

# Induction of Mortality and Malformation in *Xenopus laevis* Embryos by Water Sources Associated with Field Frog Deformities

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Water samples from several ponds in Minnesota were evaluated for their capacity to induce malformations in embryos of *Xenopus laevis*. The FETAX assay was used to assess the occurrence of malformations following a 96-hr period of exposure to water samples. These studies were conducted following reports of high incidences of malformation in natural populations of frogs in Minnesota wetlands. The purpose of these studies was to determine if a biologically active agent(s) was present in the waters and could be detected using the FETAX assay. Water samples from ponds with high incidences of frog malformations (affected sites), along with water samples from ponds with unaffected frog populations (reference sites), were studied. Initial experiments clearly showed that water from affected sites induced mortality and malformation in *Xenopus* embryos, while water from reference sites had little or no effect. Induction of malformation was dose dependent and highly reproducible, both with stored samples and with samples taken at different times throughout the summer. The biological activity of the samples was reduced or eliminated when samples were passed through activated carbon. Limited evidence from these samples indicates that the causal factor(s) is not an infectious organism nor are ion concentrations or metals responsible for the effects observed. Results do indicate that the water matrix has a significant effect on the severity of toxicity. Based on the FETAX results and the occurrence of frog malformations observed in the field, these studies suggest that water in the affected sites contains one or more unknown agents that induce developmental abnormalities in *Xenopus*. These same factors may contribute to the increased incidence of malformation in native species. **Key words:** amphibian malformations, environmental sentinels, FETAX, teratogenesis. *Environ Health Perspect* 106:841–848 (1998). [Online 18 November 1998] <http://ehpnet1.niehs.nih.gov/docs/1998/106p841-848burkhart/abstract.html>

Sporadic occurrences of morphological limb abnormalities in wild populations of several species of amphibians have been reported in the past (1–3). Increased incidences of supernumerary hindlimb appendages predominate the historical record, although there are a few cases of missing limbs. In general these occurrences have involved specific sites and species, with the suggestion that in some cases exogenous factors, possibly viral, associated with cohabiting fish species may have contributed to the observed limb abnormalities (4). The increase in the frequency of malformations reported over the last few years, combined with the more widespread occurrence of ectromelia (missing limbs) and ectrodactyly (missing digits), may, however, be unique in recent environmental history (5). Identified affected species currently include the northern leopard frog (*Rana pipiens*), green frog (*Rana clamitans*), bullfrog (*Rana catesbeiana*), and mink frog (*Rana septentrionalis*). Limited evidence suggests that the incidence of malformations among different species at a single site may be related in some way to the relative time each species spends in the aquatic habitat, with increased residence time in the water being

associated with increased incidence of malformation (6).

External abnormalities include supernumerary and ectopic limbs, ectromelia, ectrodactyly, and missing or misplaced eyes. Internal abnormalities are currently being characterized. It is not understood how the apparent increase in abnormalities relates to the overall rate of amphibian decline or what may be the more far-reaching environmental implications. It is also not clear how the increased attention given to the issue, along with the involvement of press and lay public, may be influencing our estimation of the magnitude of the problem relative to the few existing historical records. However, the geographic range and broad spectrum of abnormalities in natural sentinel species, combined with the pivotal importance of aquatic environments, indicate a need to investigate the phenomenon within a broader context of both ecological and human health (7).

Several factors with the potential to produce frog deformities have been identified and/or proposed. Various groups have supported theories based on changes in predation, endoparasite infestation and disease factors, ultraviolet (UV) radiation,

mineral depletion (e.g., calcium, magnesium), and the ability of natural or man-made chemicals to cause malformation under certain conditions in the laboratory. Mechanical disruption in the form of beads placed in developing limb buds to mimic metacercaria (trematode cysts) has been shown to produce limb abnormalities (8). The action of microbial infection or naturally occurring toxicants cannot be ruled out, especially given the potential for changing ecosystems to influence niches and acquired pathogenesis. Natural compounds produced by aquatic microbes (or by soil bacteria and runoff) may have the capacity to alter complex patterns of cell growth, differentiation, and apoptosis in water-reared embryos (9–12).

The direct action of UV radiation may have a role in anuran population decline and, perhaps, in the induction of developmental abnormalities; however, the data are conflicting (13). Enhanced UV-B has been shown to increase frog embryo mortality, but there are several mitigating factors such as species sensitivity related to UV damage repair, water penetration of UV radiation, and pigmentation of embryos and larvae, which may have significant effects on dosimetry (14,15). Recent laboratory experiments have demonstrated that it may indeed be possible for direct UV to cause limb abnormalities in *Rana pipiens*, although the extent to which the abnormalities are consistent with field observations and dose is not clear (16). There is the possibility for synergistic action of UV irradiation with agents in the water or UV modification of compounds to products that may be more toxic than the parent compound. Methoprene, an insect juvenile hormone analog, can photoisomerize to forms with

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varying binding avidity to the retinoid receptor. Methoprene photoisomers have been shown to be more potent teratogens than the parent compound in the FETAX assay (17). Exposure of carbaryl to UV causes photoenhancement of toxicity in early-stage amphibians in the laboratory (18). Supernumerary and ectopic limbs have been induced by *trans*-retinoic acid (RA) in both frogs and mammals (19,20). Local administration of RA in regenerating limbs of amphibians can lead to pattern duplication, and tadpoles of *Uperodon sytoma* with amputated tails can generate hindlimbs instead of tails when cultured in water containing retinyl palmitate (21,22). Limb malformations have been induced in *Xenopus* by exposure of the developing embryos to RA (D.J. Fort, personal communication). It has also been established that  $Mg^{2+}$ , and perhaps  $Ca^{2+}$ , can alter the embryotoxic effects of metals such as  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Zn^{2+}$ , and  $Cd^{2+}$ , which are teratogenic in *Xenopus* (23). A number of studies demonstrate possible association of agricultural chemicals with abnormalities and with DNA damage such as variation in cell DNA content and strand breakage among frogs in areas with significant agricultural chemical usage (24–26).

