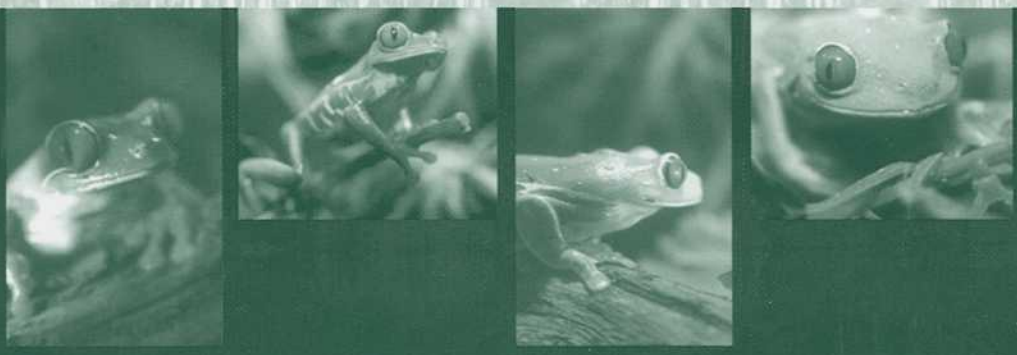
Multiple Stressor Effects in Relation to Declining Amphibian Populations



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Multiple Stressor Effects in Relation to Declining Amphibian Populations

G. Linder, S. Krest, D. Sparling and E. Little, editors

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Foreword

This publication, *Multiple Stressor Effects in Relation to Declining Amphibian Populations,* contains papers presented at the symposium of the same name held in Pittsburgh, PA, on 16–17 April 2002. The symposium was sponsored by ASTM International Committee E47 on Biological Effects and Environmental Fate. The symposium co-chairpersons were Greg Linder, USGS/BRD/CERC, Sherry Krest, US Fish and Wildlife Service, Ed Little, USGS/BRD/CERC, and Don Sparling, USGS/BRD/Patuxent Wildlife Research Center.

Contents

Overview

This book represents the work of several authors who participated in the symposium entitled "*Multiple Stressor Effects in Relation to Declining Amphibian Populations*" convened 16–17 April, 2002, in Pittsburgh, Pennsylvania. Declines of amphibian populations of varying severity have been observed for many years, and in the last 8 to 10 years considerable progress has been made in documenting the status and distribution of a range of amphibian species. Habitat alteration and destruction are likely linked to many amphibian declines, but a variety of other factors, both anthropogenic and natural, have been observed or proposed to have caused declines or extinctions of amphibian populations. Unfortunately, determining the environmental causes for the decline of many species has proven difficult. The goals of this symposium were three-fold. First, highlight ASTM's historic role in providing a forum for the standardization of amphibian toxicity test methods and the characterization of adverse effects potentially associated with chemical stressors. Second, demonstrate through case studies the current state of technical "tools" available to biologists, ecologists, environmental scientists and natural resource professionals for assessing amphibian populations exposed to various environmental stressors. And third, characterize a process that brings a range of interdisciplinary technical and management tools to the tasks of causal analysis, especially as those relate to a multiple stressor risk assessment "mind-set." As part of the symposium, scientists and resource management professionals from diverse fields including ecotoxicology and chemistry, ecology and field biology, conservation biology, and natural resource management and policy contributed oral presentations and posters that addressed topics related to declining amphibian populations and the role that various stressors have in those losses.

The papers contained in this publication reflect the commitment of ASTM International committee E47 on Biological Fate and Environmental Effects to provide timely and comprehensive information to the technical community and the lay-public who have become increasingly aware of amphibians and their current plight. Common themes emphasized throughout the symposium can be found in this issue, including papers focused on (1) toxicity assessment, (2) integrated field and laboratory studies, and (3) causal analysis. In addition to historical accounts of the development of amphibian test methods and contemporary studies illustrating current applications of these methods, this publication addresses future needs by providing contributions focused on research requirements in these areas.

Toxicity Assessment

Contributed papers on the application of toxicity assessment methods effective for amphibians reflect the range of ASTM International E47's involvement with the standardization process. Such methods range from long-established laboratory approaches for evaluating adverse chemical effects to amphibians to methods that link chemicals in surface waters, sediments, and soils with adverse effects observed among amphibians in the field. In addition, potential ASTM International standards were proposed during the symposium. These candidate tools and refinements are beginning to be added to existing standards and will likely be applied more frequently to future evaluations of chemical stressor effects on amphibians.

Field and Laboratory Studies

A series of case studies illustrated the importance of integrated field and laboratory studies in the evaluation of multiple stressor effects that may lead to declining amphibian populations. A range of laboratory and field studies of chemicals, such as herbicides, insecticides, chlorinated organic compounds, metals and complex mixtures were presented. The methods applied in these studies emphasized the role that ASTM International E47's standards play in current hazard and risk assessment practices. These contributed papers also clearly indicated that a number of additional assessment tools need to be developed in the future.

Causal Analysis

Nowhere are research needs more clearly identified than in our third theme, causal analysis. Here, a series of contributed papers illustrate the range of tools currently available for evaluating "cause-effect" relationships between environmental stressors and declining amphibian populations. Research needs identified in these presentations clearly informed the methods development community of methodological issues that must be advanced, if solutions for amphibian deformity and decline are going to be attained.

The editors of this collection of contributed papers would like to thank Dorothy Fitzpatrick, Hanna Sparks, and Maria Langiewicz from ASTM International Headquarters for their help in developing this STP. We are also grateful to the participants of this symposium. This publication would not have been possible without your interest in amphibians and concern for the quality of their widely diverse habitats.

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Toxicity Assessment

James N. Dumont,[1] John A. Bantle,[2] and Greg Linder[3]

The History and Development of FETAX (ASTM Standard Guide, E-1439 on Conducting the Frog Embryo Teratogenesis Assay-*Xenopus*)

Reference: Dumont, J. N., Bantle, J. A., and Linder, G., **"The History and Development of FETAX (ASTM Standard Guide, E-1439 on Conducting the Frog Embryo Teratogenesis Assay–*Xenopus*),"** *Multiple Stressor Effects in Relation to Declining Amphibian Populations, ASTM STP 1443,* G. Linder, S. Krest, D. W. Sparling, and E. Little, Eds., ASTM International, West Conshohocken, PA, 2003.

Abstract: The energy crisis of the 1970's and 1980's prompted the search for alternative sources of fuel. With development of alternate sources of energy, concerns for biological resources potentially adversely impacted by these alternative technologies also heightened. For example, few biological tests were available at the time to study toxic effects of effluents on surface waters likely to serve as receiving streams for energy-production facilities; hence, we began to use *Xenopus laevis* embryos as test organisms to examine potential toxic effects associated with these effluents upon entering aquatic systems. As studies focused on potential adverse effects on aquatic systems continued, a test procedure was developed that led to the initial standardization of FETAX. Other than a limited number of aquatic toxicity tests that used fathead minnows and cold-water fishes such as rainbow trout, *X. laevis* represented the only other aquatic vertebrate test system readily available to evaluate complex effluents. With numerous laboratories collaborating, the test with *X. laevis* was refined, improved, and developed as ASTM E-1439, Standard Guide for the Conducting Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Collabrative work in the 1990s yielded procedural enhancements, for example, development of standard test solutions and exposure methods to handle volatile organics and hydrophobic compounds. As part of the ASTM process, a collaborative interlaboratory study was performed to determine the repeatability and reliability of FETAX. Parallel to these efforts, methods were also developed to test sediments and soils, and in situ test methods were developed to address "lab-to-field extrapolation errors" that could influence the method's use in ecological risk assessments. Additionally, a metabolic activation system composed of rat liver microsomes was developed which made FETAX more relevant to mammalian studies.

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Keywords: FETAX, amphibians, toxicity, teratogenicity

Introduction

In the mid- to late 1970's and early 1980s the United States faced an energy crisis characterized by increased energy use, dwindling domestic supplies of oil, and increasing reliance of foreign sources of oil. President Jimmy Carter signed the United States Synthetic Fuels Corporation Act of 1980 (Public Law 96 - 294; 94 Stat. 633 et seq.) in order to develop alternative fuels (then called, "synfuels") which were intended to relieve the country's sole reliance on imported oil. Synfuels were those that could have been produced from the country's supply of oil shale and tar sands, or from liquifaction or gasification of coal by exposure to high temperatures and pressures (Bergman 1980, Epler 1980, Griest et al. 1981, Mahlum 1981). As these technologies were developed, however, concern for their environmental impacts heightened, since conversion of oil shale and tar sands, and the liquifaction or gasification of coal, yielded effluents and production byproducts that were complex mixtures of organic and inorganic compounds and potentially toxic. The development of these energy technologies, as well as the disposal and management of their derivative complex chemical mixtures, gained the attention of regulatory agencies (e.g., Environmental Protection Agency), academic researchers, national laboratories (e.g., Oak Ridge National Laboratories), and industry. Through their combined efforts, energy technologies and tools for evaluating hazards and risks associated with these technologies were developed. For example, among the biological tests developed for assessing hazards were the Ames Test for evaluating a material's carcinogenicity and mutagenicity (Ames et al. 1975) and tissue culture assays for evaluating other adverse biological effects (e.g., Hsie et al. 1978, Li et al. 1983). Aquatic toxicity tests were developed, including those that relied on macroinvertebrates such as *Daphnia magna* or *D. pulicaria,* (e.g., Biesinger and Christensen 1972, Buikema and Cairns 1980, Geiger et al. 1980) and fishes such as fathead minnow (*Pimephales promelas*) and cold-water fishes (e.g., Sprague 1969, 1970, 1971, Committee on Methods for Acute Toxicity Tests with Aquatic Organisms 1975, McKim 1977). ASTM standards (e.g., E-729 on the Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians) initially published in 1980 were instrumental in setting the stage for developing tools for toxicity assessment. Contemporary with others whose work focused on developing aquatic toxicity test methods (e.g., Mount and Brungs 1967, Cairns and Dickson 1973, Committee on Methods for Acute Toxicity Tests with Aquatic Organisms 1975, Birge 1976, Birge and Black 1977, Peltier 1978), Dumont and others were working with *X. laevis* in laboratories at Oak Ridge National Laboratory (ORNL; Oak Ridge, Tennessee) and began to use amphibian embryos to examine the toxicity of coal and oil shale conversion technologies. Much of this early work provided the starting point for FETAX.

Early Studies with Complex Synfuel Mixtures

Effluents and byproducts of synfuel energy technologies were complex mixtures of a wide range of chemicals, i.e., inorganic chemicals (e.g., arsenic, selenium, sulfur, and heavy metals such as lead, copper, cadmium, and zinc) and organic chemicals (e.g.,

polynuclear aromatic hydrocarbons, aromatic amines, pyridines, phenols, furans, quinolines). For evaluating the biological activity associated with these mixtures, water-soluble materials were diluted and used directly in tests with *X. laevis* embryos. Many materials, however, were less soluble in water, e.g., tars from sources such as electrostatic precipitators. These materials were extracted by stirring an 8:1 mixture of water and test material at room temperature for 16 hours. Extracts were then filtered through glass wool, diluted, and used for testing with amphibian embryos.

One of the first samples studied was "sour water," which was produced from polishing fuel derivatives of the coal gasification process (Dumont and Schultz 1980). These early 96-hr tests used amphibian embryos tested in acute static exposures (no renewal) with four replicates of 50 embryos each in 250 ml test solution. Results exhibited concentration-dependent effects on mortality and abnormal development, growth inhibition, altered pigmentation, and reduced motility in surviving embryos. In these early tests, changes in pigmentation and motility were rated subjectively, although techniques currently available quantify such observations (e.g., Buchwalter et al. 1996). Effects observed in these tests were both acute and chronic. For example, many embryos presented blistering and edema, but when removed from exposure solution, these reversible effects regressed to apparent recovery. In contrast, abnormalities from which embryos did not recover included severe tail flexion, and abnormal visceral and cephalic development (Dumont and Schultz 1980).

These early studies were extended using aqueous extracts of electrostatic precipitator tar from coal gasification processes (Shultz et al. 1982). Interestingly, different abnormality types were found with this material (Figure 1). In Figure 1, the embryo on the left was exposed to control conditions (e.g., culture water) and that on the right had been exposed for 96-hr to 0.5% aqueous extract of tar. Extract-exposed individuals characteristically presented large blisters and epidermal hyperplasia on the dorsal fin (indicated by arrows). In addition these embryos presented malformations of the developing face and jaw.

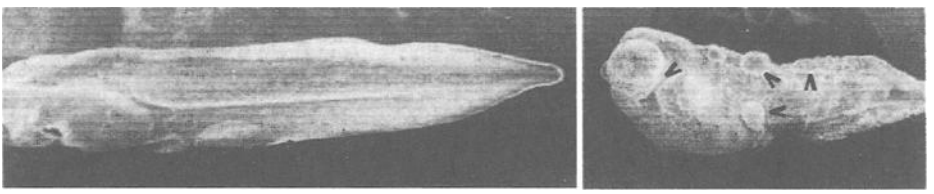

Fig. 1--*Control embryo on left presents typical external morphology, with total length between 9.0mm and 10.0mm at the end of 96-hr test. Exposed embryo on right presents reduced growth, failure to attain typical 96-hr development stage, and blistering and edema along length of body.*

Scanning electron micrographs in Figure 2 illustrate a control embryo (left) and a treated embryo (right). The treated embryo displays significant malformations of the head. In the control embryo the developing papillae, nasal placodes, and the elongate mouth with the oral sucker on the lower jaw are clearly visible. In contrast, the treated embryo shows malformations of the head and mouth in addition to abnormally developing jaws (indicated by arrow).

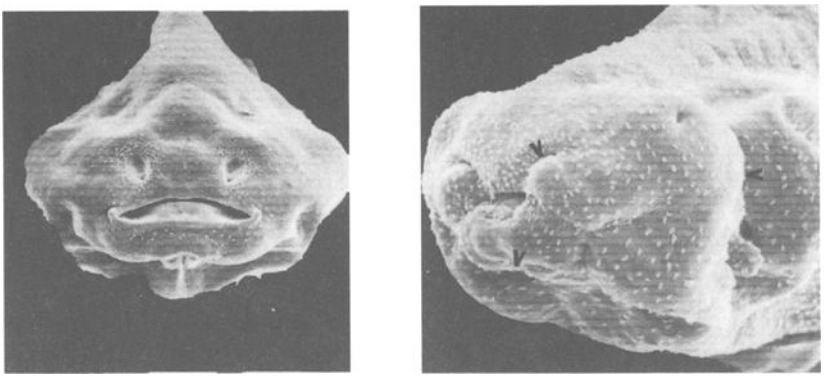

Fig. 2–*Control embryo on left displays typical facial morphology in this frontal aspect SEM micrograph. Exposed embryo on the right displays abnormal facial features described in text, as indicated by arrows.*

Oil shale extracts also produced marked developmental abnormalities, including extreme dorsal flexion of the tail (Figure 3). The embryo illustrated in Figure 3 was exposed to 2.5% aqueous extract of materials derived from oil shale processing, with this scanning electron micrograph showing abnormalities of the developing head, face and mouth.

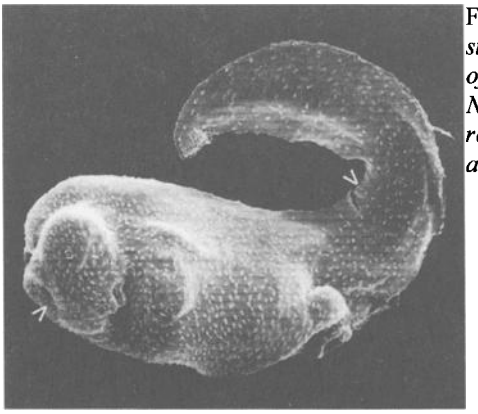

Fig. 3–*Exposed embryo at termination of standard 96-hr exposure to aqueous extracts of derivatives from oil shale processing. Note marked abnormalities on head and recurvature of axial skeleton indicated by arrows.*

A number of similar studies ensued using synfuels (Dumont et al. 1982), fractionated or variously treated raw materials (Schultz et al. 1982a, 1982b, Schultz et al. 1983, Schultz et al. 1984), and pure compounds known to be present in the complex mixtures (Davis et al. 1981, Dumont et al. 1979). In all of these studies concentration-response LC50s (median lethal concentrations) were determined and abnormalities identified. Throughout these studies, developmental abnormalities were observed that were characteristic of the complex chemical mixtures to which the embryos had been exposed. These observations suggested that amphibian embryos would be similarly responsive to

toxicants having similar modes of action, a condition in a vertebrate test system that must be satisfied in order to warrant its use as a screening tool (Rogers and Kavlock 1996).

Studies with Pure Compounds - Teratogens and Non-teratogens

Spurred by these observations and the need for rapid, cost-effective screening tests, workers at ORNL tested *X. laevis* embryos with a series of pure compounds, including known mammalian teratogens (Shepard 1986). In total, 41 mammalian teratogens and ten non-teratogens were tested. Of the non-teratogens, only three compounds yielded false positive results when compared to their status as mammalian teratogens (i.e., were teratogenic to *X. laevis* embryos but not recognized as mammalian teratogens). Of the 41 mammalian teratogens, 85% were identified as teratogenic to *X. laevis* embryos, with these responses characteristically being concentration-dependent. Abnormally developing embryos presented terata characteristic of abnormalities induced by the same chemicals in mammals, which suggested that similar modes of action for developmental toxicants may affect these vertebrate embryos similarly (Rogers and Kavlock 1996).

Other chemicals such as plant secondary compounds were also evaluated with respect to their biological activity in the amphibian test that used *X. laevis* embryos (Schultz et al 1985). Lathyrigens such as semicarbazide are compounds commonly found in vetch and sweet peas, and were originally characterized for their adverse effects on development of muscle, connective tissue and skeletal elements in mammals (Shepard 1986). When *X. laevis* embryos were exposed to lathyrigens (e.g., thiosemicarbazide, thiourea, ethylene thiourea), the developing amphibian embryos presented foreshortening of the long axis and a wavy notochord consistent with the terata developed in traditional mammalian models (Figures 4a and 4b). Here, Figure 4a provides examples of embryos exposed to 100 mg/L thiosemicarbazide and consistently presented axial skeleton anomalies, most frequently characterized by the embryo's bent tail in these photomicrographs. Figure 4b illustrates a cross section through the tail region typical of this abnormality. Here, a scanning electron micrograph of a cross section through the tail region of a treated embryo exhibits the spongy central core the notochord surrounded by muscle tissue. Rather than a symmetrical, intact cord, the sheath of the notochord is weakened and has ruptured (indicated by arrow).

Inorganic chemicals, including metals and metalloids, were also examined using *X. laevis* embryos (e.g., Browne et al. 1979, Dumont et al. 1983a, 1983b). Lithium (Figure 5) produces shortening and curvature of the long axis of developing embryos, and cranial and facial abnormalities. Severe malformation of the gut is also presented by the embryo in Figure 5. Similarly, Figure 6 is an embryo exposed to lead acetate, with marked anomalies being observed about the embryo's head and along the long axis which shows ventral flexion of the tail.

