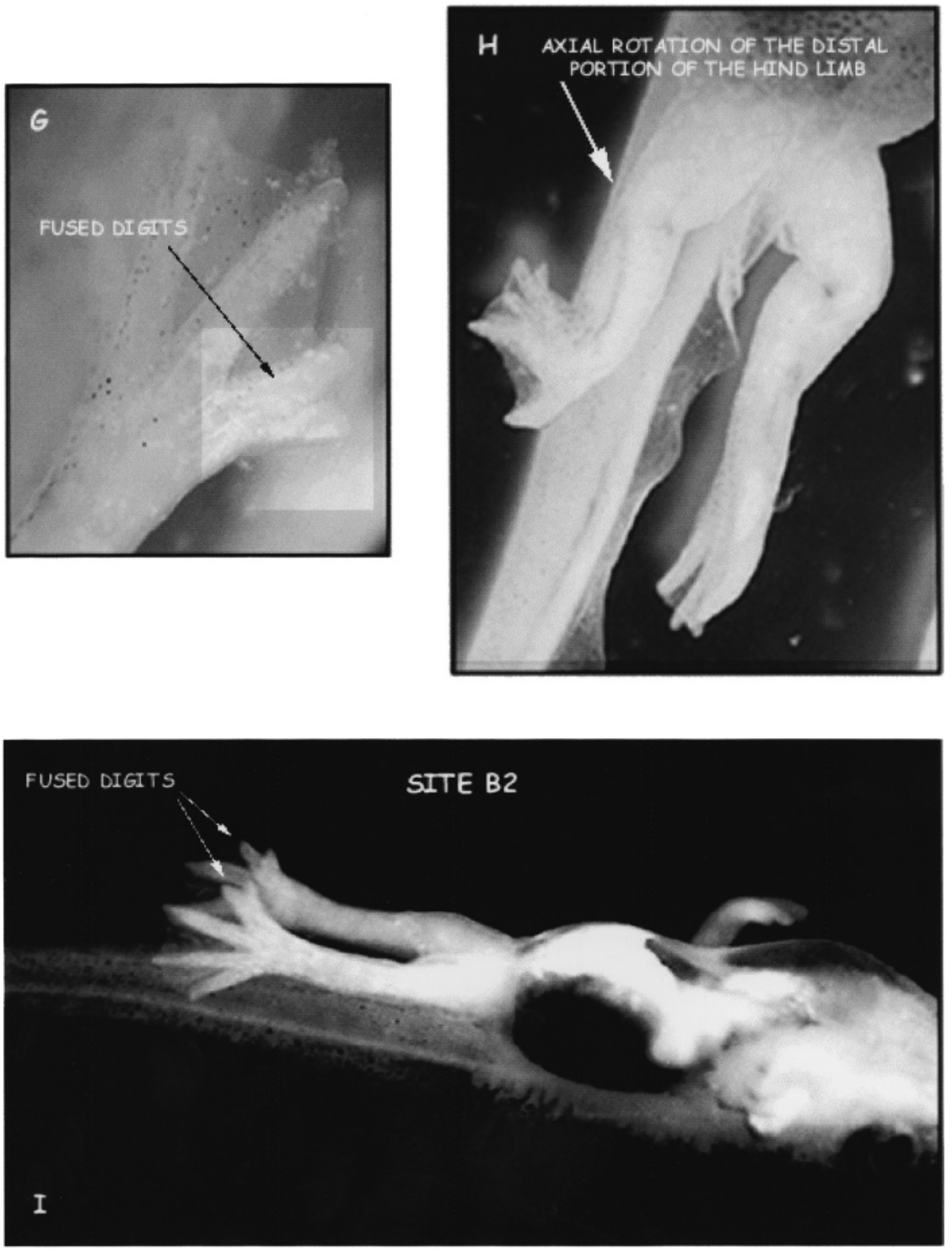


**Figure 2.** Continued.

Biochemically slowing the development has similar developmental consequences to performing the assay at cooler temperatures: slows development, opens critical developmental windows and thus increases sensitivity to particular toxicants. These results corroborate results from studies in Minnesota, which indicated that impairment of thyroid activity did not directly result in maldevelopment but exacerbated the toxicity of the samples. In Minnesota,[5,6] there appeared to be two primary factors that affect the rate of development: the ionic matrix; and naturally occurring organic compounds, including phytollic acids.