Because frogs develop in an aquatic environment, it is necessary to test the hypothesis that increased incidences of abnormal frogs can be directly associated with exposure to the water from sites where abnormalities occur. It is clear that exposure to natural and man-made agents can result in malformations in test species. However, if such chemicals are responsible for malformed frogs, agents leading to malformations in one area may not be the same as those inducing abnormalities in another locale. In North America, the geographic range of reports of abnormal amphibians now includes regions of Canada and several U.S. states including Minnesota, Wisconsin, and Vermont. A national reporting center has recently been formed to compile and evaluate incidences of deformities among natural frog populations ([www.npwrc.org/narcam](http://www.npwrc.org/narcam)).

The most extensive information on malformed frogs in the United States is available from Minnesota, where specific sites have been monitored for the incidence of abnormalities (mostly in *Rana*) for three consecutive seasons. Large-scale laboratory studies would be difficult with *Rana* because of seasonal breeding characteristics, food requirements, and uncharacterized genetic diversity that might influence results. However, there have been relatively extensive studies of *Xenopus* embryo development using known teratogens. Testing with *Xenopus* can be conducted rapidly without the constraints

associated with seasonal breeding. In the present study, the 96-hr frog embryo teratogenesis assay in *Xenopus laevis* (FETAX) was used to test water, sediments, and sediment extracts from a number of Minnesota sites that had been previously characterized on the basis of malformed frog incidences in field sampling. Groundwater and interface (sediment/pond) water were also collected from shallow wells installed at one of the pond sites identified as having a high incidence of abnormal frogs. In addition to the results of experiments reported here, chemical analyses, water fractionation, and a panel of biological activity assays are underway to assess the environmental status of water samples from specific sites where both high incidences of abnormalities have been observed and where the water produces abnormal *Xenopus* embryos. Because the assays as applied here were scored at 96 hr, there was no expectation that the malformations observed in *Xenopus* would be identical to limb abnormality observed in the natural *Rana* populations. However, occurrence of malformation in *Xenopus* does allow for identification of sites of concern and for the design of studies to determine causative agents.

## Methods

**Site selection and field sampling.** Using field data from the previous two seasons, six ponds were classified as either “affected” based on reported high incidence of abnormality, or “reference” where there has been no clear evidence of malformation. Selection was based only on frog data without consideration of land use, runoff, topography, or limnology. Reference and affected sites were paired by county proximity and coded in this presentation to maintain the agreement of confidentiality with the landowners. Pairs have been designated here as Af (affected) or R (reference) and a numerical suffix denoting the specific pair. Where there was more than one reference for an affected site, it is distinguished as “a” or “b.” These sites are focal points for efforts to identify causal factors. The potential for changes in both water chemistry and natural frog populations over the course of a breeding season was anticipated.

Beginning in late April 1997, water samples were collected at 2- to 3-week intervals for the purpose of chemical analysis and, intermittently, for assays of biological activity in FETAX. Water samples were collected in clean, amber glass bottles (VWR, TraceClean, VWR, Marietta, GA) for organic chemical analysis and in plastic bottles for metals analysis (VWR, TraceClean). Sediment samples were collected once from each site; the upper 2–5 cm of sediment was collected from several

stations in the shallow water along the site edge and placed in clean bottles (VWR or I-Chem). Sampling equipment was cleaned between sites with site water and acetone followed by hexane. Collectors’ gloves were changed between sites. Samples were transported back to the lab on ice and maintained in a cold room (4°C) until overnight shipment to the analytical laboratory. For the FETAX assay, 4 liters of site water was collected in 1-liter amber glass bottles. Upon receipt at the lab, the water was combined in a 4-liter plastic container that had been rinsed in dilute HCl followed by three successive rinses with deionized water prior to use. Water was held at 4°C in a cold room until the FETAX assay was conducted. During the 96-hr FETAX assay, the water was replaced daily. At one site, designated Af1, subsurface pond water was collected with new silicon tubing via a variable-speed peristaltic pump connected to a screened aperture. Groundwater was pumped 0.5 m in fine sand below the littoral shore line, whereas the pond/ground water interface required a flat 20-cm screen placed 1 cm into the littoral sediment 1 m from the shore (27).

Field biologists from the Minnesota Pollution Control Agency and the University of Minnesota monitored the frog populations of the two groups of ponds/wetlands to estimate the frequencies and types of malformations. Animals were scored by species, stage of development, and gross examination for abnormalities, which were classified by type such as missing limbs or digits, missing eyes, extra limbs, and fused limbs/digits and released. Statistical analyses were performed using the Prism software package. Affected sites were compared to their respective control sites, which were located in the same county, using Student’s *t*-test. A grouped *t*-test was also performed to compare percent deformities within all affected sites to grouped deformities within all control sites. A limited number of animals were also collected for more extensive diagnostic examination of internal abnormalities and parasitic or microbial infection.