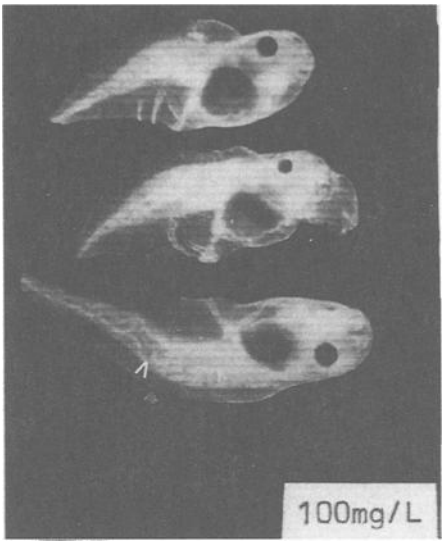

Fig. 4a–*Embryos exposed to 100 mg/L thiosemicarbazide for 96 hours consistently presented reduced growth and axial skeleton anomalies, most frequently characterized by the embryo's bent tail.*

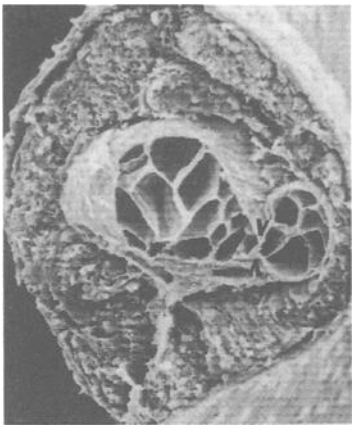

Fig. 4b–*Cross section through strongly bent region of the tail in Fig. 4a. The spongy central core the notochord is surrounded by muscle tissue, but rather than a symmetrical, intact cord, the sheath of the notochord is weakened and has ruptured (indicated by arrow).*

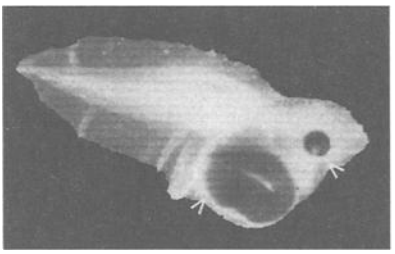

Fig. 5--*Lithium-exposed embryos produces shortening and curvature of the long axis with cranial and facial abnormalities after 96-hr exposures. Severe malformation of the gut is also presented by the embryo.*

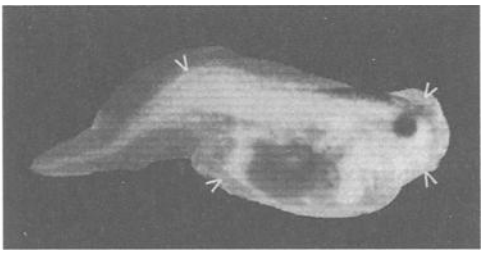

Fig. 6–*Embryo exposed to lead acetate for 96 hours presents marked anomalies about the head and the long axis which shows ventral flexion of the tail.*

Origins of the ASTM Standard Guide

As a result of these early studies, a preliminary standard testing procedure was developed at ORNL for testing with *X. laevis* embryos. That standard procedure specified test duration (96-hr), stage of embryos used to initiate test (late blastula to mid-gastrula), number of embryos per test replicate (20 or 25), incubation temperature (22°C), standardized culture solution, and the use of at least three mating pairs per test to mitigate differences between adult pairs. Since the developing embryos were relatively transparent, internal anatomy could be easily observed and by 96-hr of age most of the organ systems have differentiated and could be evaluated as test endpoints.

With its initial publication as ASTM E-1439 in 1991, FETAX became available as a standard aquatic toxicity test complementary to others available at the time (e.g., fathead minnow toxicity test and fathead minnow teratogenicity test). However, FETAX presented greater leverage to the user, especially those focused on evaluations of teratogenicity, since *X. laevis* brought with it a long history of use in developmental biology (Shi 2000) and examined both toxicity and developmental endpoints. Also, *X. laevis* presented life history attributes amenable to laboratory testing, since the species was readily available, was easily maintained in the laboratory, could be hormonally induced to reproductive condition throughout the year (unlike North American amphibians which are seasonally reproductive), and could provide large numbers of embryos for testing. Experimentally, the test procedures could be applied to other amphibian species. Many species have been tested and have yielded results consistent with those obtained with *X. laevis*, although species sensitivities vary (Birge et al. 2000). In the early studies with amphibian embryos, testing insoluble materials was accomplished by using low concentrations (usually less than 2%) of solvents such as ethanol, dimethyl sulfoxide, propylene glycol, acetone or others as constituents of FETAX solution (as later specified in E-1439). As specified in E-1439, FETAX or modifications of FETAX using alterative species or modified exposure conditions could be used to help evaluate hazards and risks associated with chemicals released to the environment.

Endpoints

Endpoints measured in FETAX are easily collected, and critical to the hazard and risk assessment process. For example, mortality is recorded daily and at termination of the test the number of abnormal embryos is noted along with the type of abnormality. These data provide LC50 and EC50 (median effective concentration for terata based on number of abnormal embryos among the survivors) data, which may be applied to derivative measures as outlined in ASTM Standard E-1439 (e.g., teratogenic index, LC50/EC50). Embryo growth is measured at the end of the test and is an important indicator for the general response of the developing embryos. Reduced growth is a common response in exposures to teratogens, and growth rates of exposed embryos may also be affected; hence, the stage of development reached in 96-hr is a critical endpoint to record.

Behavioral endpoints related to motility (e.g., avoidance behavior, increased or decreased response to external stimuli) and changes in pigmentation may be endpoints

critical to the evaluation of some chemicals, depending on their mode of action, e.g., neurotoxicants.

Pulsed Exposures and Modified FETAX

As suggested in ASTM Standard E-1439, FETAX may be modified to address chemical-specific or application-specific issues related to exposure or test species, e.g., the examination of sensitive development stages. For example, pulsed exposures to the developing embryos may be administered at various times during the 96-hr static renewal. Figure 7 pictures embryos exposed to the DNA synthesis inhibitor hydroxyurea

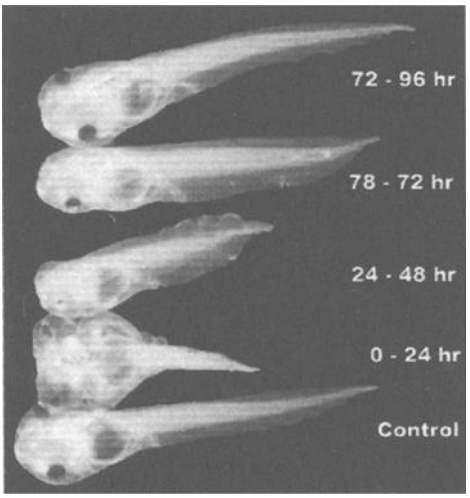

Fig. 7–*Embryos exposures to hydroxyurea at different intervals during the 96-hr exposure manifested time-dependent effects, with embryos treated in earlier stages (e.g., 0-24 hours of test) presenting abnormalities in development of the eye, marked edema, and growth inhibition. These abnormalities were not reversible, despite subsequent renewals consisting of FETAX solution without hydroxyurea, and were not as marked in embryos exposed to 24-hr pulses during later renewals of the 96-hr exposure.*

at specified time intervals during the 96-hr exposure (Dumont et al. 1983c). During these periods the exposure medium included hydroxyurea, while untreated media was used throughout the other renewal periods in the test. As suggested by these examples of treatment groups, those embryos exposed during the first 24-h were most sensitive to hydroxyurea exposures, while those exposed during later stages of development are progressively less sensitive as far as adverse effects on growth are concerned. Embryos treated in earlier stages also present abnormalities such as abnormal eye development and edema in addition to growth inhibition. As these pulsed exposure tests suggest, the flexibility of FETAX as outlined in ASTM Standard E-1439 set the stage for future derivative tests focused on extended exposure periods and critical periods during development, e.g., tail resorption tests and others focused on hormonal disruption and its influence on normal metamorphosis (e.g., Fort and Stover 1997, Fort et al. 2003a in this volume).

Further Development of FETAX

Defining FETAX solution and alternative exposure regimens

After the initial development of ASTM E-1439, work with FETAX continued with an emphasis on defined media and different methods of exposure. For example, after FETAX was initially described by Dumont et al. (1979) and Dumont and Schultz (1980), standardizing the exposure medium was addressed, and FETAX solution was more fully developed and characterized in order assure more comparable test results with pure compounds (Dawson and Bantle 1987). As summarized in E-1439, the phosphate buffer system provides a foundation for FETAX solution that complements the role that pH plays in the solubility of many toxic chemicals, including metal ions. For some test materials, e.g., lead compounds such as lead acetate, the salts in FETAX solution are too concentrated to achieve complete solubility, but in these cases, FETAX solution may be diluted with distilled water without adversely effecting test performance, owing to the tolerance of the test embryos. *X. laevis* embryos characteristically present a wide range of sensitivity to salinity. Experiments may be conducted with brackish waters without confounding interpretation of test results, since control embryos develop normally between 0.5x to 2x FETAX solution. When surface waters are being evaluated within the context of ecological assessments, tests are often modified by using alternative amphibian species and substituting surface waters for the standard FETAX solution (Linder et al. 1991; see also Linder 2003 in this volume).

Originally, FETAX required 8-10 ml of test solution and 20 to 25 embryos per exposure, which is reflected in ASTM standard guide E-1439. However, early efforts in developing amphibian tests (e.g., Birge et al. 1985, Linder et al. 1990) and contemporary studies using modified FETAX (e.g., see Linder 2003 in this volume) clearly illustrate the flexibility available to the user of ASTM standard guides, and the potential confounding effects when minimum test specifications are exceeded (e.g., Schuytema et al. 1991). Test volumes and number of embryos per exposure may be modified to the specifications required of any particular study design, and if greater embryo-to-volume ratios are desired, exposure conditions can be modified following ASTM E-729, Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians or E-1192, Standard Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians. For short-term 96-hr tests, however, the guidance in E-1439 has a long history of attaining the test's specified performance standards as reflected in the published results of the interlaboratory tests (Bantle et al. 1994a, 1994b, 1996, 1999a, Fort et al. 1997).

Modifications to Standard FETAX Solution and Static Renewal Exposure

Early work centered on issues of solubility of test materials and the use of carrier solvents suggested that the ASTM standard could be enhanced by better characterizing steps to address poorly water-soluble chemicals. The early work was continued by Rayburn and others (Rayburn et al. 1991), and a number of candidate co-solvents were evaluated to address solubility issues associated with hydrophobic substances intended

for testing with FETAX. Much of this work indicated that the use of co-solvents would be highly problematic because interactions between test material and co-solvent would confound test results, and if co-solvents were used as a constituent of FETAX, ample controls must be provided to account for any interactions that may occur (Rayburn et al. 1991). Testing volatile materials was also addressed by using either continuous-flow dilution methods or, when quantities of test materials were limited, a static system employing a 250 ml sealed glass jar with a teflon-lined cap. After the introduction of FETAX solution, the solution was oxygenated via bubbler until a dissolved oxygen concentration of 8 mg/L was reached. Embryos and test material were then added, the jar sealed, and conducted for 96 hours as usual. In any of these examples, the various implementations and flexibility characteristics of E-1439 are evident, and variations from standard FETAX procedure were reported as indicated by the standard.

Routine animal husbandry, scoring abnormal embryos, and metabolic activation system

To provide a common starting point for early developmental testing with amphibians, ASTM E-1439 provides details on animal care related to routine husbandry (e.g., breeding and feeding) along with methods of data analysis and standardized forms. Negative and positive controls were defined as well as acceptable performance limits. To assist with the identification of malformations, the "Atlas of Abnormalities" was published and updated (Bantle et al. 1999b). Additionally, the 1998 revision to ASTM E-1439 included guidance on the application of an optional in vitro metabolic activation system which had been developed and validated since the standard's original publication in 1991. Similarly, to reinforce the standard's application to evaluations of chemical risks to amphibians, other studies using alternative test species were characterized and compared to the available data generated from tests with *X. laevis*. While differences in exposure length, temperature and stage of development at test initiation demonstrate the flexibility of FETAX when applied to alternative test species or exposure scenarios, these differences may confound direct interspecies comparisons. From the literature currently available, *X. laevis* appears to be a moderately tolerant test organism compared to other amphibians (see Birge et al. 2000), although work completed by DeYoung and Bantle (1996) indicated that *X. laevis* is more sensitive to a larger number of developmental toxicants than fathead minnows when experimental factors were comparable.

Also during this time in the method's development, an interlaboratory study on repeatability and reliability of FETAX was initiated with multiple laboratories (six to eight laboratories collaborated throughout the study). Results from the interlaboratory study were used to improve the standard guide, which is reflected in the 1998 revision of ASTM E-1439. For example, the revised standard guide included procedures for handling soils, sediments and volatile materials. Since the method's initial standardization, field studies had also been conducted that included modifications of the amphibian test for work in situ, e.g., Bruner et al. (1998) worked with bullfrog and toad embryos in tandem with FETAX and demonstrated that the laboratory method was a reliable predictor of toxicity in the field. Earlier integrated field and laboratory studies (see Linder 2003 in this volume) had indicated these and other field studies (Bishop and Martinovic 2000) could make significant contributions to the hazard and risk assessment process for amphibians and wetland habitats (Lemly et al. 1999). FETAX had also been

used to assess the developmental toxicity of UV light (Bruggeman et al. 1998) and microorganisms (Genther et al. 1998).

FETAX Interlaboratory Study

Following the publication of ASTM E-1439 in 1991, an interlaboratory study (ILS) was undertaken to quantitatively characterize the repeatability and reliability of FETAX, following ASTM Standard E-691. ILS was accomplished through the collaboration of six to eight laboratories over a three phase testing program.

Phase I testing indicated that FETAX, as conducted by inexperienced users, would yield acceptable results, but training was acknowledged as a factor critical to optimizing test performance (Bantle et al. 1994a). In response to Phase I testing, training workshops were conducted for all participating laboratories, and the "Atlas of Abnormalities" (Bantle et al. 1999b) was published. These efforts were essential to standardizing training and reducing the variability apparent from Phase I testing. Because FETAX requires trained and experienced users, especially in the evaluation of embryo malformations, the lack of experience is often reflected in test variability associated with characterization of malformed embryos, particularly when the toxicant is a weak teratogen that does not cause dramatic abnormalities.

Phase II was conducted using highly water-soluble chemicals and suggested that training and prior knowledge of test concentration ranges enhanced test performance (Bantle et al. 1994b). Parkhurst et al. (1992) had previously demonstrated that whole embryo toxicity test yielded acceptable results when coefficients of variation values were less than 50 percent, and FETAX performance under Phase II testing easily conformed to these expectations. Bantle et al. (1994b) completed an analysis of interlaboratory test results following ASTM Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method, ASTM E-691 and found little or no variation outside acceptable limits, which clearly indicated that FETAX could be performed repeatedly and reliably, given training and experience with the test.

Phase III, Part 1 testing demonstrated a limitation in FETAX, as well as other toxicity tests based on designs that relied on unspecified dilution series (Bantle et al. 1996). In Phase III, Part 1 each collaborating laboratory determined their initial concentration ranges for testing. Some laboratories chose very wide concentration ranges, while others selected very narrow test ranges. Interlaboratory variation was higher in Phase III, Part 1 testing and prompted greater specification in range-finding and definitive testing procedures in ASTM E-1439 as revised in 1998.

Phase III, Parts 2 and 3 employed the metabolic activation system (MAS). Although the study was reduced to three laboratories in this final phase, MAS did not appear to cause any additional variation in FETAX despite the inclusion of rat liver microsomes and generator system (Bantle et al. 1996, Fort et al. 1997, Bantle et al. 1999a). Overall, results of the ILS helped optimize FETAX and suggests that test performance as defined by variability is consistent with other whole animal toxicity tests (e.g., fathead minnow or *Ceriodaphnia dubia*).

Sediments and Soils

As suggested by integrated field and laboratory studies completed in parallel with development of laboratory methods (Linder et al. 1991), amphibians may serve as species-level indicators of wetland health, which lead in part to the development of sediment and soil testing procedures for FETAX. Simple aqueous extractions of sediments and soils were incorporated into ASTM E-1439, and tests with amphibian embryos could be completed using the extracts as the test materials. In addition to tests completed with aqueous extractions of sediment and soil, ASTM E-1439 could be modified to test with the solid matrix directly (Fort et al. 1996). For example, amphibian embryos could be directly exposed to sediments or soils by suspending embryos over a 50 g sample in a 250 ml sealed wide mouth jar (Figure 8). In this modification of ASTM E-1439, embryos rest on an inert 100-micron stainless steel mesh suspended above the sediment in a 55 mm Pyrex glass pipe. The test apparatus provides a non-invasive method of exposure and does not damage the embryos. As in experiments using volatile materials, these tests were usually conducted with the test apparatus sealed to minimize loss of volatile organics, and oxygen depletion was avoided through aeration. Tests with sediments and soils indicated that toxic constituents were not necessarily tightly bound to the solid-phase material. Hence, amphibian tests on sediments and soils potentially bring pertinent information to the evaluation of ecological risks and remedial decisions (e.g., Hutchins et al. 1998, Fort et al. 2003b).

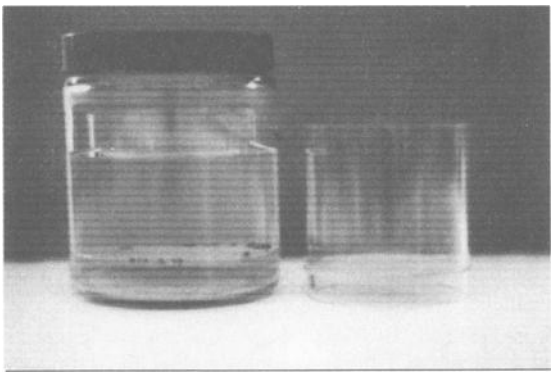

Fig. 8–*Exposure apparatus for modified FETAX when sediments are being evaluated with amphibian embryos.*

In Situ Studies and Use of North American Species

At many instances, modified FETAX may be conducted in the field with native amphibians. Field testing eliminates the possibility of sample changes attendant upon collecting and storing samples, and helps reduce "lab-to-field" extrapolation error. While temperature fluctuations and waters varying widely in tonicity and pH and must be

considered in field testing (Linder 2003 in this volume, as well as general guidance provided by ASTM E-2122 Standard Guide for Conducting In-situ Bioassays with Marine, Estuarine, and Freshwater Bivalves), certain questions, for example, those related to multiple stressors and field conditions potentially altering (ameliorating or exacerbating interactions), can only be answered with field studies. Various exposure "cages" may be used in conducting field tests (see Linder 2003 in this volume, Bishop and Martinovic 2000 for overview of field test methods), but regardless of apparatus, early-stage embryos can be collected from reference areas and placed in areas of concern for evaluating adverse effects in the field

Parallel to field tests, FETAX may be completed in the laboratory using indigenous species. Among them *Rana catesbiena, Rana pipiens, Bufo fowleri* and *Bufo americanus* are recommended in ASTM E-1439, but other species may also be used. FETAX requires that the test start at early blastula and that continuous exposure occur through primary organogenesis. This standard should be maintained so that data are comparable. However, development is highly temperature-dependant and varies greatly among species, so test exposure times will also vary for each species and developmental stage at test termination should be a recorded endpoint. A number of FETAX or modified FETAX studies have been conducted with native species and show distinct species differences in sensitivity to certain toxicants. On the basis of the available comparative toxicity data, *X. laevis* sensitivity appears to lie mid-range within the species tested and shows the difficulty of making species comparisons when developmental times and, therefore, exposure times vary (Dumont unpublished, Birge et al. 2000).