In addition, the incidence of limb malformations was also reduced by the co-administration of thyroxine. Low doses ($\leqslant 10\ \mu g\ l^{-1}$) of thyroxine will typically stimulate normal advanced development, including limb emergence and tail resorption. However, increasing concentrations of thyroxine at stages $<54$ may induce various forms of maldevelopment, including asymmetrical limb emergence (Fig. 2). We have observed similar trends in *Rana pipiens*. In contrast to the overall development delay detected in each sample, organisms exposed to water samples from ponds FW and W actually resorbed their tails faster than control organ-

 

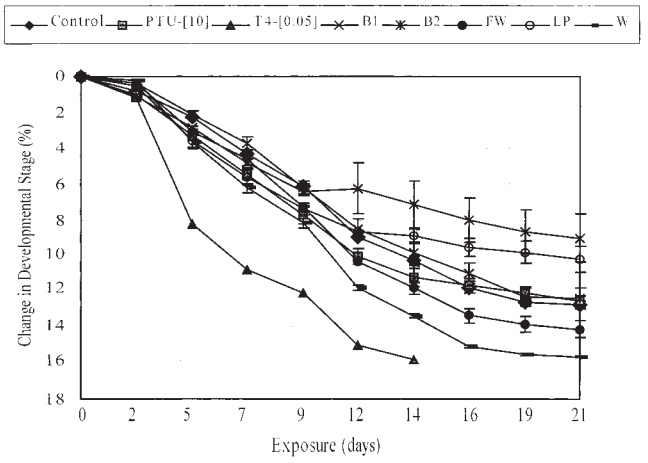


**Figure 3**. Change in developmental stage relative to day 0 versus days of exposure to five water samples from New Hampshire, USA, 6-propythiouracil (PTU), thyroxine (T4) and control. Initial exposure stage 57.[19]

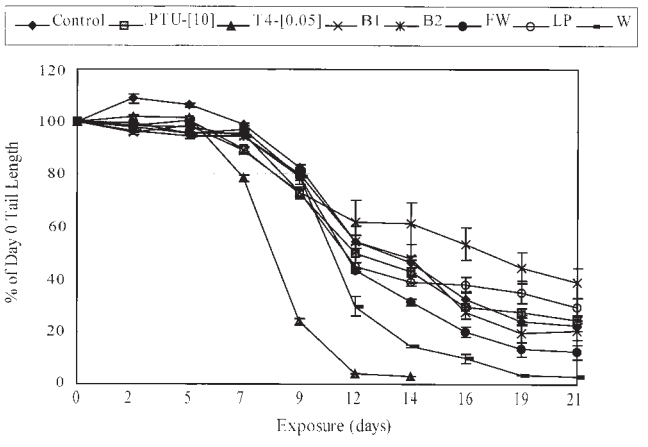


**Figure 4**. Percent of day 0 tail length versus days of exposure to five samples from New Hampshire, USA, 6-prophythiouracil (PTU), thyroxine (T4) and control. Initial exposure stage 57.[19]

isms. Delayed resorption was noted in the B2 and LP samples. Thus, the broad developmental delay observed may be the result of different toxicological mechanisms than those responsible for affecting metamorphic climax. Because exogenous thyroxine is capable of increasing development, an effect on the thyroid axis cannot be ruled out. It is also possible that exposure to the samples resulted in changes in the biochemical responsiveness of sensitivity to thyroxine. Overall, more work needs to be performed to explain fully the existing data and eventually to develop mechanistic clues.

Separate screening studies conducted with *R. pipiens* confirmed that sediment extracts from ponds B2, W, FW and LP induced mouth, eye and craniofacial defects. Samples from pond B1 (reference) did not induce abnormal development in *R. pipiens*. *Rana clamitans* collected from ponds B2 and W were found to possess both eye and limb defects (missing digits), whereas *R. catesbeiana* possessed missing digits. The overall deformity rate in both species at both sites was ca. 10%. Later collections found that several *R. catesbeiana* and *R. clamitans* possessed incompletely resorbed tails and several of the *R. clamitans* collected from the B2 site possessed abnormally resorbed tails. Again, the contrasting effects observed between field observations at site W and the effect on tail resorption observed in the laboratory during metamorphic climax may be due to longer term exposure and to exposure at critical stages of development, which result in extensive developmental delay and thus lengthened time to completion of metamorphosis. Alternatively, thyroid active components of W surface water may also induce the increased rate of tail resorption during metamorphic climax and abnormal resorption in the field specimen. Because similar effects were observed in *X. laevis* and *R. pipiens*, it does not appear that the different responses between the present laboratory-based study and the field specimen are the result of a difference in species responsiveness, although differences in sensi-