**Sample characterization.** General parameters such as dissolved oxygen, pH, ammonia, nitrate, alkalinity, hardness, and conductivity were determined using standard methods for all samples (28). Multimetals analysis was conducted by Midwest Research Institute (Kansas City, MO) using cold vapor AA for Hg, graphite furnace AA for As, Pb, Se, and Tl, and inductively coupled plasma–AES for all other analytes tested. Ion exchange and activated carbon chromatography was conducted using an automatic ion chromatograph/high pressure liquid chromatograph (29).

***Xenopus* embryo assays.** FETAX is based on exposure of early-stage embryos to test water and scoring of free-swimming

larvae at 96 hr postfertilization for mortality and morphological abnormalities (30,31). Tests were performed according to the American Society for Testing and Materials (ASTM) Standard Guide and the prepared Atlas of Abnormalities for the FETAX assay (32,33). Incidence of malformations reported here is based on percentage of live embryos at each observation time. Samples were given an additional code and scored blind. Typically 40–60 embryos are scored for each test sample, dilution, or replicate and accompanied by scoring of a negative control (FETAX control solution) and positive controls using two concentrations of 6-aminonicotinamide. Types of abnormalities are classified for each embryo to develop a malformation spectrum that may be related to action of specific waterborne contaminants. Some of the samples giving positive FETAX results were either filtered or boiled for 30 and 60 min to exclude possible direct effects from microorganisms or from volatile and thermolabile compounds. In some cases the water from affected sites was diluted with water from reference sites instead of the control solution.

Sediments were tested by overlaying sediment with the FETAX control solution containing the embryos or by using an aqueous extract of the sediment derived by extraction (1:4 w:v in FETAX solution) for 48 hr followed by centrifugation and filtration to remove particulates. FETAX solution is a reconstituted water medium suitable for the culture of *Xenopus* embryos. For these studies the FETAX control solution was prepared with reverse osmosis/deionized water with a conductivity of less than 3  $\mu\text{mhos}$  [ $\mu\text{mhos}(\Omega^{-1}\text{cm}^{-1})$ ] containing NaCl (Sigma S-7653; Sigma Chemical Company, St. Louis, MO),  $\text{NaHCO}_3$  (Sigma S-6297), KCl (P-9333),  $\text{CaCl}_2$  (Sigma C-5080),  $\text{CaSO}_4$  (Sigma C-3210), and  $\text{MgSO}_4$  (Sigma M-7506) to final concentrations of 272 mg/l  $\text{Na}^+$ , 15.64 mg/l  $\text{K}^+$ , 19.6 mg/l  $\text{Ca}^{2+}$ , and 14.88 mg/l  $\text{Mg}^{2+}$ , followed by clarification by centrifugation (34,35). The supernatant was then used in the FETAX assay. The ion simulations, matched to certain affected sites, were based on these four cations because they are the only ones known to be significant in the FETAX control solution. Other ions present in the control solution or in the ion simulations would be as contaminants of these highly purified reagents.

## Results

**Field studies.** Sites previously characterized as affected or reference retained the overall designation in 1997, although a strict interpretation is difficult for such studies due to

**Table 1.** Incidence of malformation among frogs at study sites

Site	Date (1997)	No. collected	No. normal	No. abnormal	Percent	Affected vs. reference
A1	Aug 12	148	139	9	6.1	A1:R1b $p = 0.108$
	Aug 19	112	101	11	9.8	
	Sep 08	110	84	26	23.6	
Total		370	324	46	12.4	
A2	Jul 22	115	101	14	12.2	A2:R2 $p = 0.039$
	Jul 23	57	51	6	10.5	
	Aug 13	103	78	25	24.3	
	Sep 08	96	83	13	13.5	
Total		371	313	58	15.6	
A3	Jul 18	104	99	5	4.8	A3:R3 $p = 0.090$
	Aug 18	144	134	10	6.9	
	Aug 25	90	84	6	6.6	
	Sep 11	245	229	16	6.5	
	Sep 19	61	53	8	13.1	
	Sep 29	115	94	21	18.3	
Total <sup>a</sup>		759	693	66	8.7	
R1b	Aug 07	52	51	1	1.9	
	Sep 29	69	67	2	2.9	
Total		121	118	3	2.5	
R1a	Aug 19	24	24	0	0	
	Sep 22	14	14	0	0	
Total		38	38	0	0	
R2	Jul 22	100	100	0	0	
	Sep 08	47	44	3	6.4	
Total		147	144	3	2.0	
R3	Jul 17	104	99	5	4.8	
	Sep 11	99	97	2	2.0	
Total <sup>b</sup>		203	196	7	3.4	

Abbreviations: A, affected; R, reference, SE, standard error.

<sup>a</sup>Grouped  $\pm$  mean = 12.02; SE = 1.80;  $p = 0.0065$ ;  $n = 13$ .