Summary

Since its inception in the 1980's, FETAX has undergone numerous modifications and improvements which are reflected in ASTM E-1439, Standard guide for conducting Frog Embryo Teratogenesis Assay-Xenopus. FETAX is flexible in its implementation by the biological testing community, and it has been useful as a short-term, cost-effective screen for evaluating chemical effects in aquatic environments, including wetlands, and has been useful in supporting hazard and risk assessments for a variety of applications. Although originally developed with *X. laevis* as the primary test organism, users have applied modified FETAX to other amphibian species and adapted procedures accordingly, especially within ecological contexts. Users have implemented FETAX to evaluate various complex environmental samples, aqueous extracts of insoluble materials ranging from insoluble chemical complexes such as tars to soils and sediments, and the myriad of pure compounds that enter the marketplace annually. The addition of MAS to FETAX potentially extends the test to examine early developmental effects on mammalian systems. FETAX can serve as a basis of many environmental testing needs, and can identify both embryotoxicity and potential teratogenic effects.

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The FETAX of Today – and Tomorrow

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Abstract: Frog Embryo Teratogenesis Assay – *Xenopus* (FETAX) – was originally developed in the mid-1980s as a developmental toxicity screening test for pure chemicals and complex mixtures in the laboratory. The longevity and success of the FETAX model can be attributed to several factors including the overall reliability of the assay, method standardization, and the versatility of the model system. Until recently, the versatility of the FETAX model had not been exploited. Today, however, developmental toxicity screening is one of many different applications of the FETAX model. This model is now used to evaluate modes of biotransformation, detoxification, and understand mechanisms of actions; as a model for studying limb development; a model for evaluating endocrine disrupting chemicals, including those acting on the thyroid axis; more advanced ecotoxicological evaluation including the use of alternative species; *in situ* monitoring; impacts of multiple stressors, and more complicated lab-to-field extrapolations; as a model for studying nutritional essentiality and nutritional toxicology; as a system for evaluating mixtures, mixture interactions, and developing structure-activity relationships; and as a model for evaluating reproductive toxicity. Several of these applications of the FETAX model now include a multiple endpoint approach utilizing a combination of whole embryo-larval morphological endpoints with suborganismal and molecular markers with the goal of obtaining more substantive mechanistic information. For example, a tail resorption and limb emergence assay morphologically marking thyroid activity coupled with thyroid hormone and thyroid receptor binding assays are being used to evaluate toxicological impact on the thyroid axis. Most recently, development of new partial lifecycle methods and a new full life-cycle test protocol was developed.

Keywords: frog embryo teratogenesis assay – *Xenopus*, applications, chemical screening, endocrine disruption, limb development, biotransformation, mixtures, nutrition

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The origin, ASTM standardization, and validation of the frog embryo teratogenesis assay – *Xenopus* (FETAX) – model as a short-term developmental toxicity model was described in the present STP (see Dumont and Bantle, STP 1443). Historically, this period spanned approximately 20 years, although the process of validating this assay continues today. The true merit of alternative model systems like FETAX lies in their versatility as models. The capacity to be broadly applied to different areas of research dramatically increases the value of an alternative model system. In this section, we will discuss the milestones achieved in the development of FETAX, the ramifications these milestones have on the future of FETAX, review recent applications of the FETAX model, and discuss the future of FETAX.

FETAX Milestones

The Four Primary Milestones

Although many milestones in the development, validation, and application of the FETAX model could be described, the most significant milestones can be summarized in four events: ASTM standardization (ASTM 1998), publication of the Atlas of Abnormalities (Bantle et al. 1998), review by the Interagency Coordinating Committee for the Validation of Alternative Test Methods (ICCVAM) (NIEHS 1997), and acknowledgement of the need for and appropriateness of amphibian test methods in screening for potential endocrine disrupting agents by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) and incorporation within the Endocrine Disruptor Screening Program (EDSP) (U.S. EPA 1997). Although the EDSTAC did not specifically recommend the use of FETAX in EDSP, inclusion of amphibian assays to assess reproductive, developmental, and thyroid dysfunction was a positive step in the use of derivations of the FETAX concept of using amphibians in ecotoxicological monitoring.

ASTM Standard Guide and Atlas of Abnormalities - The continued use of the FETAX model in many different forms can be attributed to the development of a consensus guide to the performance of FETAX through ASTM. ASTM not only provided peer-reviewed guidance on the development of the FETAX standard, but also provided assistance on the appropriate methods for validating the test method. Since method validation is never truly complete and standardization is a continually evolving process, the FETAX Standard Guide will continue to be reviewed at least every seven years by ASTM to ensure the adequacy of the standard and to incorporate new research findings. The FETAX Standard Guide (ASTM 1998) is currently in its second edition.

The Atlas of Abnormalities (Bantle et al. 1998) was originally developed as a pictorial companion document to the ASTM Standard Guide. The Atlas of Abnormalities describes the malformation syndromes observed in *Xenopus* and provides a key to scoring abnormalities in developing amphibians. It is likely that this document will be incorporated into the ASTM Standard Guide during its next revision in 2005.

ICCVAM - ICCVAM was established in 1994 by NIEHS to develop a report recommending criteria and processes for validation and regulatory acceptance of toxicological testing methods that would be useful to Federal agencies and the scientific

community. In late 1998, ICCVAM initiated an evaluation of the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). The ultimate objective of the ICCVAM evaluation was to determine applications in which FETAX may be successfully used and to determine areas of use, which require further validation. Ultimately, the outcome of the ICCVAM evaluation prioritized further research needs and validation efforts to maximize understanding of how FETAX can be most effectively used as an alternative test method. To assist the ICCVAM expert panel in its evaluation of the validation state of FETAX, a background review document was prepared based on the existing data available from FETAX validation studies prior to a workshop to discuss the validation state of FETAX. The workshop topics included a detailed discussion of test method status, performance, reliability, environmental applications, ongoing research, and further research needs.

A summary of the meeting was provided in an article prepared by BNA News (Andrew M. Ballare, BNA News, May 1998).

"A frog embryo toxicity test method being reviewed by a scientific review panel is difficult to evaluate without better information on what it will be used for," members of the panel said May 16. According to Nigel A. Brown, a science professor with the University of London, the desired uses for and outputs from the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) are important to determining whether the in vitro toxicity testing method is appropriate and effective. Brown made his comments before an expert panel convened by the National Institute of Environmental Health Sciences. He is the co-chair of the subgroup charged with evaluating the method's performance. FETAX originally was developed in 1983 by workers at the Oak Ridge National Laboratory. The assay involves toxicity testing of chemicals on the Xenopus frog species' embryos. Methodologies that included additional testing and procedures were developed by John A. Bantle, Zoology Professor at Oklahoma State University, Douglas J. Fort, VP at The Stover Group (presently President, Fort Environmental Laboratories), and other scientists and were published from 1988 to 1995.

EPA Request

In May 1998, the Environmental Protection Agency requested that the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluate the effectiveness of the Xenopus method for toxicity testing.

ICCVAM formed an expert panel that is meeting May 16-18 in Durham, NC, to evaluate the test method. Groups were formed to evaluate the protocol, reliability, performance, and potential environmental applications of the method, as well as research and development issues related to it. "Currently, the performance of FETAX is an unknown quantity," Brown told panelists. He said the effectiveness of the method depends on its intended use, which was not clearly defined by federal regulators. In addition, Brown told the group, whether the method was to be used to determine effects on humans, environmental effects, or the potency of certain chemicals also was important to know to evaluate its appropriateness and reliability. According to Brown, his

group's initial recommendations also included that the raw data be run through another prediction model that could be used in a new validation study.

Concerns About Results

Members of the panel also expressed concern about the range of results between labs and experiments that used the FETAX method. According to Susan Hurt, program manager with Rohm & Haas Co.'s toxicology department and co-chair of the reliability group, the variability in the analysis of toxic effects across and between various labs can have a significant effect of many reliability issues.

*"Any time you have variability, you develop problems with transferability," she told the group. Another area of concern for the reliability group members is that the method only uses three pairs of frogs to produce the embryos in the study, according to Hurt. More pairs should be used because "genetic variability might be a major contributor," she said. However, despite panel members' initial concerns, Hurt said, "**there's something here that needs to be built upon.**"*

The ICCVAM report provided limited information on the validation status of FETAX primarily because the intended use of FETAX in a regulatory context was not well defined prior to the meeting. As noted above, "FETAX is an unknown quantity." FETAX was one of the first alternative assays and the first alternative developmental toxicity model to be reviewed by ICCVAM. In retrospect, the ICCVAM review process suffered from two key problems aside from the lack of intended use definition including, 1) inability of the environmental/ecotoxicology and mammalian toxicology subgroups to agree on mutual criteria for successful alternative assays that are broadly applicable for both arenas, and 2) lack of a "gold standard" to compare FETAX results. In the case of the latter, it was identified that comparison of FETAX results to existing mammalian test methods was difficult since results in rodents and rabbits were equivocal. In fact, the compounds selected for the validation study produced primarily equivocal results in the *in vivo* mammalian models. Thus, this represented a poor selection of test materials for validation purposes. When the review process was complete, the results were somewhat anticlimactic; however, several lessons were learned during the ICCVAM review. First, clearly establishing an intended use for an assay is essential when reviewing validation data. Second, validation of assays intended for regulatory use should be done under stringent quality assurance (QA) requirements, preferably good laboratory practices (GLP). Third, valuable research insights into improving FETAX should be considered. The ICCVAM workshop participants recommended two primary areas of research focus. These recommendations included the use of alternative species, including close relative *X. (Siluria) tropicalis* and native U.S. species, and the development of lifecycle test methods with both reproductive and developmental endpoints.

Current research has identified the following advantages and disadvantages of using *X. tropicalis* in the FETAX model (Table 1). Use of *X. tropicalis* effectively allows FETAX to be conducted in ca. 2-d as opposed to 4-d with *X. laevis*. However, the increased rate of development in *X. tropicalis* needs to be evaluated thoroughly as it may have an impact on the sensitivity of the test. Often species that develop at cooler

temperatures develop more slowly than those at greater temperatures and thus, are more sensitive presumably due to longer developmental windows (i.e., larger exposure targets). Since FETAX currently uses an exogenous metabolic activation system (MAS) consisting of rat liver microsomes, the increased temperature will increase the effectiveness and efficiency of the MAS. The diploid genome of *X. tropicalis* also represents a genetic advantage over *X. laevis*, which is oligotetraploid.

Table 1 – *Comparison of Xenopus laevis and Xenopus tropicalis*

Consideration	X. laevis	X. tropicalis
Rate of Development (time to complete FETAX test)	4-d	2-d*
Culture Temperature	23°C	26-28°C*
Ploidy	Oligotetraploid	Diploid*
Chromosomes		
Genome Size		
Egg/Larvae Size	Larger	Smaller*
Ease of Breeding	Easy*	Easy to moderate
Clutch Size	1500/female	2500/female*
Transgenic Capacity	Moderate	Good*
Time to Sexual Maturity	2 years	4 months*
Capacity to Develop Inbred Lines	Little	Great*
Disease Susceptibility	Relatively Low*	Low to Moderate
Literature Available	Large database*	Growing database

*Represents a significant advantage.

The smaller size of *X. tropicalis* compared to *X. laevis* presents practical advantages for the assay. Less test material is required for the smaller organism and more organisms can be accommodated in each replicated treatment. *X. laevis* produces a greater quantity of waste products during the assay than *X. tropicalis* [ca. 1.5 mg/L NH_3-N vs. <0.5 mg/L NH_3-N] Fort, unpublished data). Increased clutch size also creates a significant production advantage for the FETAX model. One of the most exciting advantages is the increased capacity to create transgenic lines of *X. tropicalis* and the use of cDNA microarray technology. It is also possible to create inbred strains of *X. tropicalis*, which will likely decrease the genetic variability observed in *X. laevis*. Developing inbred lines of *X. laevis* has been difficult at best.

In addition to the aforementioned advantages of *X. tropicalis,* the shorter life-cycle allows more expedient and practicable lifecycle, reproductive toxicity studies, and endocrine evaluation of the thyroid axis during metamorphosis. Currently, methods are being developed that address lifecycle, reproductive toxicity, and endocrine disruption chemical (EDC) screening assays. To specifically address the ICCVAM workgroups request to develop lifecycle test methods with *Xenopus,* two methodological approaches have been developed. The first involves a *X. tropicalis* embryo-to-reproductively mature adult exposure. A subset of the reproductively mature adults are necropsied to evaluate development and to study reproductive parameters. A separate subset is bred in a cross-over design, and the progeny (F_1 generation) are cultured for varying lengths

to evaluate transgenerational effects. The cross-over breeding design establishes which sex is contributing to transgenerational effects in the progeny. The second design (Fort et al. 2001a) approach involves exposure of reproductively mature *Xenopus* to a test substance or mixture for a period of approximately 30- to 45-d. The specimens are initially flushed of eggs to establish a baseline breeding performance and so that egg maturation dynamics can be studied. At the end of the exposure period, the frogs are subdivided for necropsy and breeding, as described for the first method. In this method, the progeny are also cultured to evaluate transgenerational effects. We are currently working on adaptation of these methods in ranid species. This work, however, has been substantially more challenging due to the length of time required to achieve sexual maturity.

Studies comparing the sensitivity and practicability of use of *X. tropicalis* relative to *X. laevis* are nearing completion. However, it is too early to determine whether global replacement of *X. laevis* with *X. tropicalis* in FETAX will be appropriate.

Recommendation of the use of native species with a modified FETAX model was reasonable. Since FETAX was designed as a model system, the use of species other than *Xenopus* was fully intended. The rationale for use of other native species or species was based on two primary premises: 1) "the Amphibian Bill of Rights" and 2) the need for site- and case-specificity. We have coined the phrase the "Amphibian Bill of Rights", which is the reverse of our constitutional Bill of Rights that suggests that all men are created equal. With amphibians, we must recognize that there is diversity amongst amphibian species and that different species possess different life history traits. Thus, the responses of different amphibian species to different environmental stressors can vary widely. Today, alternative short-term developmental toxicity protocols have been established for several anuran species, including *Rana sp.* and *Bufo sp.*, as well as several urodeles.

EDSTAC and EDSP - Concerns regarding both the presence of endocrine disruptors in food, water, or other environmental media and the potential risk they pose to humans and wildlife have been growing in recent years. Passage in 1996 of the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) reflected these concerns and required EPA to:

"... develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen or other such endocrine effect as the Administrator may designate."

Specifically, EPA was required to develop a screening program by August 1998, to implement the program by August 1999, and to report to Congress on the program's progress by August 2000. In 1996, EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), charging the Committee to provide advice on how to design a screening and testing program for endocrine disrupting chemicals. The committee recognizes that the science of endocrine disruption is rapidly and continually evolving. EPA will need to incorporate the results of on-going research and recent publications when implementing the Committee's recommendations.

The EDSTAC was composed of individuals representing various stakeholder groups and scientific expertise. The members included scientists and other representatives from EPA, other federal agencies, state agencies, various sectors of industry, water providers, worker protection organizations, national environmental groups, environmental justice groups, public health groups, and research scientists. The Committee began their deliberations in October 1996 and completed their recommendations in July 1998.

The conceptual framework provides the structure for the EDSTAC's recommendations for screening and testing. The Committee determined that a tiered approach would be most effective in utilizing reasonably available resources to detect endocrine disrupting chemicals and quantify their effects. The core elements of the approach include initial sorting, priority setting, Tier 1 Screening (T1S), and Tier 2 Testing (T2T). A chemical entering the framework would go through initial sorting based on existing data. An evaluation and analysis of this information would direct the chemical to one of four categories. The first would lead to the "hold box," indicating that the chemical is not likely to interact with the estrogen-androgen-thyroid (EAT) hormone systems and no further analysis is required at this time. The second category contains chemicals without sufficient data to make a determination to proceed to T2T or hazard assessment. These chemicals enter the priority setting and, from there, proceed to the T1S portions of the endocrine disruptor screening and testing program (EDSTP). The EDSTAC anticipates that most chemical substances and mixtures entering the program will fall into this category. The third category includes chemicals with sufficient existing data to move directly to T2T (i.e., existing data meet Tier 1 requirements). The fourth category consists of chemicals with sufficient existing data to move directly to hazard assessment (i.e., existing data are adequate for both Tier 1 and Tier 2 requirements). Amphibian test methods were recommended by the EDTAC in T1S (amphibian metamorphosis assays) and T2T (amphibian full and partial lifecycle assays) and have been tentatively incorporated into EDSP.

The EDSTAC recognized that biological effects data are incomplete or lacking for most chemicals, a condition which makes priority setting difficult. To help address this problem, the EDSTAC recommended that some of the Tier 1 Screening (T1S) assays be conducted in a high-speed, automated fashion to provide preliminary hormonal or biological activity information. This approach is called "high throughput pre-screening" (HTPS) where, rather than following traditional manual sample preparation, handling, and analysis procedures, automated techniques are used to accelerate the assay process. Such a process permits a large volume of chemicals to be tested in a short period of time. The EDSTAC recommends that HTPS be conducted on: (1) all chemicals with current production volumes greater than 10,000 pounds per year (estimated to be approximately 15,000 chemicals); (2) all pesticide active ingredients and formulation inerts; and (3) all chemicals that are proposed to bypass either T1S or both T1S and Tier 2 Testing (T2T) for any reason. If HTPS validation is successful, the EDSTAC believes that HTPS can be a powerful, cost effective tool in the EDSP. Work is currently underway to evaluate the most practicable amphibian tools for addressing endocrine disruption in amphibians (see Recent Applications of FETAX section) including HTPS models.

Recent Applications of FETAX

Currently, the FETAX model may not be appropriate for use as a regulatory mandated developmental toxicity assay for human health assessment based on the review by ICCVAM, until further validation studies are completed. However, this has not hindered research in the broad use of the FETAX model for a variety of applications. Several of these FETAX applications are listed in Table 2.