**Table 6. General water quality characteristics**

| Sample set | Sample type | Sample location | DO (mg $l^{-1}$) | pH (S.U.) | tHardness (mg $l^{-1}$) as $CaCO_3$ | tAlkalinity (mg $l^{-1}$) as $CaCO_3$ | Conductivity ($\mu$Mhos $cm^{-1}$) | $NH_3$-N (mg $l^{-1}$) | Residual chlorine (mg $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Water | B1 | 10.8 | 7.2 | 42 | 22 | 220 | <0.1 | <0.1 |
| | | B2 | 10.3 | 7.2 | 44 | 42 | 183 | <0.1 | <0.1 |
| | | FW | 10.1 | 7.2 | 52 | 20 | 127 | <0.1 | <0.1 |
| | | LP | 10.9 | 7.3 | 74 | 12 | 193 | <0.1 | <0.1 |
| | | W | 10.9 | 7.4 | 58 | 64 | 56 | <0.1 | <0.1 |
| 1 | Sediment extract | B1 | 6.5 | 6.4 | 120 | 32 | 1790 | 0.2 | <0.1 |
| | | B2 | 7.3 | 6.7 | 180 | 82 | 1218 | 7.5 | <0.1 |
| | | FW | 2.5[a] | 6.6 | 192 | 100 | 1770 | 4.0 | <0.1 |
| | | LP | 4.8[a] | 6.4 | 410 | 48 | 1590 | 1.3 | <0.1 |
| | | W | 3.5[a] | 6.0 | 440 | 32 | 1150 | 9.0 | <0.1 |
| 2 | Water | B1 | 8.3 | 6.3 | 34 | 16 | 198 | <0.1 | <0.1 |
| | | B2 | 8.2 | 6.5 | 36 | 32 | 161 | <0.1 | <0.1 |
| | | FW | 7.5 | 6.5 | 42 | 14 | 113 | <0.1 | <0.1 |
| | | LP | 8.6 | 7.1 | 62 | 6 | 174 | <0.1 | <0.1 |
| | | W | 8.4 | 6.6 | 14 | 52 | 42 | <0.1 | <0.1 |

[a]Aerated to DO = 7.2–7.5 prior to use.
DO—dissolved oxygen; S.U.—standard limits.

tivities likely exist. *Rana clamitans* collected from ponds FW and LP primarily suffered eye malformation, including size asymmetry and malpositioning, but this was more significant in the FW site than in the LP site. No malformed *R. clamitans* were collected from the B1 site.

Amphibians represent a critical class of animals in the ecosystem and a key sentinel for the health of the environment. Although the scientific community has been aware of global declines in amphibian populations for many years, recent studies in the USA that identified significant numbers of deformed amphibians in Minnesota and Vermont, as well as several other states, increased awareness of the sensitivity of amphibians to a variety of environmental stressors. The incidence and severity of deformities among amphibians surveyed in Minnesota and Vermont were appreciably greater than those recorded elsewhere in recent history.[2,3]

The first anecdotal evidence for a possible increase in the incidence of abnormal frogs in Minnesota began to appear in 1994 and 1995.[4] By 1996 the Minnesota Pollution Control Agency (MPCA) had received reports from over 100 locations with approximately 20 sites confirmed by biologists. The abnormalities in six species of frogs and toads were primarily in the form of missing, reduced and misshapen rear limbs, and a few animals with extra limbs or missing eyes. The incidence rates ranged from 4.8% to 24.3%, with >2000 specimen collected.

In the late summer of 1996, malformed frogs were reported by the general public to the Vermont agency of Natural Resources (VTANR) from 12 sites in five counties within the Lake Champlain Basin.[4] Of 290 Northern Leopard frogs (*R. pipiens*) examined, the incidence of malformations averaged 13.1%, ranging from 5 to 23%. Malformations were primarily missing and partial hindlimbs. In late July 1997 the Northern Leopard frog (*R. pipiens*) was targeted and 1475 metamorphs were collected and examined. Roughly 8.0% of the frogs had malformations, with rates ranging from 2.0 to 45.4%. Categories of malformations were primarily missing/partial limbs and shortened/missing digits. Missing or partial hindlimbs comprised 57% of the malformations encountered, followed by 11.2% with shortened hindlimbs.

In these previous studies, a high incidence of amphibian mortality and malformation has been reported in the field, suggesting that toxic and/or bioactive agents are present in the environment of the affected amphibians.[3] Recent studies[5,6] provided evidence for this hypothesis, because it applies to several affected ponds in Minnesota and Vermont. Three developmental bioassays were carried out on samples from three reference and three test sites, or one reference and three test sites, in Minnesota or Vermont, respectively. The bioassays utilize *Xenopus* as a model system, measuring altered developmental patterns during the first 4 days of development (FETAX), hindlimb development over a 30 day period and tail length resorption over a 14 day period. Strong correlations were observed between the results for all three bioassays, as well as between adverse developmental effects in the laboratory and in the field.

The present studies demonstrated that the sample sets collected from New England were capable of adversely affecting amphibian development and metamorphosis. Results from the present study showed some resemblance to those obtained from Minnesota. In the current study, a strikingly greater incidence of eye and brain malformations was noted in early development and a reduction deficit limb malformation in advanced development than observed in the Minnesota studies. The other major difference between the present study and studies in Minnesota and Vermont was the dichotomy between the marked and consistent developmental delay observed and the varying rates of metamorphosis climax, with two samples actually accelerating this process. Further study will be required to determine the specific cause(s) of the effects.

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