<sup>b</sup>Grouped  $\pm$  mean = 2.25; SE = 0.84;  $n = 8$ .

biological and environmental variation. Table 1 shows the overall incidence of malformations observed in the 1997 season among newly metamorphosed frogs collected at sites defined as affected or reference on the basis of the previous 1995 and/or 1996 field studies. The data presented here are based on metamorphs because of adult migration and the possibility that malformed individuals might not have survived over the first winter. With the exception of the R1b site, all of the metamorphs scored here are *Rana pipiens*. The R1b site represents *Rana clamitans* (50.4%) and *Rana septentrionalis* (49.6%) metamorphs because no *Rana pipiens* were captured. Data collected from extensive sampling of different species at the A1 site by Dave Hoppe (University of Minnesota) indicate that the incidence of malformation may vary by species, but general trends are similar among *Rana pipiens*, *Rana clamitans*, and *Rana septentrionalis*.

Differences in  $n$  values between sites and collections (Table 1) as well as differences in the frequencies of affected animals make the data statistically inconclusive at the  $p = 0.05$  level for some of the sites, but nonetheless suggest differences between sites. There was

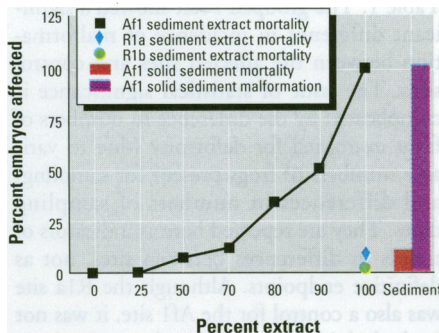
no statistical difference in incidence of malformations in native *Rana* species between the affected site Af1 and its control site R1b. There was a significant difference between the affected Af2 site and its control site R2 ( $p = 0.039$ ). There was no significant difference in incidence of malformations between the affected site Af3 and its respective control R3. Exact probabilities are reported in Table 1. The grouped  $t$ -test showed a significant difference in incidence of malformation between the affected sites and control sites. The issue of statistical significance is complicated by the difference in numbers of frogs examined for deformity (due to variable numbers of frogs present for sampling) and differences in numbers of sampling dates. They are reported here as indicators of trends in differences between sites, not as definitive endpoints. Although the R1a site was also a control for the Af1 site, it was not included in the statistical comparison because there was a small sample size, and no error could be attached where no abnormalities were observed.

**FETAX assays.** A high frequency of FETAX embryo abnormalities and mortality was induced by pond water from the three affected sites. Boiling and filtration to remove

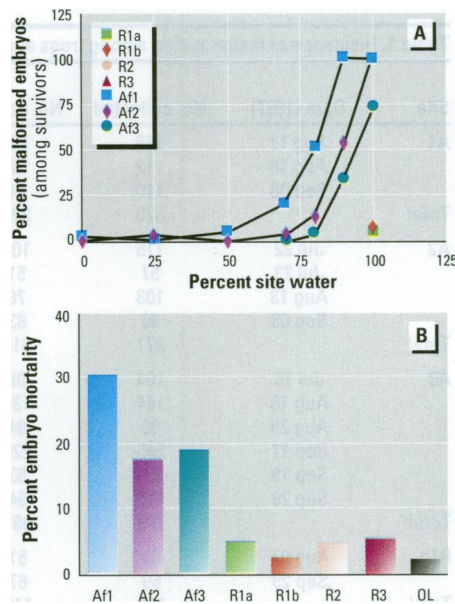
microbial contamination had no effect on the initial results, and no further such treatments were done in subsequent experiments. Figure 1 shows the percent morphological abnormality (Fig. 1A) and mortality (Fig. 1B) induced in *Xenopus* embryos reared in water from affected sites Af1, Af2, and Af3 and reference sites R1a, R1b, R2, and R3. Because of the high frequencies of abnormalities, dilution of water from the Af1, Af2, and Af3 sites with the control FETAX solution was conducted to assess dose–effect relationships. The incidence of lethality and malformation in embryos is dose dependent with dilution of water from affected sites. The water from the Af1 site is more toxic in this set of experiments than water from the Af2 or Af3 site. Water from reference sites did not have any effect above the negative controls.

The possibility that biological activity of pond water might vary over the course of the summer was examined by conducting the FETAX assays on samples collected at intervals for one of the sites (Af1) with high incidences of abnormalities. Figure 2 demonstrates morphological abnormality (Fig. 2A) and mortality (Fig. 2B) associated with three separate samplings of water from the CW site over a period of approximately 1 month. Morphology was scored in dilutions of the site water while mortality was scored in undiluted pond water. Single field samples from two reference sites, R1a and R1b, and the Af2 affected site were included in each Af1 test as a measure of continuity of assay effects and stability of the stored water samples. The July 19 sample from Af1 was originally tested at dilutions of 25, 50, 75, and 100% site water. In later tests, the dilutions were changed to 25, 50, 70, 80, 90, and 100% to provide better characterization.