Table 2 – *Applications of the FETAX Model*

1. Chemical Screening/Prioritization of Discovery Chemicals
2. Modes of Biotransformation, Detoxification, and Mechanisms of Action
3. Model for Limb Development
4. Model for Evaluation of Thyroid Disruption
5. Ecological Hazard/Risk Assessment
6. Nutritional Toxicology
7. Mixtures/QSAR

Chemical Screening/Prioritization of Discovery Chemicals

Although the general focus of this paper is to describe the applications of FETAX beyond chemical screening, derivations in the use of chemical screening information including advanced testing prioritization, structure-activity relationships (SARs), and site of action prediction in higher animal models has been successful (Fort et al. 2000). Discovery chemical prioritization is useful in determining which newly developed chemicals should be tested first in the traditional developmental toxicity models and which ones should be set low on the priority list (Table 3). Structure-activity relationships have allowed the determination of specific chemical moieties within chemicals that are bad actors and permitting chemical reconstruction in some cases. FETAX has also been used to predict sites and mechanisms of action (Fort et al. 2000a). This enabled a detailed prioritization and histological review of specific organ systems in mammals during subsequent testing. It has also provided a means of keying on specific embryological mechanisms of action.

Biotransformation and Detoxification

The original intent of incorporating an exogenous rat liver metabolic activation system into the FETAX model was to increase the relevancy to mammalian model systems (Bantle et al. 1988). The use of an exogenous MAS has been valuable in evaluating modes of biotransformation and detoxification. To date, FETAX with the complimentary MAS, has been used to evaluate modes of biotransformation and its effect on the developmental toxicity of cyclophosphamide, nicotine, diphenylhydantoin, isoniazid, acetaminophen, trichloroethylene, caffeine, coumarin, 2-acetylaminofluorene, benzo(α)pyrene, 4-bromobenzene, thalidomide, and most recently ethanol and

Table 3 – *Prioritization of Discovery Chemicals for Further Evaluation using FETAX*

Chemical Family	Compound	Endpoint		Teratogenic Hazard Ranking[3]
		TI[1]	MCIG[2]	
1	A	6.2	10	1
	B	1.0	100	4
	C	1.6	100	3
	D	2.6	35	2
	E	1.0	100	5

[1] TI (Teratogenic Index) = LC50/EC50(malformation).

[2] MCIG - Minimum Conentration to Inhibit Growth (ANOVA, Bonferonni t-test, $p<0.005$). Expressed as % of LC50 value.

[3] 1 represents greatest hazard and 5 represents the least potential hazard.

thioacetamide (Dawson et al. 1988, Fort et al. 1988, 1992, 1993, 1996a, 1996b, 1998a, 2000b, in press a, Fort and Bantle 1990a, 1990b, Propst et al. 1997). One of the greater assets of the FETAX model is the robustness of the embryos. An example of the use of different rat liver MASs with the notorious proteratogen thalidomide is provided in Table 4 (Fort et al. 2000). Without addition of the exogenous MAS, particularly the isoniazid-induced (induces P-450 isozyme CYP2E1) or post-isolation mixed (Aroclor 1254:isoniazid) MAS, thalidomide was not developmentally toxic. Addition of the INH-induced or mixed MASs, however, induced developmental toxicity to developing *Xenopus* embryos. Only the 3-amino-1,2,4-triazole- and cyclohexene oxide-inhibited MASs were effective at reducing and dramatically increasing the developmental toxicity of thalidomide, respectively. 3-amino-1,2,4-triazole inhibited P-450 isozyme CYP2E1 and cyclohexene inhibited epoxide detoxification enzyme epoxide hydrolase. Overall, these results indicated that CYP2E1 was responsible for the bioactivation of thalidomide, and a highly toxic arene oxide intermediate was likely involved in this process.

The final approaches used liver MASs from other species, including humans, and used supplements, including for example, glutathione/glutathione-S-transferase or alcohol dehydrogenase. Combined together, these approaches have been successful in evaluating models of biotransformation, bioactivation, and detoxification using the FETAX model.

Table 4 – *Developmental Toxicity of Thalidomide with and without Differently-Induced or Inhibited Exogenous Metabolic Activation Systems (MAS)*

MAS	LC50 (mg/L)	EC50 (mg/L)	TI
Unactivated[1]	>300.0	>300.0	---
Aroclor 1254-Induced[2]	285.1	227.3	1.3
INH-Induced[3]	207.4	105.5	2
Mixed[4]	183.7	105.9	1.7
α-N-MAS[5]	208.8	86.6	2.4
ATZ-MAS[6]	>300.0	245.8	1.2
CHO-MAS[7]	36.3	29.5	1.2
DM-MAS[8]	144.5	95.9	1.5

[1]No MAS.
[2]Aroclor 1254-induced rat liver microsomes MAS.
[3]Isoniazid-induced.
[4]Post isolation 1:1 mixed Aroclor 1254 and INH-induced MAS.
[5]α-napthoflavone-inhibited Aroclor 1254-induced MAS.
[6]3-amino-1,2,4,-triazole-inhibited mixed MAS.
[7]Cyclohexene oxide inhibited mixed MAS.
[8]Diethylmaleate inhibited mixed MAS.

Limb Development Model

Increasing interest in amphibian limb development due to the identification of limb abnormalities in indigenous species across the U.S. has warranted further development and refinement of amphibian limb development models (Burkhart et al. 1998). However, creation of limb development models using a FETAX–like approach dates back to 1996 (Fort and Stover 1996). Two different approaches have been developed to establish potential modes of action (Figure 1). The first, more traditional approach exposed blastula stage *Xenopus* embryos for ca. 30-d until hind limb development was complete at approximately stage 54 (Nieuwkoop and Faber 1975). This represented an all-inclusive exposure scenario covering the organization of the hind limb bud cells, establishment of embryonic limb fields, proximal-to-distal limb growth, and differentiation of the toes. However, because some developmental toxicants that alter limb development, such as thalidomide and retinoic acid, may affect early limb development in the organizational stages, the use of an early exposure and a pulsed exposure approach was developed to better establish possible mechanisms of action. In the case of activated thalidomide, both exposure scenarios induced phocomelia (Fort et al. 2000) (Figure 2). The only difference in the response between the two exposure scenarios was that complete femur development was achieved with the early pulsed exposure, whereas continual exposure for 30-d to activated thalidomide yielded a stumped femur. Further investigation of the early limb bud mass in activated thalidomide exposed larvae indicated that the cell mass was highly irregular and substantially disorganized indicating that thalidomide altered the organization (cellular

migration and communication) of hind limb bud cells. Similar methodologies are currently being developed for other amphibian species.

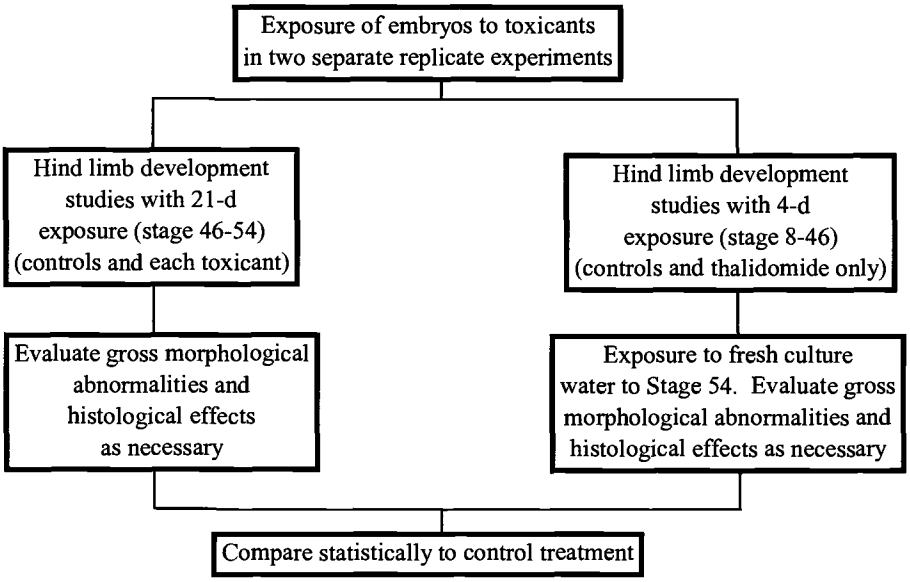

Figure 1 – *Approaches for Evaluating Limb Development using an Extended FETAX Model*

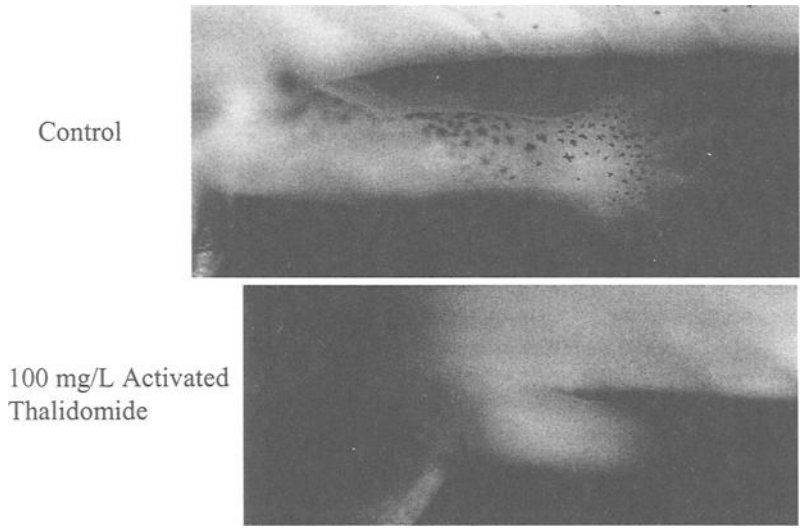

Figure 2 – *Effect of Activated Thalidomide on Limb Development in X. laevis*

Thyroid Disruption Model

The use of amphibians to evaluate the role of the thyroid gland in metamorphosis dates back to the early 1900s (Gudernatsch 1912, Kendall 1915). However, due to the complexity of the thyroid axis, confounding endocrine axes, and external factors, development of appropriate models for evaluating the toxicological impacts of toxicants and complex mixtures on the thyroid, along with the effects of thyroid disruption on amphibian development has been relatively difficult. The original model used to evaluate thyroid disruption and its impact on metamorphosis was the *Xenopus* tail resorption assay (Figure 3) (Fort et al. 2000c). In its original form the assay measured the rate of tail resorption during metamorphic climax, which spans a period of ca. 14–d in *Xenopus* (stage 58-66). During this time, digital photographs and digitizing software recorded the resorption of the tail (Figure 4). Several confounding factors with the use of the14-d frog metamorphic climax assay (Fort et al. 2000c), including the natural variability in tail resorption and the focus on only the final stages of metamorphosis, have warranted evaluation of modified approaches to measuring thyroid disruption.

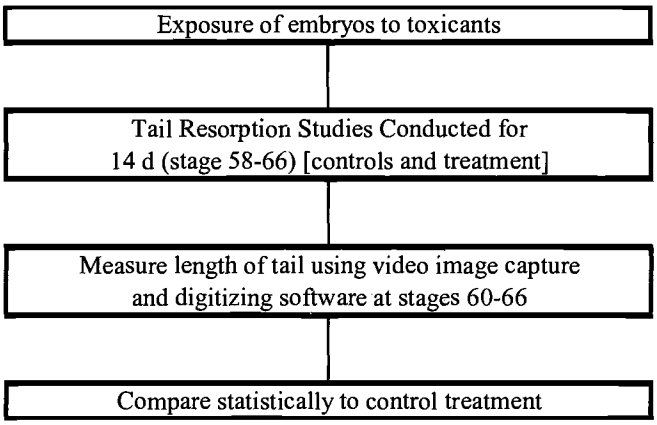

Figure 3 – *Original Metamorphic Climax Assay Approved*

The new approach to evaluate thyroid disruption and its impact on metamorphosis includes a suite of assays composed of morphological tests, biochemical analyses, and molecular tests. Of the proposed morphological tests using *Xenopus*, an assay including the entire period of metamorphosis, which spans nearly 28-d in *X. laevis*, is currently being evaluated (OECD 2001). A similar test format in *X. tropicalis* that requires a 14-d culture period is also being evaluated. Methods of thyroid hormone (TH), TH precursors, and auxiliary enzymes, including deiodinase, analysis using radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography-gas chromatography with mass selective detection (LC/GC-MS) are also being evaluated. The newest additions to the suite of assays include the molecular

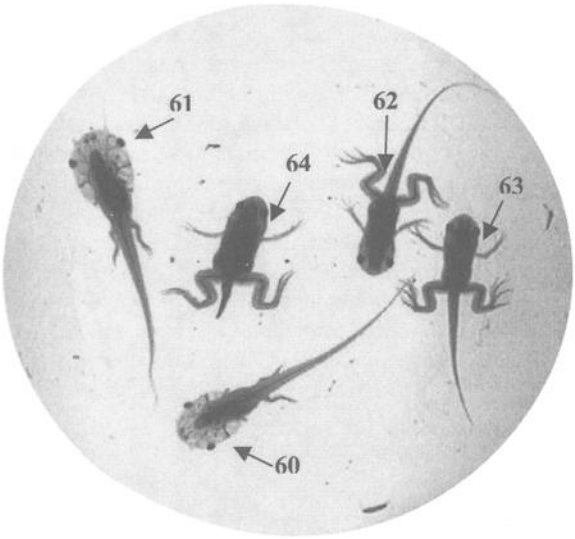

Figure 4 – *Metamorphic Climax in Xenopus laevis with Stage Development Indicated (Nieuwkoop and Faber 1975)*

tests. These tests include assays monitoring gene expression, such as differential display and ribonuclease protection assays (RPAs), development of transgenic animal lines (knockouts); and cell and organ culture lines. Amphibian cell lines with transfected thyroid axis response elements are currently being contemplated. Whole tail organ culture assays from wild and transgenic *X. tropicalis* are also being studied for potential use in this suite of assays. Obviously, a strong research focus has been placed on the development of this suite of assays. However, much work needs to be completed before selection of the most suitable assays can be made.

Ecological Hazard/Risk Assessment

FETAX or modified FETAX assays have been incorporated into various forms of ecological risk assessments (Dawson et al. 1985, Bantle et al. 1989, Fort et al. 1995, 1999a, 1999b, 2001b, Fort and Stover 1997, Linder et. al. 1991). Most of the studies conducted have done an adequate job of addressing ecological hazard. However, one of the more interesting outcomes from hazard assessment work in Minnesota (Fort et al. 1999a, 1999b), Vermont (Fort et al. 1999a), New Hampshire (Fort et al. 2001b), Washington (Fort et al. 1995), South Texas (Fort et al., in press b) was the development of a conceptual model (Figure 5) that described the multiple stressors that influenced or caused amphibian deformities and how these stressors interacted with each other. A common link in each of these cases was the impairment of thyroid function in developing larvae. Although thyroid repression has not been directly implicated as the cause of deformities in any of these cases, impaired thyroid function appeared to increase the sensitivity of local specimen to other causative stressors in these areas.

Previous studies (Fort et al. 1999a, 2001b) have shown that thyroid impairment increases the sensitivity of both *Xenopus* and ranid species to anthropogenic chemicals. New research also suggested that disruption of thyroid function in developing *Xenopus* and ranid species increases the larval susceptibility to uv light-induced toxicity (Burkhart and Fort, in press). In a more anecdotal sense, a connection between the sensitivity of amphibians to the perturbation of endogenous retinoid homeostasis, retinoid mimics, and impaired thyroid function in the developing tadpole might be linked to the relationship between the retinoid RXR receptor and the thyroid hormone receptor (TR). In amphibians, the TR dimerizes with the RXR receptor to form an active receptor complex (Ogryzko et al. 1996, Spencer et al. 1997, Blanco et al. 1998, Chen et al. 1997). The least understood feature of the conceptual model is the relationship between thyroid status and immunological status, including susceptibility to parasite infections. The use of amphibians and reptiles in general, and derivations of the FETAX model, in ecological risk assessment has been addressed by Birge et al. (2000) and Meyers-Schöne (2000); and is further discussed in the present standard technical publication (Fort and McLaughlin, in press).

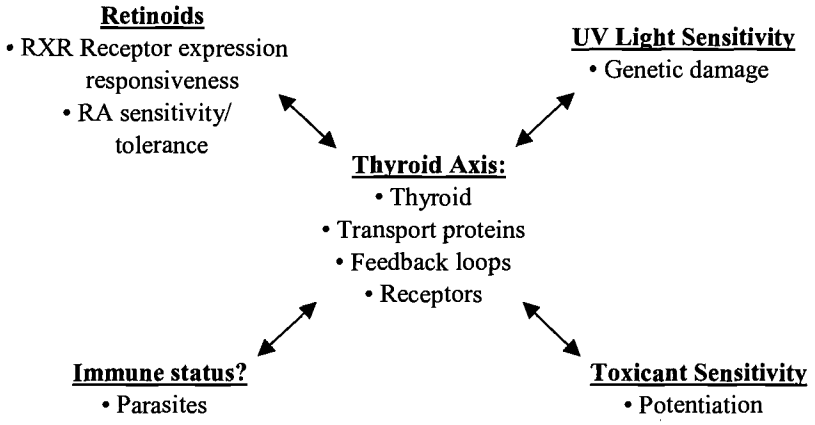

Figure 5 – *Conceptual Multiple Stressor Working Model – Possible Causes of Amphibian Deformities*

Nutritional Science and Toxicology

The FETAX model has also been applied to the field of nutritional science. In this capacity, the challenge was not to simply evaluate the toxicity of micronutrients but to evaluate developmental and reproductive effects associated with micronutrient deficiency. To date, the FETAX model and derivations of the FETAX model have been used to study the nutritional essentiality of copper (Fort et al. 2000d, 2000e), zinc (Fort et al. 2000), and boron (Fort 2002, Fort et al. 1998b, 1999c, 1999d, 1999e, 2000e,). Studies with the nutritional essentiality of the established micronutrients copper and

zinc led to the establishment of classical u-shaped concentration-response curves (Figure 6). However, the nutritional essentiality of boron was unconfirmed in vertebrate animals prior to 1996 warranting evaluation in an established vertebrate development and reproduction model system. Results from an on-going research program evaluating the nutritional essentiality of boron, found that *X. laevis* fed a low B diet in a low B culture media produced a substantially higher number of necrotic eggs and fertilized embryos than frogs fed a boron sufficient diet. Markedly decreased embryo cell counts at mid-blastula transition and an increased frequency of abnormal gastrulation were also noted in embryos from adult frogs fed the B-deficient diet. By 96-h of development, the majority of the larvae collected from the B-deficient adults and maintained in low boron culture media developed normally. Multigenerational effects, including limb malformation (Figure 7) were also observed in the F_2 generation. Reproductive effects associated with B deficiency in female *Xenopus* included ovary atrophy, oocyte necrosis, and incomplete oocyte maturation. In males, a decrease in testis weight and sperm count was noted. These studies suggest that these adverse effects resulting from B deficiency could be found during gametogenesis, gamete maturation, embryonic development, and larval maturation. The studies also confirmed that B deficiency was capable of interrupting the *X. laevis* lifecycle. Additional studies evaluating the role of B in the thyroid axis and the oocyte plasma membrane progesterone receptor provide the first line of direct evidence for a biochemical role of boron in *X. laevis*. Combined together, this research program provides firm evidence that B is nutritionally essential in *X. laevis*.

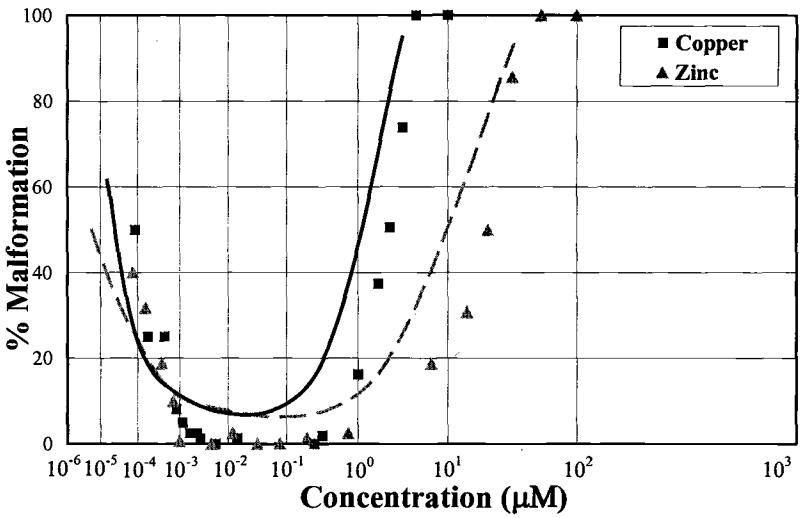

Figure 6 – *U-shaped Dose-Response Curves Developed with X. laevis Characterisitc of Essential Micoronutrients*

Figure 7 – *F2 Generation Boron Deficient X. laevis with Forelimb and Hindlimb Malformation*

Mixtures and QSAR

The FETAX model has proven useful in evaluating the toxicity of chemical mixtures (Dawson and Wilke 1991a, 1991b) and in establishing qualitative structure activity relationships. Much of the focus on mixture assessment has been on establishing a joint binary mixture modeling approach that incorporates toxic units (TU). This approach is potentially useful in not only determining the significance of interactive and non-interactive responses between compounds, but also similarities and differences in sites and mechanisms of action.

In TU analysis, joint action or potential interaction of binary mixtures was determined by standardizing toxicity to that of each individual component and was used to evaluate the potential for toxicological interaction between two compounds or mixtures. The 4-d EC50 (malformation) of each chemical alone is defined as 1.0 TU because of exposure to that chemical specifically. The concentration of each component at 50% effect for a chemical A:chemical B mixture is given as a fraction of its EC50 (malformation). The sum of the chemical A and chemical B TU gives a TU value for each particular ratio of the joint mixture. Mixtures with TU values near 1.0 (fiducial intervals overlap 1.0) have concentration additive rates of malformation. Thus, the toxicity of the mixture is equal to the sum of toxicities of each component when expressed in equivalent terms and adjusted for relative potencies. A mixture TU value slightly and substantially >1.0 represents response addition (toxicants that act in varying degrees of dissimilarity and are non-interactive) and antagonism (negative interaction), respectively. A mixture TU value of <1.0 may be indicative of positively interactive toxicants or synergism/potentiation. An isobole diagram was coupled with TU analysis to better evaluate the potential for interactive or non-interactive joint responses. As an

example, results from joint mixture studies with ethanol and acetaldehyde are presented in Figure 8. Results from this joint action study indicated that the joint response was non-interactive and characteristic of response addition.

In mixture toxicity, toxicants may cause joint effects in either non-interactive (most common) or interactive manners. Non-interactive toxicants that cause toxicity by the same or very similar modes of action typically show additive (concentration addition) toxicity when expressed on equipment terms and adjusted for relative potencies. For toxicants that exert toxic action on different systems or affect the same system differently, the combined toxicity is expected to be less than additive (response addition). Response addition represents a combined toxic effect greater than for either compound alone, but less than that for a concentration additive response. This appeared to be the case for ethanol and acetaldehyde (Figure 8). Toxicants that act interactively may produce greater than concentration responses (synergism) or less than response additive effects (antagonism). The differences in these types of toxic responses can be more easily identified and understood using the toxic unit interaction model coupled with an isobole diagram. In the toxic unit model, statistical differences in response patterns may be evaluated using calculated fiducial intervals.

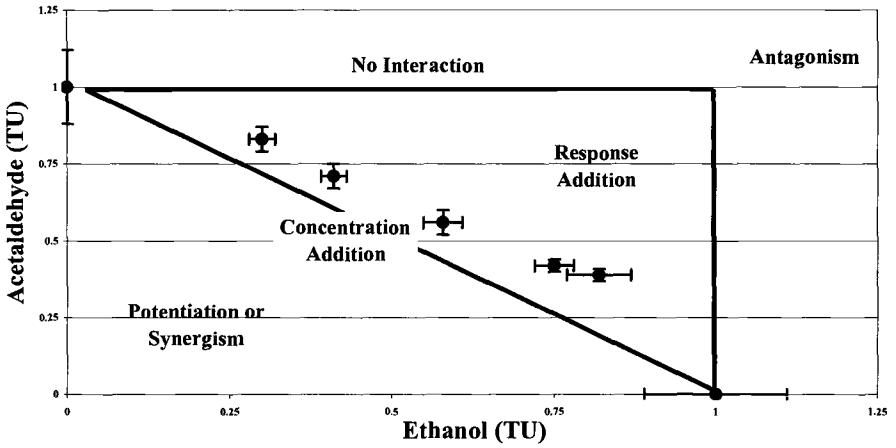

Figure 8 – *Isobole Diagram of Joint Toxic Action of Ethanol and Acetaldehyde*

The Future of FETAX

The future of FETAX lies in its utility and versatility as a model system for the study of amphibian development. The need for a model system like FETAX warrants continued research. Future investigation will continue to improve and augment FETAX and derivations of the FETAX model.

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AMPHITOX: A Customized Set of Toxicity Tests Employing Amphibian Embryos

Reference: Herkovits, J. and Pérez-Coll, C. S., **"AMPHITOX: A Customized Set of Toxicity Tests Employing Amphibian Embryos,"** *Multiple Stressor Effects in Relation to Declining Amphibian Populations, ASTM STP 1443*, G. Linder, S. Krest, D. Sparling, and E. Little, Eds., ASTM International, West Conshohocken, PA, 2003.

Abstract: Based on a large number of toxicity studies of single chemicals and complex mixtures, a set of four toxicity tests utilizing amphibian embryos (AMPHITOX) was developed (Herkovits and Pérez-Coll 1999). In this contribution, the versatility of AMPHITOX for the evaluation of the toxicity in 36 environmental samples is reported. AMPHITOX can be customized to acute (AMPHIACUT), short-term chronic (AMPHISHORT), and chronic (AMPHICHRO) exposure periods. By plotting the LC_{10} (or NOEC), LC_{50} and LC_{90} (or LC_{100}), the toxicity profile (TOP) curves from 24 hr to 14 days of exposure can be obtained allowing the visualization of concentration- and time-exposure thresholds, as well as the range of concentrations which exerts adverse effects in each case. By employing the early-life-stage test (AMPHIEMB) it is also possible to evaluate malformations. The environmental samples studied were obtained from surface and ground water, leaches, industrial effluents and soils. Data from acute, short-term chronic and chronic tests, were expressed as LC_{50}-96 h, NOEC 168 h (7 days) and NOEC 336 h (14 days). The maximal value for acute toxicity was 0.5 % V/V (in a leach), while the lower toxicity was 85 % V/V corresponding to the NOEC/14 days for a leach sample. In 6 samples (4 provided from reference places) no toxicity was detected. By applying the AMPHIEMB test in a water sample providing from Gutierrez stream in Lujan, Buenos Aires Province, 100 % lethality with concentrations over 50 % within 7 days of exposure was obtained and the surviving embryos exhibited reduced body size, delayed development and malformations. Malformations including microcephaly, abnormally developed tail and severe flexures, were proportional to the concentration of the sample. The results point to the possibility of evaluating the toxicity of a wide diversity of environmental samples. This may be accomplished by selecting the most appropriate AMPHITOX test according to the toxicity of the sample and the end point of major relevance. Ultimately amphibian embryos can be used in toxicity studies as indicators of environmental quality for wildlife protection purposes.

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Keywords: AMPHITOX, toxicity test, amphibian embryos, environmental pollution, Buenos Aires metropolitan area

Introduction

The recent growing pressures on natural resources and life support systems results from escalating human numbers and activities combined with increasing environmental pollution and inappropriate environmental management (Strong 1994, Cairns 1994, Herkovits et al. 1996). These facts are of major concern because the declines in environmental quality and biodiversity could severely affect ecosystem services essential to the quality of the biosphere and human health (Black 1994, Cairns and Niederlehner 1994, Costanza et al. 1997, Herkovits 1995, 1997). From an evolutionary perspective, chemical stress could be related to the development of life on earth including major mass extinctions such as the Cretaceous-Tertiary event, an exceptional ecologic crisis usually considered as devastating the biosphere and resulting in a dramatic change in the family assemblages which occur about 65 million years ago (Herkovits 2001 a, b). In present day conditions it is well-documented that degradation of air, water and soil quality could result in lethal effects (Herkovits 1995; Jones 2001; Cacciola et al. 2002, Cocco 2002, Krieger and Higgins 2002, Mannino 2002, Ritter et al. 2002) while sublethal impacts, from teratogenesis (Pérez-Coll et al. 1986, Hu and Kulkarni 2000, Prati et al. 2002) to endocrine disruption (Hong et al. 2000, Wilson et al. 2001, Schantz and Widholm 2001) could be also a threat for wildlife and human health. The studies on the fate and transport of pollutants in the environment point out the close-in problems for air, soil, sediments, surface and groundwater as interrelated compartments. The aquatic environment is particularly important as a recipient, transporting medium and sink for the majority of xenobiotic chemicals (Cairns 1994). In order to cope with such a wide range of environmental situations and to compare results from different matrices, there is a need for sensitive, cost-effective and short-term biological test methods.

The development of FETAX (ASTM 1992, Bantle et al. 1994) represented a significant contribution for the evaluation of lethal as well as teratological effects of pollutants on amphibians within an acute exposure period. As stewardship for environmental and human health protection increases the development of a more flexible and customized toxicity test for amphibians seems to be of high priority. For instance, by focusing on the most sensitive life-cycle stages, EPA suggested that chronic toxicity tests could be shortened to 7-day exposure protocols for the evaluation of industrial effluents (U.S.EPA 1991). Moreover, for water quality-based toxicity control purposes, the U.S.EPA found a good predictive correlation between embryo-larval survival and independent ecological parameters, such as species richness and diversity (U.S.EPA 1991). In an ecotoxicological study of the Reconquista River, located in the northern area of metropolitan Buenos Aires, the hazard and risk assessment was reported based on results obtained with amphibian embryos as the most susceptible among the three taxa evaluated in that study (fish, crustacean and amphibian) to the pollutants in the water column (Herkovits et al. 1996).

A worldwide decline of amphibian populations linked to the increasing environmental degradation is widely documented and adverse effects, such as malformations, could be related either to the presence of developmental toxicants or the absence of essential micronutrients (Simms 1969, Cook 1981, Baringa 1990, Blaustein et al. 1994, Boyer and Grue 1995, Fort et al. 1999, Burkhart et al. 1998, Burkhart et al. 2000, Tietge et al. 2000, Bridges et al., 2002). By exploring the response of *Bufo arenarum* embryos (a native amphibian specie of a large area in South America) to environmental pollutants, it was found that they are highly sensitive to single chemicals and complex mixtures (Herkovits et al. 1997a, 2000, Herkovits and Helguero 1998). Since the toxicity of a substance or complex mixture depends on the concentration and exposure time, threshold values taking into account these parameters are reported as Toxicity Profile curves (TOP) providing, within a systemic toxicity approach, a more appropriate set of data for hazard and risk assessment purposes (Holcombe et al. 1987, Bantle et al. 1989, Herkovits and Helguero 1998, Herkovits et al. 1996, 1997a, 2000). AMPHITOX is a standardized test employing amphibian embryos that can be used to evaluate toxicity for acute, short-term chronic, chronic, and early life stage exposure to hazardous substances and samples (Herkovits and Pérez-Coll 1999). By means of AMPHITOX the toxicity of different environmental samples such as surface, groundwater, soils, leaches and industrial effluents can be evaluated by adjusting the exposure period to the toxicity of the sample (Herkovits et al. 2002). In this contribution we expand the potential of AMPHITOX by providing an example of TOP in the case of these environmental samples and the teratogenesis exerted by pollutants in the surface water of a stream in the neighborhood of Lujan City, Province of Buenos Aires, Argentina.

Experimental Method

Sample collection. Environmental samples were obtained from the neighborhood of Buenos Aires City and were grouped by: a) surface water, b) groundwater, c) industrial effluents, d) leaches (obtained from landfills and solid industrial wastes), and e) soils. Samples of surface water were collected at the following stations: five samples from the Reconquista River (R1-R5), the first of them (R1) a reference sample, five from Moron Stream (MS), a tributary of Reconquista River, nine from the Matanza-Riachuelo River and tributaries (MR1-MR9) one from Gutierrez Stream (GS), tributary of Lujan River (LR). Surface water samples were collected near the shore by means of a manual pump at a depth of 0.5 m. At the sample stations no industrial or municipal discharges were observed. Groundwater samples were collected in two reference (G4 and G5) and three presumably polluted stations (G1, G2 and G3). Industrial effluents (E1-E4), leaches (L1-L4) and soil samples (S1, S2) were provided to our facility by a consultant firm in clean glass bottles maintained at 4°C. S1 was a reference sample and the industrial effluents were provided as blind samples from different activities. In the cases of soil and solid industrial wastes, a 24-hour extraction procedure was applied (U.S.EPA 1980) and

toxicity tests were performed with the processed material. The samples were obtained during 1994-1997 and, with the exception of surface water, they were provided to the laboratory as coded samples. For each, 4 L or 1 kg (in the case of solids) were transported to the laboratory and maintained at 4°C; the toxicity tests were accomplished within the next 96 hours.

Obtainment of Bufo arenarum (Hensel) embryos. Bufo arenarum adults were collected in Lobos (Buenos Aires Province). Ovulations were induced by intraperitoneal injection of a suspension of one female homologous hypophysis in 2 mL of AMPHITOX's solution, (AS, Table 1). Oocytes were fertilized "in vitro" with a sperm suspension in AS; the embryos provided from different couples. After fertilization, embryos were maintained in AS at 20+/-1° C. For early-life stage studies exposure started with embryos at neurula (S.16, Del Conte and Sirlin 1951), while acute to chronic tests occurred at the end of their development (S. 23-25). Table 1 provides a comparative view on the main features of the maintaining media for FETAX, artificial water and AMPHITOX.

Table 1. *Comparison of FETAX, artificial water and AMPHITOX solution water**

Ion	FETAX solution (mg/L)	Artificial water (mg/L)	AMPHITOX solution (mg/L)
Na^+	272.0	0,72	14,75
K^+	15,7	0,72	0,26
Ca^{2+}	19,4	8,48	0,36
Mg^{2+}	15,2	1,57	0
Cl^-	403.0	0,66	22,71
SO_4^{2-}	93,3	26,5	0
NO_3	0	0,04	0
HCO_3	69,7	1,88	1,45
Embryos/volume	25/10 mL		10/50 mL

*Modified from Tietge et al. 2000

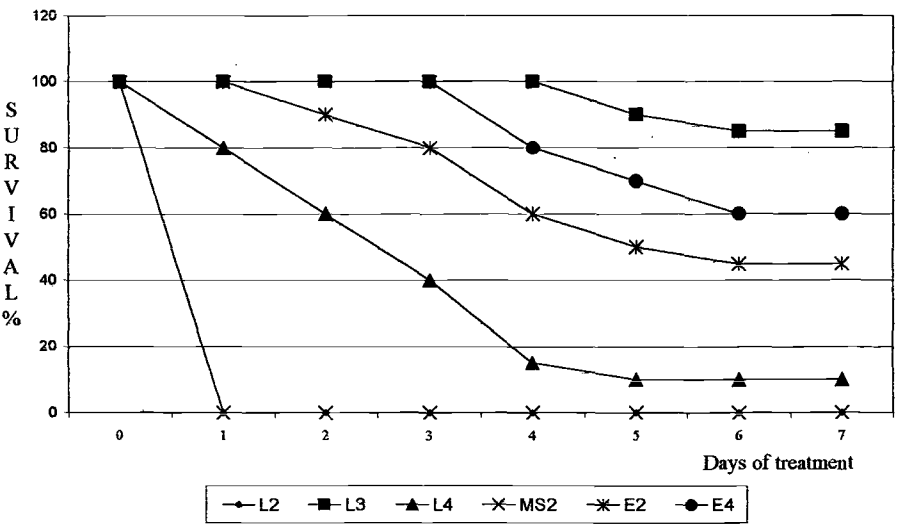
Toxicity tests. The toxicity tests conducted to evaluate the environmental samples were performed according to the standardized conditions of AMPHITOX (Herkovits and Perez-Coll 1999): 96 h exposure, AMPHIACUT; for short term chronic (7-day exposure), AMPHISHORT, for chronic (14 day-exposure), AMPHICHRO; and AMPHIEMB (7 day-exposure) as the early-life stage toxicity test. Batches of 10 embryos, in triplicate, were placed in 10 cm diameter glass petri dishes containing 40 mL of medium and maintained at 20 +/-1°C. Conductivity and pH of the samples were measured with a Luftman C400 conductimeter and a Luftman P300 pH meter, respectively and proper control bioassays for these parameters were conducted. Solutions were renewed each 24 hours. Survival and embryonic malformations were recorded daily and dead embryos were removed. The exposure period was determined by the preliminary toxicity data obtained from each sample. In samples exerting a high toxicity, acute and short-term chronic data are reported. For samples with low toxicity, the exposure period was extended up to 14 days. Data were statistically analyzed by

means of PROBIT analysis (U.S.EPA 1988) and transformed into acute Toxicity Units and short term-chronic/chronic Toxicity Units. Abnormalities were identified according to the Atlas of Abnormalities, a Guide for the Performance of FETAX (Bantle, Dumont, Finch and Linder 1985). Additional information on the significance and use of AMPHITOX test including, methodologies, water for culturing embryos, test material and organisms, procedures, acceptability of test, records, safety precautions, references and information on amphibian species can be consulted in the standardized protocol for AMPHITOX (Herkovits and Pérez-Coll 1999). Some embryos with typical malformations were selected and prepared for Scanning Electron Microscopy (SEM) and observed in a Jeol microscope operated at 5 kW.

Results and Discussion

The toxicity of the samples from the metropolitan area of Buenos Aires city ranged in a wide spectrum (Figure 1) and represent results obtained from the surface water, leaches and industrial effluents.