The pH of the Af1 water sample was low (5.6), and there was some question as to whether the effects in the *Xenopus*



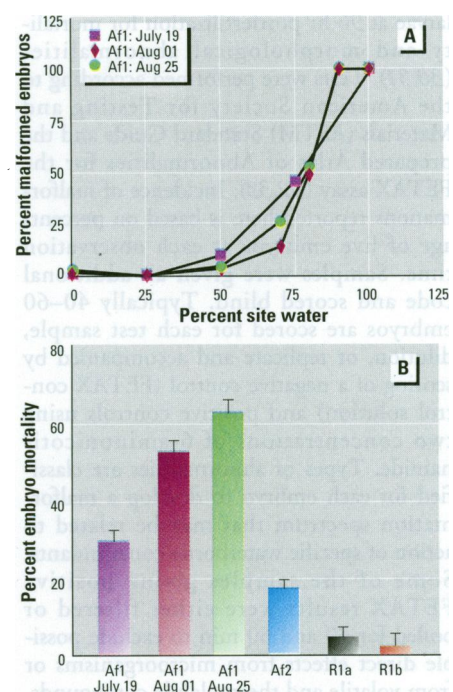
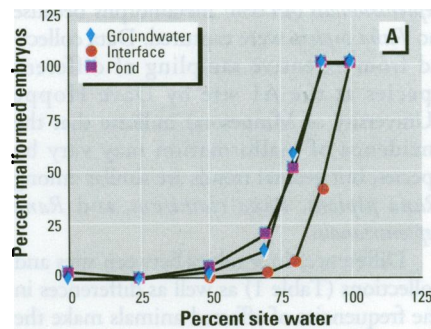
**Figure 3.** The percent affected embryos (mortality or morphology as indicated) induced by aqueous extracts of Af1 and R1 sediments. Abbreviations: Af, affected; R, reference. Dilution of the Af1 extract is with control solution. Bars show percentage of embryos with mortality and malformation induced by exposure to Af1 sediments without extraction.



**Figure 1.** The incidence of (A) malformations and (B) mortality among *Xenopus* embryos exposed to pond water from affected (Af) and reference (R) sites. Each point represents the average of two replicates for a single water sample and 40–60 embryos per replicate. Percent malformations is based on survivors only. Control in panel 1B denotes mortality associated with the FETAX solution.

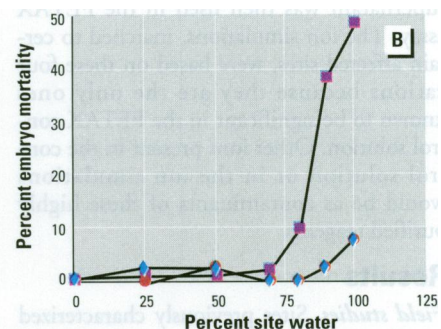
embryos could simply be a response to pH and independent of agents in the water. In addition, metabolic activation and detoxification are important components of biological response to chemical agents. The July 19 sample from Af1 was combined with a metabolic activation system (MAS) in the form of rat liver microsomes to test for the influence of mammalian biotransformation (29). Adjustment of the Af1 water from 5.6 to neutral and/or the addition of the MAS had little effect on the toxicity of the sample (not shown).

Many types of agents in an aquatic environment bind to sediments and become an important route of exposure for species individually and in the food chain. Figure 3 demonstrates that sediments and their aqueous



**Figure 2.** The percentage of embryos with (A) malformations and (B) mortality after exposure to Af1 site water collected at three time intervals. Abbreviations: Af, affected; R, reference. Dilutions in panel A are with the FETAX control solution. The Af2, R1a, and R1b samples in B are 100% site water and represent three replicates of a single water sampling for each site.

extracts from the affected site induce mortality, whereas sediments and extracts from reference sites do not. The responses to the sediments were different from the responses to the sediment extracts. Incubation of the embryos with sediments induced primarily malformation, and response was similar to the pond water. Aqueous extracts of Af1 sediment were more lethal than the undiluted pond water. The data do not allow any distinction between different causal factors or doses. Sediments and sediment extracts from the reference sites R1a and R1b had little effect on embryo development or mortality.



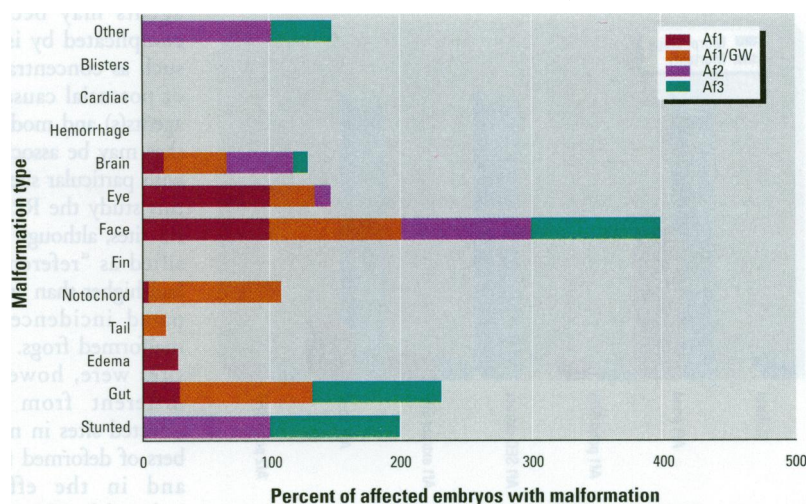
**Figure 4.** Proportion of embryos with (A) malformations and (B) mortality after incubation with dilutions (control solution) of surface, ground, and interface water from the Af1 site (affected).

In the natural aquatic systems examined in Minnesota, the pond water was derived from a mixture of subsurface and surface runoff and precipitation sources. The surrounding watersheds tended to be small, allowing for relatively long hydraulic residence and mixing. Figure 4A compares the teratogenic potential of pond water, groundwater, and the interface water. The groundwater has teratogenic potential similar to the pond water, and both are different from the interface. Figure 4B compares the mortality induced by the same samples. Lethality associated with groundwater is much lower than the water in the pond and suggests possible environmental transformation to a more active form (e.g., microbial action or UV light) or suggests that the induction of enhanced lethality has its origin in multiple water sources, synergism with factors already present in pond water, or matrix effects. The observed differences in mortality and malformation between pond, ground-, and interface water further emphasizes roles for potentially different causes or modifiers in the lethality and malformation effects.