Figure 1. *Examples of toxicity values obtained from surface water, leaches and industrial effluents by means of AMPHITOX*



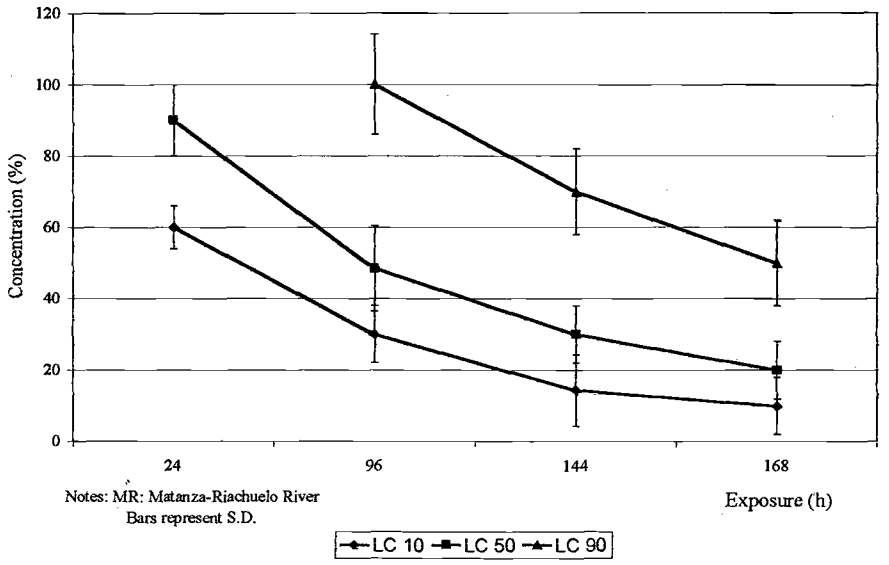
Concentrations in which the plotted results were obtained:
L2: 0.9 % L3: 100 % L4: 100 % E1: MS2 80 % E2: 100 % E4: 100 %
L: Leach E: Industrial effluent MS: Moron Stream

As examples of the highest toxicity found in this study, a leachate (L2) and a surface water sample (MS2) produced 100 % lethality at a concentration of 0.9 % and 80 %

respectively within 24 h of exposure. The lowest toxicity (excluding no toxic samples) was obtained in groundwater (G2), surface (R5) and soil (S2) samples which exerted slight lethality only after 12 days of exposure. The coefficient of variation for acute, short-term chronic and chronic exposure conditions ranged between 12 and 48 % which is within the accepted values for toxicity test purposes (U.S. EPA 1991). It is noteworthy that toxicity of a substance or complex mixture could be modulated by the salts in the maintaining media of the test organisms. Comparative analysis of FETAX solution, artificial water, and AMPHITOX points out the high content of different substances such as Na and Cl, in the case of FETAX, in relation to artificial water and the AMPHITOX maintaining solution. This fact should be considered for eventual differences among results reported in each case.

The TOP curves were obtained by means of the PROBIT values corresponding to the toxicity of each sample on *Bufo arenarum* embryos at stage 25. LC_{10}, LC_{50} and LC_{90}, comprising the period from 24 to 168 h of exposure were plotted. The TOP curves corresponding to the toxicity of a surface water sample from Matanza-Riachuelo River can be observed in Figure 2.

Figure 2. *Toxicity Profile of a surface water sample (MR* 3)*



In this case a concentration of 90 % (V/V) is representing the LC_{50} for 24 h of exposure and this value diminished up to 20 % as the exposure was extended to 168 h. A similar pattern occurred with the values corresponding to the LC_{10} and LC_{90} from 24 to 168 h of exposure. In many cases the standard deviation (S.D.) of LC_{50} is overlapping those of LC_{10} and/or LC_{90} for all or certain time exposure periods as was reported for the case of single substances (Herkovits et al. 1997a; 2000). Although the

most commonly used threshold in environmental toxicology is the LC_{50}/48 h the results plotted in Figure 2 confirm that the information arising from these data could not properly represent the toxicity of a substance or sample on living organisms. Regarding concentration thresholds, the LC_{10} and LC_{90} seem to be also very important for hazard assessment. For instance if the S.D. of LC_{50} is overlapping the S.D. of LC_{10} this last concentration could represent a risk to 50 % of the population. In our experience it is not unusual that the S.D. corresponding to the LC_{50} for a given substance (or mixture of chemicals) overlaps the S.D. of a concentration with a negligible effect and/or the S.D. of a concentration exerting a very severe effect. Moreover the LC_{50} by itself does not inform about concentrations exerting a negligible effect or the ones causing a very severe effect. The TOP curves provide this information and within a systemic toxicity approach also point out the proximity of LC_{100} and NOEC values reflecting the range in which a substance or sample exert toxicity as it was reported in the case of aluminum (Herkovits et al. 1997a), copper (Herkovits and Helguero 1998), and nickel (Herkovits et al. 2000).

Because toxicity involves an inverse relationship with effective concentration (EC; the lower the EC, the higher the toxicity), it is helpful to translate concentration-based toxicity data into toxicity units. The number of toxic units was defined by U.S.EPA (1991) as 100 divided by the EC measured; in the case of acute toxicity (TUa)= $100/LC_{50}$; and for chronic toxicity, (TUc)= 100/NOEC. For whole (industrial) effluent, U.S.EPA recommends maximal values of 0.3 TUa and 1.0 TUc for the most sensitive of at least three test species in both cases. In Table 2 the toxicity data for acute, short-term chronic and chronic exposure of embryos to the environmental samples evaluated are summarized. Based on the U.S. EPA criteria for water quality-based toxicity control (U.S. EPA 1991) from a total of 36 samples studied, 19 exerted toxicity within the initial 96 h of exposure, while after 7 days the number of samples exerting lethal effects rose to 27. By expanding the exposure period to 14 days, only 6 samples did not exert toxicity. As R1, G4, G5, and S1 belong to reference places almost 90 % of the non-reference environmental samples had some measurable toxicity. As observed in Table 2 in only 3 cases some toxicity was detected by expanding the exposure from 7 to 14 days. These results support the recommendation from U.S. EPA that chronic toxicity test could be shortened to 7-day exposure protocols by focusing on the most sensitive life-stages (U.S.EPA 1991). As expected, the TUc/TUa ratios were 1 or higher, and the increase in this value reflects a lower uptake rate of the pollutants, their delayed toxic effects on the amphibian embryo or both (Herkovits et al. 2002). In an ecotoxicological study in Reconquista River in the metropolitan area of Buenos Aires (Herkovits et al. 1996), it was confirmed that within single-species protocols, amphibian embryos could be among the most sensitive to environmental pollutants (Wesley et al. 1985, Pérez-Coll et al. 1988, Herkovits and Pérez-Coll 1990, Hall and Henry 1992).

Table 2. *Acute, short-term chronic and chronic toxicity data of environmental samples #*

Sample (Group)	LC50-96h sample (%v/v)	aTU (Acute test)	NOEC-7day sample (%v/v)	stcTU (Short-term Chronic test)	NOEC-14day % sample	cTU (Chronic test)	stcTU/aTU
R1(a)*	-	Not lethal	-	Not lethal	-	Not lethal	-
R2 (a)	-	Not lethal	30	3.33	10	10	-
R3(a)	-	Not lethal	50	2	20	5	-
R4(a)	-	Not lethal	80	1.25	40	2.5	-
R5(a)	-	Not lethal	-	Not lethal	80	1.25	-
MS1(a)	40	2.50	7	14.29	-	Ne	5.72
MS2(a)	30	3.33	5	20	-	Ne	6.06
MS3(a)	45	2.20	7	14.29	-	Ne	6.50
MS4(a)	70	1.43	12	8.33	-	Ne	5.87
MS5(a)	35	2.85	6	16.67	-	Ne	5.85
MR1(a)	35	2.85	20	5	-	Ne	1.75
MR2(a)	80	1.25	20	5	-	Ne	4
MR3(a)	50	2	20	5	-	Ne	2.5
MR4(a)	40	2.50	40	2.5	-	Ne	1
MR5(a)	30	3.33	10	10	-	Ne	3
MR6(a)	-	Not lethal	-	Not lethal	-	Not lethal	-
MR7(a)	70	1.43	60	1.67	-	Ne	1.18
MR8(a)	75	1.33	60	1.67	-	Ne	1.26
MR9(a)	60	1.67	60	1.67	-	Ne	1
GS(a)	-	Not lethal**	50	2	25	4	-
LR (a)	-	Not lethal	-	Not lethal	-	Not lethal	-
G1(b)	-	Not lethal	50	2	20	5	-
G2(b)	-	Not lethal	-	Not lethal	70	1.43	-
G3(b)	-	Not lethal	75	1.33		2.5	-
G4(b)*	-	Not lethal	-	Not lethal	-	Not lethal	-
G5(b)*	-	Not lethal	-	Not lethal	-	Not lethal	-
E1 (c)	40	2.50	5	20	-	Ne	8
E2 (c)	60	1.67	45	2.22	-	Ne	1.33
E3(c)	-	Not lethal	70	1.43	50	2	4.77
E4(c)	17	5.88	5	20	-	Ne	3.40
L1(d)	10	10	10	100	-	Ne	10
L2 (d)	0,5	200	100	1000	-	Ne	5
L3(d)	-	Not lethal	90	1.11	85	1.18	-
L4(d)	17	5.88	10	10	-	Ne	1.72
S1 (e)*	-	Not lethal	-	Not lethal	-	Not lethal	-
S2 (e)	-	Not lethal	-	Not lethal	90	1.11	-

(a) surface water, (b) groundwater, (c) industrial effluents, (d) leaches, (e) soil.
Ne: No evaluated due to the toxicity found in acute and short-term chronic tests.
*Reference samples ** Malformations 100 %

Modified from Herkovits et al. 2002

Table 3 compares data on lethality and malformations obtained with water samples providing from Lujan River and its tributary the Gutierrez Stream.

Table 3. *Survival and malformation data (%) of Bufo arenarum embryos exposed to different percentages surface water samples of Lujan River and Gutierrez Stream.*

	Survival (%)						
	Lujan River		Gutierrez Stream				Control (AS)
Time(hs)	75 %	100 %	25 %	50 %	75 %	100 %	
0	100	100	100	100	100	100	100
24	100	100	100	100	100	100	100
48	100	100	100	100	100	100	100
72	100	100	100	100	100	100	100
96	100	100	100	100	100	100	100
168	100	100	100	0	0	0	100
336	100	100	100	0	0	0	100

	Malformations (%)								
	Lujan River				Gutierrez Stream				Control (AS)
Time(hs)	25 %	50 %	75 %	100 %	25 %	50 %	75 %	100 %	
0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	33,33	63,33	100	100	0
48	3.33	0	3.33	0	33,33	63,33	100	100	3.33
72	3.33	0	3.33	0	33,33	63,33	100	100	3.33
96	3.33	0	3.33	0	33,33	63,33	100	100	3.33
168	3.33	0	3.33	0	33,33				
336	3.33	0	3.33	0	33,33				

In contrast to the results obtained in Lujan River with no mortality and no significant malformations even after 336 h (14 days) of exposure, in the case of Gutierrez stream the water was very toxic with teratogenic effects observed from 24 h of exposure onwards. The number of malformed embryos was proportional to dilution. It is noteworthy that a concentration of 75 % of the stream water exerted 100 % of malformations while lethality was not registered even with 100 % of the stream water within the acute exposure period (96h). However at 168 h of exposure, all embryos exposed to a concentration of over 50 % stream water died while those exposed to a concentration of 25 % survived, but 33 % of the embryos were malformed. The most common abnormalities were axial incurvations, stunted size, microcephaly and underdeveloped or abnormally developed tail, fin and eyes. These effects, as well as growth inhibition, were proportional to the concentration of the water sample. The

teratogenic effect of the pollutants in the water column of the Gutierrez stream is remarkably high since 100 % of malformations occur with 75 % of dilution, while no lethal effects were obtained even with 100 % of the sample. However, a dilution to 25 % exerted 33 % of malformations while no lethality was observed up to 14 days of treatment. At ultrastructural level the SEM study revealed a large number and prominent microridges with vaulted and folded cells and clusters of small spherical shaped cells in contrast to normal pentagonal/hexagonal glandular and ciliated epithelial cells in the flanks of control as well as of experimental embryos (Figure 3).

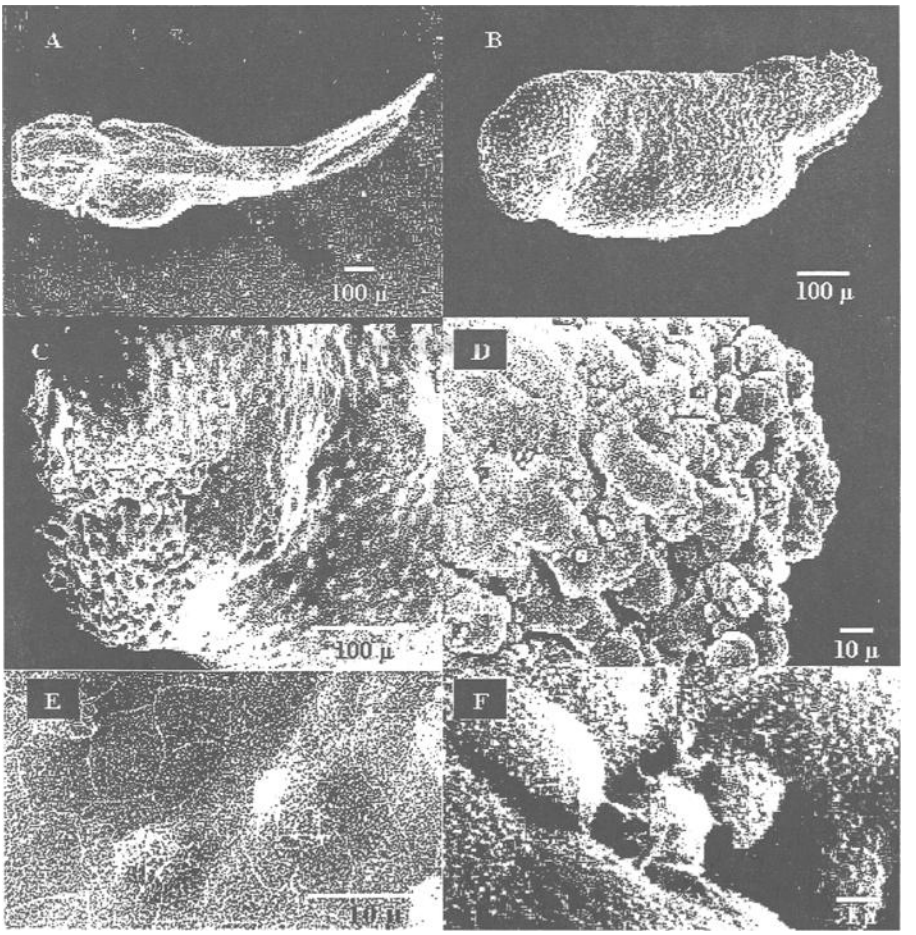


Figure 3: (**A**) Control *Bufo arenarum* embryo at S.25. (**B**) Malformed embryo due to the exposure to a sample (50 % V/V) providing from Gutierrez Stream. (C) and (**D**) show details of abnormal ridges of epithelial cells in the sucker and the tail of malformed embryo. (**E**) control epithelial cells and (**F**) vaulted shaped epithelial cells in malformed embryos.

It is noteworthy that in all cases abnormal epithelial cells occur mainly in areas with active morphogenetic processes, such as the head, gills and tail regions.

Although stage dependent susceptibility to environmental pollutants has been reported and could focus on specific adverse effects according to the cell differentiation and morphogenetic processes at each developmental stage (e.g. Pérez-Coll and Herkovits, 1990; Herkovits et al. 1997b), as a whole it seems that for environmental protection purposes a continuous treatment like the early life stage toxicity test is appropriate to evaluate teratogenic agents. In the case of this study, the treatment started from neurula stage onwards, including the most susceptible developmental stages to chemical stress. For acute, short term chronic and chronic toxicity test with amphibian embryos, AMPHITOX provides several advantages for routine toxicity screening purposes: i) the relatively constant sensitivity to xenobiotics of embryos from stage 23 onwards which allow to conduct the preliminary and definitive test with embryos providing from the same couple ii) the very low mortality baseline (less than 10 %) registered during all this period of about 20 days which facilitate to extend the toxicity assessment at least to 14 days of exposure, iii) the convenience of the biological material which allow register mortality with a limited expertise compared to teratological studies (Herkovits et al. 2002).

In a recent study, Tietge *et al* (2000) found out that inherent variability in water quality of field-collected samples could result in artifactual developmental effects when using FETAX. These artifacts were related to the low volume and high concentration of certain salts in the maintaining media of FETAX test, conditions which do not apply in the case of AMPHITOX (see Table 1). The results point out that AMPHITOX as a customized toxicity test provides the possibility to conduct a proper evaluation of the toxicity for a wide diversity of environmental samples reporting both, lethal and teratogenic effects according to the objectives of the study. By means of the TOP, as isotoxicity curves, concentration/exposure thresholds can be reported providing a better understanding on the adverse effect of single substances or complex mixtures for hazard and risk assessment. AMPHITOX tests could be conducted with other amphibian species although modifications might be necessary and the range in sensitivity to xenobiotics should be taken into account when comparing data

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Field and Laboratory Studies

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Investigation of Frog Abnormalities on National Wildlife Refuges in the Northeast U.S.

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Abstract: To address concerns about frog abnormalities, the U.S. Fish and Wildlife Service examined over 3,643 frogs and toads on National Wildlife Refuges (NWRs) in the Northeast U.S. The objectives were to: 1) determine if certain refuges had sites where abnormalities were frequently observed; 2) evaluate if the prevalence of abnormalities at a site was consistent within a season and among years; and 3) investigate possible causes. Sampling was conducted from 1999 through 2001. A complete sample from a site consisted of ≥50 metamorphs of one species. The prevalence of abnormalities ranged from 0 to 15% and fluctuated within season and among years. The most common external abnormalities were truncated limbs, and missing limbs, feet, and digits. Frogs with duplication of limb segments were rare (6). Based on radiographical examinations of 89 abnormal frogs, 55 had abnormalities due to trauma, 22 due to malformations, and 12 could not be classified. Metacercariae of the trematode *Ribeiroia* were detected in substantial numbers in two species from Iroquois NWR, with one specimen having supernumerary hindlimbs. We recommend continued sampling and integrated, causal evaluations on NWRs where the prevalence of abnormalities exceeds 5% or where the types of abnormalities warrant further study.

Keywords: frog, malformations, Northeast, Refuges

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Introduction

In the United States, attention was focused on the "malformed frog issue" in 1995, when middle school students found grossly abnormal frogs in a Minnesota wetland (Helgen et al. 2000). In the early 1990s, researchers in Quebec found abnormal frogs in agricultural ponds (Ouellet et al. 1997). This was followed by the discovery (by children in the summer of 1996) of northern leopard frogs (*Rana pipiens*) with missing hindlimbs in the Lake Champlain Basin of Vermont. These findings provided initial evidence that frog abnormalities were not a local phenomenon. In response to the findings of abnormal frogs in Vermont, the Vermont Agency of Natural Resources (VANR) examined 12 sites in the Lake Champlain Basin in the fall of 1996. The prevalence of abnormalities ranged from 5 to 23% (Fort et al. 1999).

In the summer of 1997, the U.S. Fish and Wildlife Service (USFWS) assisted the VANR in surveying sites in the Lake Champlain Basin of Vermont, including Missisquoi National Wildlife Refuge (NWR). The prevalence of abnormalities in northern leopard frogs at the sites sampled ranged from 2.0 to 45.4%, with the prevalence at Missisquoi NWR ranging from 3.1 to 5.9% (Fort et al. 1999). Also in 1997, the USFWS initiated preliminary surveys for abnormal frogs on NWRs in the Midwest and Northeast Regions. The prevalence of abnormalities at 17 NWRs in the Northeast Region (Maine to West Virginia) ranged from 0 to 8.6%.

Preliminary sampling was again conducted in 1998 at Great Bay (NH), Great Dismal Swamp (VA), Missisquoi (VT), Prime Hook (DE), and Sunkhaze Meadows (ME) NWRs. The prevalence of abnormalities ranged from 0% (Great Dismal Swamp- southern leopard frogs, *R. sphenocephala*) to 14.4% (Sunkhaze Meadows-northern leopard frogs). At Sunkhaze Meadows, abnormalities observed included skin webbing, missing limbs, one small eye, one extra foot, and a supernumerary hindlimb.[6]

In 1999, the USFWS began a multi-year study of frog abnormalities on NWRs in the Northeast Region. Our objectives were to: 1) determine if certain refuges had specific sites or "hot spots" where abnormalities were frequently observed; 2) evaluate if the prevalence of abnormalities at a site was consistent within a season and among years; and 3) investigate possible causes. In this paper, we report the results of the first three years (1999-2001) of the study. We use "abnormality" to refer to a missing or unusually-appearing body part based on field observations; "malformation" refers to such an observation with the etiology determined to be an error in development (see Methods for further discussion of etiology). This paper describes the field assessments and diagnostic examinations used to describe the nature of the abnormalities. The Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX) tests and chemical analyses conducted as part of this study are described in Turley et al. (2003). A detailed report of the project is provided in Eaton-Poole and Pinkney (2002).

[6] Katherine Converse, National Wildlife Health Center, unpublished data, July 1998.

Methods

Site Selection

In 1999, as many NWRs were sampled as possible (Figure 1). However, due to drought, many sites were dry, and, thus, sampling was limited, particularly in the southern portion of the Region. Most of the study sites were palustrine emergent and forested wetlands, and many of these were impoundments created for waterfowl.

In 1999, questionnaires were sent to the refuge managers to obtain maps of known or potential frog breeding habitat. Land use information was also requested, particularly areas which had received pesticide applications (e.g., herbicides for control of invasive plants, a variety of agricultural applications, insecticides for mosquito control) or those with hazardous waste concerns. Some refuges (including several former military facilities) have waste sites being investigated by the U.S. Environmental Protection Agency.

Field teams were established for the northern (New England, New York, and western Pennsylvania) and southern portion of the Region (New Jersey south to West Virginia). At each refuge, the teams interviewed biologists, verified frog habitats, and researched sites with known or suspected contaminant concerns. At some refuges, few sites had frogs and, therefore, all sites were sampled. In other cases, a sub-sample of sites believed to be impacted and not impacted were chosen. Originally, the intent was to develop a statistically-based survey with sites categorized by land use or contaminant concern. However, due in part to drought in 1999, it was difficult to collect adequate numbers of frogs. Thus, sites were ultimately chosen by the presence of frogs, and land-use, pesticide, or waste-site status were reported as part of the site description. In 2000, refuges and sites that had been sampled in 1999 were resampled, along with several that were targeted in 1999 but not sampled successfully. In 2001, more intensive sampling was conducted at five NWRs - Great Bay, Iroquois, Missisquoi, Rachel Carson, and Sunkhaze Meadows - where the prevalence of abnormalities exceeded 5% (based on the suggestion by Ouellet 2000 that >5% prevalence be considered "high"), or included types that warranted further investigation (e.g.; supernumerary limb parts).

Metamorph Sampling

Frogs were collected using long-handled dipnets along the edges of the breeding ponds. Collection continued until at least 100 metamorphs of one species were captured or until there were no more successes in captures (with a minimum sample size of 50 metamorphs). This method of sampling has also been used in Minnesota (Helgen et al. 2000) and in Vermont.[7] If possible, an additional sample of a second species was collected. All sampled frogs were kept in deep plastic containers with several centimeters of site water until examination.

[7] Rick Levey, Vermont Agency of Natural Resources, personal communication.

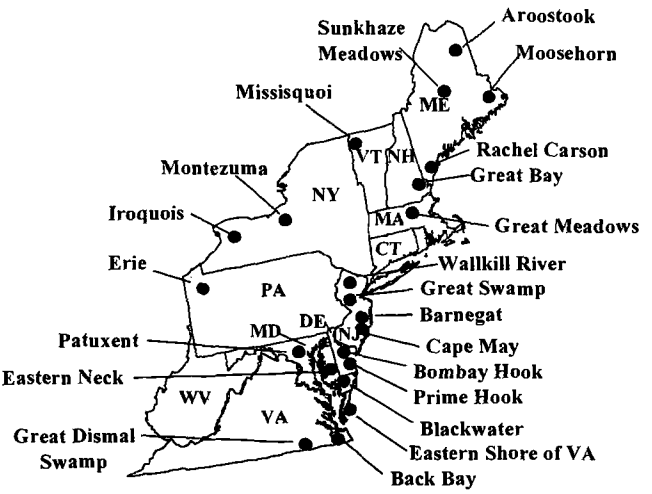

Figure 1- *Locations of National Wildlife Refuges in the Northeast U.S. that were sampled for frog and toad abnormalities in 1999.*

The snout to vent length (SVL) of each metamorph was measured and the presence of a tail was noted. Recent metamorphs were defined as frogs or toads with fully emerged forelimbs up to a maximum SVL, based on the literature (Wright and Wright 1949). Recent metamorphs were sampled rather than adults because abnormal amphibians are likely to be vulnerable to predation, and thus, less likely to survive to adulthood (Ouellet et al. 1997).

Each metamorph was examined visually for abnormalities by holding the animal under the forelimbs with the hindlimbs dangling. Eyes were examined for presence, symmetry, and proper coloration. The jaw was then examined for proper formation and alignment. The forelimbs and digits were examined, then the back, and finally the hindlimbs. If possible, the abnormal frogs were photographed. The percent of abnormals was calculated and used as the comparison among sites and years. Any abnormalities that were clearly due to trauma (open bleeding tissues) were not included in the calculation of abnormals. A subsample of the abnormal animals was sent for additional diagnostic work-up to investigate possible etiologies for the abnormalities, or to examine for the presence of parasitic infection.

Additional Diagnostic Work-Up

From sites where several abnormal individuals were collected, we obtained confirmatory diagnoses from the U.S. Geological Survey's National Wildlife Health Center (NWHC) or parasitological examinations at the University of Wisconsin-La Crosse. In general, representative grossly abnormal animals were shipped along with a few normal-appearing animals from the same site. With the exception of one site, toad

metamorphs were not submitted for diagnostic examinations because the foot bones are predominantly cartilaginous at this stage and, therefore, are essentially invisible radiographically.

At the NWHC in 1999 and 2000, the frogs were examined visually, euthanized with tricaine methane-sulfonate (MS-222), necropsied, and fixed and stored in 10% buffered formalin. Before immersion in formalin, the specimens were flattened by taping the animals to petri dishes so that optimal radiographic images could be obtained.

Virus cultures were performed on 183 frogs and toads by pooling livers and mesonephroi from one to six amphibians from each site. Homogenized tissues were inoculated onto fathead minnow cell lines. If a cytopathic effect was observed on primary cells or after one blind passage, the supernatant was examined by negative staining electron microscopy to identify virus particles by morphology. For histology, all major visceral organs and ventral skin of 184 frogs and toads were fixed in 10% buffered neutral formalin, processed routinely, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin. Fungal infections, such as pathogenic chytrid fungus (*Batrachochytrium dendrobatidis)* and *Ichthyophonus* sp., were diagnosed histologically.

Radiographs of formalin-fixed, eviscerated carcasses were taken on a Faxitron X-ray machine, (Model MX-20, Faxitron Comp., Buffalo Grove, Illinois, USA 60089), which allows 1.5-5x magnification of specimens using X-OMAT TL film (Eastman Kodak, Rochester, NY), and were examined by a pathologist. Gross and radiographical abnormalities in each frog or toad were placed into one of five possible categories: malformation, trauma, infectious disease, nutritional deficiency, and unknown.

Malformations are abnormalities in which an organ or tissue fails to form normally, such as amelia (absent limb), polymelia, polydactyly (supernumerary metacarpal bones, metatarsal and phalangeal bones), polyphalangy (supernumerary phalanges), extra vertebra, asymmetrical sacroiliac joints, misshapen lateral spinal processes, brachygnathia (short lower mandible), brachyphalangy (short or missing proximal or middle phalanges), micrencephaly (small or short head) and intersex. Trauma was the assigned category for those abnormalities that had the appearance of amputation, such as: brachydactyly (short toe), ectromelia (lower portion of limb missing), apodia (missing foot), luxation (dislocated joint), microphthalmia (small eye) and brachyphalangy (distal phalanges missing).

Abnormalities in the "unknown" category included ankylosis of a joint ("frozen" joint), taumelia (bone bridge), micromelia (small limb; all parts present), forelimb emergence failure, short femur or tibiafibula, bloat, scoliosis, bone spur, bone cysts, and skin webs. This category included abnormalities that have never been associated with a specific cause (e.g., bone cysts, forelimb emergence failure), abnormalities associated with skin, joints or cartilage that cannot be fully characterized in radiographs and histological examinations (e.g., ankylosis, skin web), and those with several possible causes that cannot be distinguished radiographically (e.g., taumelia, scoliosis, micromelia).

The infectious diseases category included eye infections, swollen rump muscles due to *Ichthyophonus* fungus and skin ulcers due to virus infection. Nutritional deficiencies consisted of two abnormalities: absence of calcium carbonate in the endolymphatic sacs and radiolucent, misshapen or missing otoliths.

Parasitological examinations on abnormal and normal metamorphs were performed in 2000 and 2001 at the University of Wisconsin-La Crosse. Frogs were euthanized by an overdose of MS-222, skinned, and examined for all parasites with a dissecting microscope. The location, prevalence (percentage of infected individuals with a specific parasite) and mean intensity (average number of individual parasites of a species per infected host) were determined. Representative metacercariae were manually excysted and photographed. *Ribeiroia* metacercariae were identified by their characteristic esophageal cecae and other features described by Beaver (1939) and Basch and Sturrock (1969). Other metacercariae were identified to genus and type by using the primary literature cited by Yamaguti (1975) and Schell (1985).

Results

Metamorph Sampling

In 1999, 51 sites at 22 NWRs were successfully sampled; in 2000, 18 sites at nine NWRs were successfully sampled; and in 2001, seven sites were successfully sampled at four NWRs (Table 1). Over 3,643 frogs and toads of eleven different species were captured and examined in the field. Radiographical examinations were performed on 195 frogs and toads (including reference animals). Parasitological examinations were performed on 53 frogs of five species collected from six NWRs. Virus cultures were performed on 183 frogs and toads from nine NWRs in 1999 and 2000, and histological examinations on non-skeletal tissues and organs were performed on 184 frogs and toads.

In 1999, prevalence of frogs or toads with abnormalities at the 22 NWRs ranged from 0 to 15.4% (Table 1). The highest prevalence was in green frogs (*Rana clamitans*) from Great Bay NWR (Lower Peverly Pond). The abnormalities affected predominantly the digits and eyes. This high prevalence was only noted during the second sample that occurred at the end of July, whereas we found no abnormalities in the first and third sampling. Another refuge with notable abnormalities was Rachel Carson NWR. Three sites each were sampled twice with prevalences ranging from 1.9 to 6.4%. The highest prevalence was at the Brown Street Impoundment. Abnormalities at Rachel Carson NWR included amelia, unilateral microphthalmia, abnormally shaped pupils, polypodia, a skin web, apodia of both hind feet, and brachydactyly.

In 2000, the prevalence of abnormalities ranged from 0 to 10.6% (Table 1). Missisquoi NWR had relatively low prevalences at three sites in July samples (0%, 2.6%, and 2.8%) but elevated prevalences at two of the three sites in August samples (10.6%, 8.1%, and 2.4%). One northern leopard frog at Missisquoi NWR had a complete extra forelimb growing from the left shoulder and facing backwards. Iroquois NWR had very low numbers of frogs but one northern leopard frog had supernumerary hindlimbs. Rachel Carson NWR also had low frog numbers at two sites and a die-off of green frogs (frogs were dying in the hand and no frogs were observed at the site later in the season) at the third site. A full sample was nearly collected at one site, the Spurwink River site, where four of 43 pickerel frogs had abnormalities, including: amelia of the hindlimb including the ipsilateral half of the pelvis; ectromelia of both hindlimbs; and a missing forelimb. Great Swamp NWR in New Jersey had an abnormality prevalence of 8.9%. Abnormalities in green frogs included anophthalmia (a missing eye), a short femur

and ectrodactyly (missing digits), a shortened foot, and ectromelia.

The prevalence of abnormalities in 2001 ranged from 0 to 4.0% (Table 1). The 4% prevalence was found at one site at Iroquois NWR. Low numbers were observed at other sites at Iroquois NWR. Low numbers were also observed at Rachel Carson NWR, such that no sites were successfully sampled.

The prevalence of abnormalities at each site was highly variable from year to year (Figure 2). Some sites with a high prevalence in one year had low numbers the following year. At the Great Bay NWR Ferry Way site, the highest abnormality prevalences for the years 1998-2001 were 8.0%, 10.6%, 0%, and 1.9%, respectively. The Missisquoi NWR Tabor Road site was not sampled in 1997 and 1998. In 1999, the highest prevalence at the site was 2.5% and in 2000 it was 10.6%. In 2001, there were too few frogs observed to attempt sampling.

The most common abnormalities observed in the field involved the hindlimbs (32.7%) and the most common specific abnormality was unilateral ectromelia. The second most common abnormality was ectrodactyly (missing or short digits)(21.5%) of either the fore or hindlimb. Third was eye abnormalities (21.0%), particularly microphthalmia, misshaped pupils, missing pupils, anophthalmia, and abnormal pigmentation of the iris. Fourth was foot abnormalities (15.2%) including amelia, ectromelia, and apodia, and clubbed and rotated feet. Supernumerary feet, limbs, and digits were relatively rare at 3.6%. Forelimb abnormalities such as amelia, ectromelia and apodia were rare (2.9%), as was brachygnathia (1.2%), and luxation of joints (1.2%). Most abnormalities were unilateral; only two frogs had bilateral hindlimb abnormalities and they were asymmetrical.

More than one species was sampled at a site when possible. An adequate sample size (>50) was collected for two species at only six sites. The abnormality prevalences were generally similar between the species (Table 2).

Additional Diagnostic Work-Up

Each frog was placed in only one suspected etiology category even though some frogs had multiple abnormalities (Table 3). Therefore, abnormal frogs with more than one abnormality were placed in only one category according to the following hierarchy: malformed, unknown cause(s), and trauma. If any of these frogs also had indications of calcium deficiency, specifically hypocalcemia of endolymphatic sacs or hypoplasia of otoliths, they were additionally classified as nutritionally deficient. Sixty-two percent (55/89) of the abnormal frogs were placed in the trauma category based on radiographic findings. These included presumptive amputations (ectromelia, apodia, ectrodactyly, and brachyphalangy), puncture wounds of the eye, crushed digits, and unilateral microphthalmia.

Malformations were diagnosed in 25% (22/89) of the abnormal frogs. Some malformations were readily detected in the field, such as amelia, brachygnathia, polydactyly, polypodia, and polyphalangy. Other abnormalities were only detectable by radiography such as abnormal (asymmetrical) sacroiliac joints, absence of pubic bones, extra vertebra, taumelia, abnormal otoliths, or absence of calcium in endolymphatic sacs. Twelve of the 89 abnormal frogs were placed in the unknown causes category. Fourteen

Table 1- *The range of abnormality prevalence observed in recently metamorphosed frogs and toads at National Wildlife Refuges (NWRs) in the Northeast U.S. from 1999-2001.*

NWR (State)	Year	Percent Abnormal (Range)	No. Sites Sampled	Species**
Aroostook (ME)	99	0.0	2	MF,NLF
Back Bay (VA)	99	0.0	1	FT
Barnegat (NJ)	99	5.1	1	FT
Blackwater (MD)	99	0.0-1.8	2	FT, SLF
Bombay Hook (DE)	99	0.0	1	GF
Cape May (NJ)	99	5.3-7.2	2	FT
Eastern Neck (MD)	99	3.0-6.0	2	FT
Eastern Neck (MD)	00	1.6	1	FT
Eastern Shore of VA (VA)	99	3.6-6.7	2	FT
Erie (PA)	99	0.0	2	AT, WF
Great Bay (NH)	99	0.0-15.4	3	GF
Great Bay (NH)	00	0.0-3.5	2	GF
Great Bay (NH)	01	0.0-3.0	3	GF
GreatDismalSwamp (VA)	99	0.0-2.0	2	FT, NCF
Great Meadows (MA)	99	0.0	1	BF
Great Swamp (NJ)	99	5.7	1	GF
Great Swamp (NJ)	00	9.0	1	GF
Iroquois (NY)	99	1.5-3.1	3	NLF
Iroquois (NY)	01	4.0	1	GF
Missisquoi (VT)	99	1.0-2.5	3	NLF
Missisquoi (VT)	00	2.8-10.6	3	NLF, GF
Missisquoi (VT)	01	0.9-1.2	2	NLF
Montezuma (NY)	99	0.0	2	NLF
Moosehorn (ME)	99	1.9-5.8	2	BF, GF
Patuxent (MD)	99	0.0-6.9	7	FT, NCF, SLF
Patuxent (MD)	00	1.0-5.7	5	FT, NCF, SLF
Prime Hook (DE)	99	0.0-2.2	4	FT, SLF
Prime Hook (DE)	00	4.8	1	FT
Rachel Carson (ME)	99	1.2-6.4	3	GF, PF
Rachel Carson (ME)	00	9.3*	1	PF
SunkhazeMeadows (ME)	99	0.0-1.6	2	NLF
SunkhazeMeadows (ME)	00	0.0-1.9	2	MF, NLF
SunkhazeMeadows (ME)	01	0.0	1	NLF
Wallkill River (NJ)	99	0.0-1.7	3	GF, WF
Wallkill River (NJ)	00	0.0-1.8	2	GF, WF

* Low sample size; only 43 animals collected.