Several forms of abnormality are scored with the FETAX assay. Figure 5 shows the spectrum of malformations produced in 100% site water from three affected pond water sites (Af1, Af2, Af3) and the spectrum produced by the Af1 groundwater sample (Af1/GW). There are marked differences between the water sources with respect to spectrum, suggesting that there may be more than one cause for the observed effects or that the effects of a single causative agent are being modified by the variation in water matrix characteristics associated with the specific sites.

#### Water matrix and characterization.

Normal development of frog embryos can be adversely influenced by depletion of Ca, Mg, Na, and K and by high levels of metals such as Zn<sup>2+</sup>, Cu<sup>2+</sup>, and Ni<sup>2+</sup>. Chemical analysis indicated that although affected and reference sites were reasonably similar, some of the sites had very low concentrations of the essential ions contained in the FETAX control solution. One initial concern was that such salt or metal imbalances could cause the positive effects in the FETAX assay, independent of any other agents. Table 2 shows that for the geographically selected affected and reference sites, metals, alkalinity, and conductivity are similar. Ammonia/pH levels were in the range of 0.005/6.7, 0.504/7.3, and 0.397/7.6 for the affected sites Af1, Af2, and Af3, respectively. Nitrate levels were <0.007 ppm for the same samples. Experiments (Fig. 6) using laboratory-synthesized water containing Ca, Mg, Na, and K reduced to levels equal to the Af1 pond water, the Af1 sediment extract, and the Af2



**Figure 5.** Different types of laboratory *Xenopus* abnormalities associated with various water sources where malformed frogs have been confirmed at unusually high frequencies. Abbreviations: Af, affected; GW, groundwater. The classification "other" includes renal hyperplasia, protruding gut, and hypognathia depending on the site.

**Table 2.** Paired site characteristics

Analyte	Metal concentration at site (ppb)					
	Af1	R1b	Af2	R2	Af3	R3
Aluminum	206.75	59.14	182.69	82.47	75.54	401.79
Antimony <sup>a</sup>	22.44	22.44	22.44	22.44	22.44	22.44
Arsenic	2.61	1.13	2.04	2.77	2.38	2.21
Barium	12.14	6.89	23.99	13.00	44.29	75.59
Beryllium <sup>a</sup>	0.34	0.34	0.37	0.34	0.34	0.34
Cadmium	2.19	2.29	4.52	2.04	4.71	3.13
Calcium	4,890	4,759	41,547	31,595	39,524	42,339
Chromium	5.21	3.93	11.83	9.53	9.63	8.89
Cobalt	4.15	3.45	7.23	4.83	4.25	5.52
Copper	5.33	4.47	5.76	9.94	9.08	7.64
Iron	331.08	220.30	156.52	147.24	193.42	3,862.80
Lead	0.45	0.45	1.13	0.34	0.34	0.57
Magnesium	1,676	1,107	14,975	8,168	20,608	14,005
Manganese	82.26	20.91	60.91	27.35	159.80	536.93
Mercury	0.22	0.20	0.20	0.20	0.20	0.25
Nickel	7.06	8.72	94.58	8.56	11.52	9.01
Potassium	1,554	2,245	3,776	2,150	2,861	2,345
Selenium <sup>a</sup>	0.79	0.79	0.91	0.91	0.91	0.79
Silver	6.66	5.83	7.40	7.82	7.93	8.70
Sodium	1,234	469	2,098	2,641	2,876	1,722
Thallium <sup>a</sup>	0.75	0.73	0.79	0.79	0.79	0.74
Vanadium	3.97	2.95	8.09	5.97	7.14	7.43
Zinc	10.68	11.42	30.96	33.92	7.14	13.15
Alkalinity (ppm HCO <sub>3</sub> )	16	14	176	190	144	163
Conductivity (µmhos)	41	36	334	502	288	294

Abbreviations: Af, affected, R, reference.

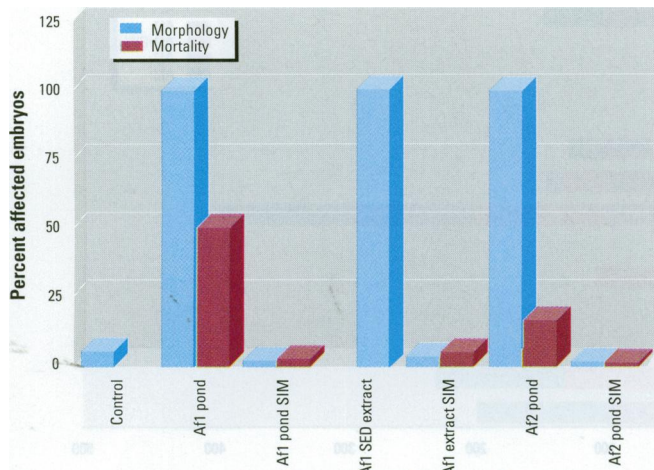
<sup>a</sup>At or below detection limits.

pond water indicate that the depletion of these ions relative to the control FETAX solution is not sufficient to explain the teratogenic effects associated with the field samples.

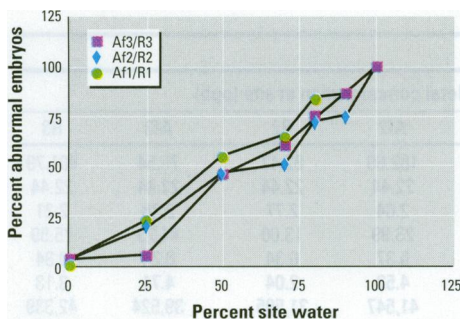
Water from three affected sites was also diluted with water from reference sites instead of the FETAX control solution. Figure 7 shows a dramatic change in slope of percent malformation versus percent dilution when compared to dilution with the FETAX control as in Figure 1A. The change in slope indicates a reduction in overall toxicity by

the control FETAX solution. Thus, water conditions such as ion concentration in the Minnesota area ponds appear to influence the observed toxicity of the affected sites.

Additional indications about the types of agents that may be involved for sites Af1, Af2, and the sediment extract from Af1 are presented in Figure 8. Mixed-bed ion exchange removes in the range of 50% of the biological activity of water and sediment extract. Both activated carbon and C-18 remove an additional proportion.



**Figure 6.** Incidence of malformations among *Xenopus* embryos reared in water simulation of field samples with respect to Ca, Na, K, and Mg. Abbreviations: Af, affected; SIM, simulation; SED, sediment.



**Figure 7.** Incidence of malformations induced by affected site water (Af) diluted with reference site water (R) for comparison with dilution by FETAX control solution (see Fig. 1A).

## Discussion

The recent appearance of deformities, and in some cases mortality, among amphibians at the sites examined here indicates a recent environmental modification. A precise measure of the extent of mortality among anurans at the study sites for a direct comparison with *Xenopus* embryos is far beyond the capacity of this study. It is unusual to see dead frogs at any site; however, in the 1997 field surveys some mortality was observed at the Af1 site. In the 1998 season, field observations at Af1 indicated delayed breeding and dramatic die-off among the current metamorphs. These observations will be part of a different report. In general, all the sites examined should probably be regarded as impacted to some degree, based on comparison of current incidence of abnormal frogs with historical data from Minnesota and other regions. The background frequency is in the range of 0.5% (36), and in the recent sampling of “reference” sites presented here, the incidence is  $2.25\% \pm 0.84$ . This also means that the concept of “reference” sites for the purpose of identifying causal

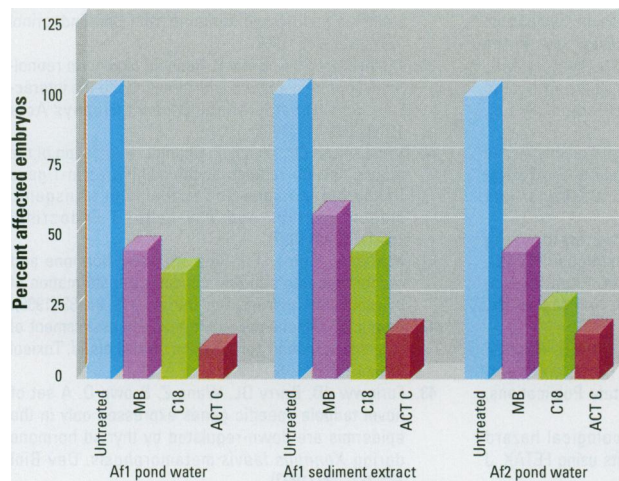
agents may become complicated by issues such as concentration of potential causative agents(s) and modifiers that may be associated with particular sites. In this study the R2 and R3 sites, although classified as “reference,” had higher than anticipated incidences of malformed frogs. The sites were, however, different from the affected sites in numbers of deformed frogs and in the effects induced by site water on laboratory animals. The overall increased incidence of abnormalities could have several

independent or interrelated etiologies including parasitic or other disease/infection, direct UV or UV-mediated chemical modification, natural or man-made chemical agents, or a change in the water matrix that has unmasked or produced a toxic effect. Although intensive study continues, a direct role for parasites and disease is not substantiated at this time by diagnostic investigation of frogs collected for histology, bacteriology, and virology as part of this study (37). Although public attention has been focused on the existence of supernumerary limbs, they are only a small subset of the range of abnormalities observed and, as noted by Ouellet et al. (5), the missing limbs and digits may be more noteworthy in comparison to the historical records for deformities among native species of frogs. Field abnormalities are consistent with modes of action involving retinoids, thyroid disruption, and lathyrogens. The interactions of thyroid hormones, retinoids, and endocrine/thyroid disrupters are complex and important in developmental control (38–40). For example, inhibition of thyroid hormone production can prevent the homeotic transformation of frog tails into legs by retinoids, indicating that thyroid hormone is required. Thyroid hormone economy can be disrupted by many different chemicals including steroids, retinoids, halogenated biphenyls, and chlorinated hydrocarbons (41,42). It has been widely speculated based on retinoid activity that methoprene, a compound used for mosquito control, could be the causative agent. Chemical analyses do not, at this time, indicate the presence of methoprene or its UV-activated metabolites. It may be possible for direct UV to have a role in some types of malformations, but it is uncertain how individual site dosimetry and characteristics might interact to produce abnormalities. We

can also speculate about the interaction of different factors such as site condition and UV sensitivity, considering that thyroid function in frogs is necessary for epidermal maturation and keratinization (43). The FETAX experiments reported here were conducted in a laboratory in the absence of UV light, and some of the more active samples were boiled and/or filtered to exclude direct microbial or viral action.

There is no assumption of parity between the abnormalities observed in the *Xenopus* 96-hr assay and abnormalities observed in the natural resident amphibians. Even though considerable genetic distance exists between laboratory *Xenopus laevis* (family Pipidae) and field *Rana* species (family Ranidae), the developmental pathways are highly conserved, and *Xenopus* is a much used model for the study of developmental biology. In addition, frogs sampled in the field are categorized as normal/abnormal after metamorphosis, and there have been no focused studies to date of mortality and morphology among early free-swimming larvae from sites where abnormal animals have been observed. It does, however, seem likely that the practical advantages of using *Xenopus* can be applied to guide further field investigation. Long-term limb development studies are being conducted in *Xenopus* using water from affected sites and lower density of embryos/water than in the normal FETAX assay.

General water matrix characteristics can influence the development of aquatic species as well as modify the effects of waterborne toxic agents. With the exception of the sediment extracts, the ionic strengths of the Minnesota waters tested here are all much lower than the FETAX control solution with respect to  $\text{Na}^{2+}$  and  $\text{K}^{2+}$ .  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentrations were low in the Af1 and R1 samples, but were much higher for the other pond samples and for the ground-water samples (not shown). The affected and reference sites are well matched in concentration for these metals and for others measured as possible contributors to induction of malformations. The only notable exception is the reference site R3, which is much higher in Fe and Mn than the paired Af3 affected site, and there were differences between sites in  $\text{Al}^{3+}$  concentration. Concern over the possibility that the low concentrations of  $\text{Na}^{2+}$ ,  $\text{K}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$  caused the malformations in *Xenopus* is not supported by the lack of effect observed with reconstituted water matched to the low site levels. However, modification of response to toxic agents present in samples is definitely indicated by the increase in toxicity using dilution with Minnesota reference site water (Fig. 7) compared with dilution using FETAX control solution (Fig. 1A). A role



**Figure 8.** The percent affected embryos after running selected pond water and sediment extracts through mixed-bed ion exchange (MB), C-18, and activated carbon (ACT C). The pond water data are percent malformations among survivors; the A1 sediment extract data are percent mortality.

for Mg in reducing the toxicity of metals such as Ni, Zn, Co, and Cd has been established, but the possibility of similar influences on embryo toxicity of chemical agents has not been sufficiently investigated (23,44). It has been demonstrated that natural water (Johnson City, TN) can modify the effects of some pesticides such as atrazine and 2,4-D to enhance or decrease the toxicity in comparison to FETAX control solution (45). A mechanism for modification of the response has not been identified.

The data reported here indicate that for the limited number of sites examined, agents exist in the water that can induce frog embryo abnormality in the laboratory. It is not known if the origin is plant, microbial, chemical, or how the water matrix of specific sites may be influencing the toxicity. The factors causing toxicity (lethality and malformation) can be significantly reduced by fractionation methods that typically remove organics from environmental samples. It does appear that specific water matrices may influence toxicity of affected Minnesota site waters in FETAX, but whether there is a parallel in native species has not been determined. The laboratory FETAX assay is now being used to identify and characterize the interaction of causal factors.

Variation in mortality for different sampling times at the A1 site concomitant with a constant incidence of abnormalities for the same samples suggests the possibility of different toxic agents, changes in concentrations, alteration of intrinsic factors, or synergism of mortality with variably present factors in pond waters. The existence of different factors or modifiers is supported by variation in the spectra of malformations

and mortality among embryos reared in the other FETAX-positive water sources. The A1 site is an example of a pond where the water may be supplied from multiple sources. Water collected from the shallow groundwater wells at the A1 site induces teratogenesis at a frequency that is similar to the pond water. However, there is much less lethality associated with the A1 groundwater in comparison to the pond water, and changes in response associated with the interface water may be due to sediment binding, environmental metabolism

(e.g., microbial action), or increased levels of ions capable of reducing the toxic effects. It is clear that increased toxicity in the form of lethality is acquired by the pond water and that highly toxic agents are liberated by aqueous extraction of the sediment from the A1 site.

Extensive investigation of agents and general characteristics of water from affected sites is still under way. The fact that there is concordance between the field data and the FETAX assays for this set of sites provides a useful means to further investigate the phenomenon. The results presented here indicate probable waterborne causal agents and suggests that 1) ionic composition of the water may influence, but is not sufficient to explain, the biological activity, 2) different agents or modifiers are likely to be involved at various sites, and 3) factors that cause *Xenopus* abnormality are transported in groundwater associated with some sites. It is also possible that the agents in the water of sites that cause teratogenesis and mortality in the FETAX assay may be interacting to increase the sensitivity of natural populations to other factors such as UV or infection (e.g., through immunosuppression) *in situ*. The possibility that agents with the capacity to disrupt morphogenesis of aquatic species are moving in the groundwater of some areas is an issue of potential importance to both natural populations and humans. As the broader investigation continues to identify agents and characterize biological activity in a number of *in vivo* and *in vitro* models, parallel studies can be focused on amphibian species such as *Rana pipiens* that are indigenous to specific study sites and, if warranted, on mammalian models.

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