** GF= Green frog; SLF= Southern leopard frog; MF= Mink frog
AT= American toad; NLF= Northern leopard frog; BF= Bullfrog
FT= Fowler's toad; NCF= Northern cricket frog; PF= Pickerel frog

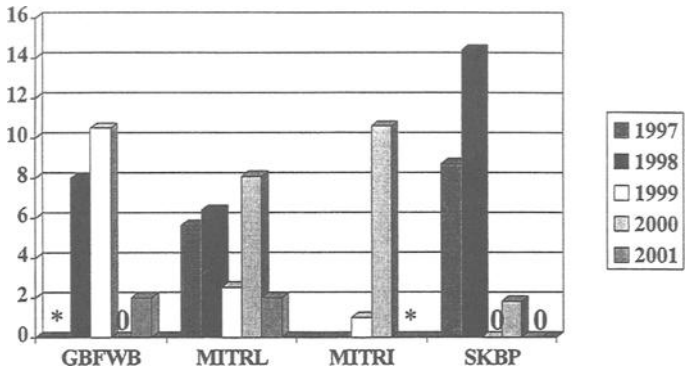

Figure 2- *Prevalence of abnormalities in frog metamorphs collected at four sites located at three National Wildlife Refuges (NWRs) in the Northeast U.S. from 1997 to 2001. * denotes years when <50 frogs were collected. 0 denotes years where the abnormality prevalence was 0%. GBFWB= Great Bay NWR (Ferry Way site); MITRL= Missisquoi NWR (Trails site); MITRI= Missisquoi NWR (Tabor Road Impoundment); SKBP=Sunkhaze Meadows NWR (Borrow Pit site).*

of the 89 abnormal frogs also had calcium deficiencies. The majority of these frogs were collected from Patuxent NWR.

The following cases indicate the diagnostic value of radiography. Eleven frogs with grossly normal musculo-skeletal systems had abnormalities that were revealed only in radiographs; these included: micrencephalies, abnormal otoliths, mild brachyphalangies, fractures of phalangeal and pelvic bones, and one cryptic case of polyphalangy. Also, ten abnormal frogs that were submitted for radiology received a diagnosis different than what was described in the field. For instance, one frog from Rachel Carson NWR was submitted with a field description of short right femur and polypodia. Radiographs identified taumelia of duplicate tibiafibulas, an abnormal ninth vertebra, and an abnormal sacroiliac joint. A frog from Patuxent NWR was submitted with a field description of short femur; the radiograph showed an absence of pubic bones and a duplicate right pelvis.
A frog from Great Dismal Swamp was submitted with short digits but the radiograph showed an extra vertebra. Radiographs also were necessary to distinguish abnormalities that appeared similar in the field; for example, a short tibiafibula (or other limb bones) could be due to taumelia, an overriding fracture, or an abnormally short bone.

In 2000, 17 of the 18 frogs shipped to the University of Wisconsin-La Crosse for parasitological examination were considered to be abnormal, and only one harbored *Ribeiroia* metacercariae. The *Ribeiroia*-positive specimen was an extra-legged northern leopard frog from Iroquois NWR that had 37 *Ribeiroia* (12 in the tail resorption site, three around the vent and 22 located at various sites extending down the limbs). Three

Table 2- *Comparison of the prevalence of abnormalities in species of frogs and toads collected from the same site at National Wildlife Refuges (NWRs) in the Northeast U.S. from 1999-2001.*

NWR/ Site	Species**	No. Collected	No. Abnormal	Percent Abnormal
Erie/				
Muddy Creek	WF	65	0	0.0
	AT	13	0	*
Great Bay/				
Ferry Way	GF	52	0	0.0
	PF	32	0	*
Missisquoi/				
Trails	NLF	103	5	4.9
	GF	58	2	3.4
Moosehorn/				
Daly Flowage	BF	52	3	5.8
	GF	53	1	1.9
Patuxent/				
Bluegill Pond	FT	130	9	6.9
	NCF	68	4	5.8
Duvall Pond	SLF	37	2	*
	NCF	70	0	0.0
Harding Spring	SLF	62	0	0.0
	GF	23	1	*
Mabbott Pond	AT	51	2	3.9
	FT	22	3	*
Reddington Lake	FT	67	1	1.5
	SLF	22	1	*
	NCF	22	0	*
Prime Hook/				
Wood Duck Pond	FT	89	2	2.2
	SLF	19	0	*
Rachel Carson/				
Brown Impoundment	GF	78	5	6.4
	PF	52	3	5.8
Spurwink River	GF	10	2	*
	PF	54	1	1.9
Sunkhaze Meadows/				
Borrow Pit	NLF	54	1	1.9
	MF	52	1	1.9

* Percent abnormal not calculated when sample size <50.

** See table 1 for species abbreviations.

Table 3- *Diagnostic data on abnormal metamorphs collected at National Wildlife Refuges in the Northeast U.S. in 1999 and 2000 sent to the National Wildlife Health Center. Although each metamorph may have multiple abnormalities, they were placed in only one suspected etiology category in order of the following hierarchy: malformed, multiple, unknown, and trauma. Metamorphs with nutritional deficiency (hypocalcemia of endolymphatic sacs or hypoplasia of otoliths) are also listed.*

NWR	No. Abnormal	No. Malformed	No. Unknown	No. Trauma	No. Nutrition
1999:					
Great Bay	10	3	2	5	0
Great Dismal Swamp	4	3	0	1	3
Great Swamp	4	1	0	3	0
Iroquois	3	1	1	1	0
Patuxent	14	2	2	10	8
Rachel Carson	12	6	0	6	2
Wallkill River	7	1	3	3	0
2000:					
Great Swamp	4	0	2	2	0
Missisquoi	24	4	0	20	0
Rachel Carson	7	1	2	4	1
Total frogs:	**89**	**22**	**12**	**55**	**14**

southern leopard frogs and three gray treefrogs (*Hyla versicolor*) from Patuxent NWR, three green frogs from Wallkill River NWR, six northern leopard frogs from Missisquoi NWR, and one bullfrog (*R. catesbeiana*) from Sunkhaze Meadows NWR lacked *Ribeiroia*. In 2001, frogs from three refuges (11 green frogs from Iroquois NWR, 12 green frogs from Great Bay NWR and 12 northern leopard frogs from Missisquoi NWR) were examined for parasites. Nine of 11 frogs from Iroquois NWR were infected with *Ribeiroia* (mean intensity = 18.8) and two of these (with five and nine *Ribeiroia*) had abnormalities in a hindlimb. Only one *Ribeiroia* was found in a slightly abnormal northern leopard frog from Missisquoi in 2001. None of the Great Bay NWR frogs harbored *Ribeiroia* and two of these frogs had abnormalities (one with a bony triangle in the radioulna and expanded width of a single digit of one front limb, and the second missing a digit on the left front limb).

During five years of field surveys in this study (1997-2001), four die-offs of frogs and tadpoles have been observed. A die-off of frogs at the Great Bay site in 1997 was not investigated. Iridoviruses were isolated from sick and dead frogs at Great Bay NWR (Ferry Way site) and Rachel Carson NWR (Brown Street Impoundment) in 1999. A die-off at the Rachel Carson site again in 2000, was investigated diagnostically, but no viruses were isolated from eight frogs. However, most of these frogs died during shipping and consequently may have been unsuitable specimens for virus cultures.

Chytrid fungus infections were detected histologically in nine of 10 northern leopard frogs from Missisquoi NWR (Trails site) in 2000. Chytrid fungal infections were not detected in 170 frogs examined from eight other NWRs in 1999 and 2000.

Ichthyophonus fungal infections were detected at necropsy and confirmed by histological examinations in frogs at Missiquoi NWR (Trails site) and Sunkhaze Meadows (Carlton Pond site). Green frogs from Missisquoi NWR (Trails site) had characteristic swollen soft tissues over the rump and urostyle in the region of the recently resorbed tail. This swollen tissue consisted of masses of minute cyst-like fungal elements surrounded by extensive granulomatous and fibrosing inflammation. Radiographically, there was no involvement of skeletal structures.

Discussion

The first objective, extent of the phenomenon, was addressed by sampling 22 NWRs, using a standard protocol, between 1999 and 2001. Ouellet's (2000) review of the literature concluded that morphological abnormalities and injuries normally occur at low frequencies in wild populations of amphibians, ranging from 0 to 2%, and he suggested that 5% be considered "high". The following refuges had sites that with an abnormality prevalence that equaled or exceeded 5%: Great Bay, Moosehorn, Rachel Carson, Wallkill River, Great Swamp, Missisquoi, and Patuxent. In addition, Sunkhaze Meadows NWR was added to the list based on a 14.4% prevalence in 1998.

In addressing the second objective, we found that the prevalence of abnormalities was highly variable between years (Figure 2). Variability in abnormality prevalence from year to year was also observed in Minnesota (Helgen et al. 2000) and Quebec (Ouellet et al. 1997). Although our data are limited, it appears that abnormalities tended to be more prevalent later in the season, a trend also observed in Minnesota (Rosenberry 2001). Fluctuations in the prevalence of abnormalities within a season have also been observed in the Lake Champlain Basin.[8] Such variability in abnormality frequencies necessitates multiple sampling events each season to properly document incidences of abnormalities.

The third objective, investigating possible causes, is being addressed through the diagnostic work-ups and FETAX/chemistry studies described by Turley et al. (2003). Twenty-five percent of the 89 abnormal frogs examined were found to have malformations. However, many malformations were internal and found only in radiographs, such as extra vertebra, taumelia (bone bridges), or absence of pelvic bones. Some of the normal-appearing reference frogs were also found to have internal malformations, therefore, the prevalence of abnormalities at any given site will be higher if a population is subjected to radiographical examinations in addition to gross examinations in the field. One unexpected result of the radiographical examinations in this study was the detection of new, previously unreported internal abnormalities (Meteyer et al. 2000), such as extra vertebra, misshapen and missing otoliths, and asymmetrical sacroiliac joints.

Trauma was the ascribed category for 62% of the 89 frogs based on radiographic examinations. The prevalence of trauma, mostly missing hindlimb segments that resemble amputations, is suggestive of attacks by invertebrate or vertebrate predators. However, Meteyer et al. (2000) pointed out that the hindlimbs of developing tadpoles lie close to the body and tail making it difficult to visualize how a developing limb could be amputated without additional injury to adjacent tissue or the whole tadpole. However, if injury does occur during the tadpole stage it is possible that healing could occur before metamorphosis.

Potential predators were not investigated in this study. However, Johnson et al. (2001) reported a suspicion that sticklebacks (*Gasterosteus aculeatus*) were responsible for missing limbs and digits in western toads (*Bufo boreas*). Johnson et al. also reviewed German literature that identified the common leech (*Erpobdella octoculata*) as responsible for the high frequencies of limb abnormalities in the common toad (*Bufo bufo*). Johnson et al. (1999, 2001) further demonstrated that experimental *Ribeiroia* infections can induce many of the types of limb abnormalities observed in this study. However, they also observed a much higher rate of supernumerary limbs in Pacific tree frogs (*Hyla regilla*) (6.5 to 25.5%) and western toads (1.6-3.3%) than have been observed in the Northeast.

Moreover, *Ribeiroia* has not been found everywhere that it might be expected if it were the primary cause of our observed abnormalities. Of the frogs submitted to the University of Wisconsin-La Crosse for parasitology, *Ribeiroia* was only identified in frogs from two NWRs, Iroquois and Missisquoi. *Ribeiroia* was found in large numbers in two species (green frogs and northern leopard frogs), including one specimen with supernumerary hindlimbs collected from Iroquois NWR in 2000. This was, in fact, the only frog in this study with supernumerary limbs or limb segments that was infected with *Ribeiroia.* The prevalence and mean intensity of *Ribeiroia* at Iroquois NWR were similar to infections seen at several malformation hotspots in Minnesota where *Ribeiroia* has been incriminated in causing frog abnormalities (Lannoo et al. 2002). At Missisquoi, however, only a single individual of *Ribeiroia* was found in one abnormal northern leopard frog in 2001. No *Ribeiroia* were found in the other ten northern leopard frogs sampled from the same site that year or the six abnormal northern leopard frogs collected in 2000. More data are needed to determine if *Ribeiroia* could be linked with the abnormalities observed at Missisquoi.

Diagnoses and attribution of an etiology for most skeletal abnormalities are subjective. While many abnormalities in our frogs and toads resemble the abnormalities induced by experimental infections of tadpoles with metacercariae of *Ribeiroia* (Johnson et al. 1999, 2001), some abnormalities induced by *Ribeiroia* can have other etiologies. For example, ectromelia is an abnormality induced by *Ribeiroia,* that can also result from traumatic amputation by a predator. Johnson et al. (1999, 2001) did not report radiographic examinations of their frogs and toads, hence, it remains unknown whether there are any radiographic features that might distinguish ectromelia caused by *Ribeiroia* from cases of traumatic amputation. Taumelia (bone bridge or bony triangle) is another problematic abnormality. Although taumelia can be induced by metacercariae of *Ribeiroia,* it has also been associated with genetic mutations and certain retinoids in endotherms (Gardiner and Hoppe 1999), and can occur subsequent to a folding fracture of a limb bone.

One benefit of this project has been having biologists systematically examine the presence and condition of frogs and toads at NWRs. From this effort and the diagnostic follow-up, we have identified several NWRs with disease concerns. Die-offs have been documented at three refuges: Iroquois, Great Bay, and Rachel Carson. An iridovirus was cultured from frogs from Great Bay and Rachel Carson (although not in the same year as

[8] Rick Levey, Vermont Agency of Natural Resources, personal communication.

the die-off). While some virus infections of mammals are associated with musculo-skeletal malformations in the fetus and newborne, such an association between iridoviruses and amphibians has not been reported. Furthermore, culture attempts on abnormal metamorphosed amphibians probably will not detect viruses that infected tadpoles and induced abnormalities several weeks prior to capture. Serological tests on abnormal amphibians may be necessary to detect prior exposure to viruses and other infectious agents. *Ichthyophonus* fungus was observed in frogs at Missisquoi Trails site and Sunkhaze Meadows Carlton Pond site in 2000, and a specific abnormality of the rump and urostyle region was associated with this organism. The pathogenic chytrid fungus of amphibians was also identified in frogs from the Missisquoi Trails site in 2000, and while this organism has no known association with musculo-skeletal abnormalities of amphibians, it may be associated with population declines (Green et al. 2002).

It was difficult to collect adequate numbers of frogs from suitable habitats at several refuges. Iroquois NWR, in particular, where *Ribeiroia* was identified in large numbers, has been difficult to sample adequately. We acknowledge that long-term sampling efforts are required to distinguish population declines due to anthropogenic stressors from natural fluctuations (Heyer et al. 1994). Nevertheless, dramatic or sudden declines in frog numbers in one year, or die-offs of amphibians at any site are worrisome findings worthy of intense surveys and diagnostic examinations. We remain concerned about the diseases and die-offs we have observed and with refuges that have apparently suitable habitats but consistently sparse frog numbers. Although population sampling has not been an objective of this project, we hope that these observations will stimulate increased interest in monitoring frog populations at these NWRs.

We recommend that focused investigations continue on refuges where there have been unusual abnormalities, mortality events, apparent low numbers of successful metamorphs (i.e., poor recruitment), or fluctuating frequencies of abnormalities above the 5% level. The investigation of possible causes requires an integrated approach that includes surveys, *in situ* exposures, diagnostic radiography, microbiology, parasitology, extended FETAX tests, analytical chemistry, and endocrinology (Sower et al. 2000, Hayes et al. 2002).

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Evaluation of the Potential Impact of Water and Sediment from National Wildlife Refuge Sites Using a Modified Frog Embryo Teratogenesis Assay- *Xenopus* (FETAX)

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Abstract: Over the past five years, comprehensive annual surveys by U.S. Fish and Wildlife Service (USFWS) personnel have identified sites with a high prevalence of abnormal native amphibians. A number of these sites are located within National Wildlife Refuges (NWR) in the Northeast. In conjunction with the field surveys, prolonged (140-d) FETAX assays were performed with sediment and surface water samples from three potentially affected NWR sites: 1) Ferry Way Beaver Pond, Great Bay NWR, Newington, New Hampshire, 2) Brown Street Impoundment, Rachel Carson NWR, Wells, Maine and 3) Black Creek (Trails Site), Missisquoi NWR, Swanton, Vermont. Endpoints used to assess the effects of site sediment and surface water exposure on *Xenopus* development included survival, ability to complete metamorphosis, time to complete and size at metamorphosis, and frequency of malformations.

Exposure to Rachel Carson sediment and surface water had no significant ($p > 0.05$) detrimental effects on *Xenopus* (*X. laevis*) development, relative to control exposures. Exposure to Great Bay water and sediment caused a significant ($p \leq 0.05$) reduction in *Xenopus* embryo survival, and significantly inhibited and delayed development and metamorphosis. Exposure to Missisquoi water, and sediment exposures with overlying Missisquoi water caused significant ($p \leq 0.05$) embryo mortality and significantly inhibited metamorphosis in *Xenopus* embryos. There were no severe malformations observed in metamorphs from any of the NWR site exposures. Slight to moderate malformations were observed in embryos/tadpoles that died in the Great Bay and Missisquoi exposures.

Detectable concentrations of pesticides were measured in the Great Bay and Missisquoi sediment samples. The presence of pesticides, and low concentrations of essential ions in site water may have contributed to the adverse developmental effects

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observed in *Xenopus* in the Great Bay and Missisquoi exposures.

Keywords: *Xenopus*, FETAX, amphibian development, metamorphosis, malformation

Introduction

Over the past decade there has been a growing concern over declining amphibian populations and increased incidences of abnormalities among native frog populations. In the fall of 1996, scientists from the Vermont Agency of Natural Resources verified the presence of deformed northern leopard frogs (*Rana pipiens*) at four sites along Lake Champlain, including sites very near the Missisquoi National Wildlife Refuge (NWR) in Swanton, Vermont (Eaton-Poole and Pinkney 2001). The types of abnormalities reported included missing limbs, reduced limb segments, missing digits, misshapen or underdeveloped feet and legs, and missing eyes (Burkhart et al. 2000; Meteyer et al. 2000). These were the first verified reports of abnormal frogs in the U.S. Fish and Wildlife Service's (USFWS) Region 5, which encompasses the Mid-Atlantic and Northeastern United States, ranging from Maine to Virginia.

Due to concerns that the abnormal frog problem may be widespread throughout Region 5, wetlands in numerous NWRs have been monitored from 1997 through 2001. Preliminary sampling and subsequent monitoring were designed to determine if abnormal frogs and toads were present at NWR sites, and to identify and verify potentially impacted sites. In addition to the monitoring, diagnostic analyses of abnormal native amphibians, and *Xenopus*-based laboratory studies have been conducted to determine whether causal factors could be identified at potentially impacted sites. The results of the field sampling and diagnostics are given by Eaton-Poole et al. (2002). Here we describe the results of the *Xenopus* tests and supporting chemical analyses.

Several potentially impacted sites have been identified. Three of these sites, Ferry Way Beaver Pond at Great Bay NWR, Newington, NH, Brown Street Impoundment at Rachel Carson NWR, Wells, MA and Black Creek (Trails Site) in Missisquoi NWR, Swanton, VT, were selected for further investigation using *Xenopus*-based laboratory studies. The site at Great Bay had a major "die-off" event in 1997 and subsequent monitoring has yielded incidences of abnormal green frogs of 3.4% to 10.0%. The site at Rachel Carson was monitored in 1999 and 2000. Sampling in 1999 yielded a prevalence of abnormal green frogs (*Rana clamitans*) and pickerel frogs (*Rana palustris*) ranging from 3.6% to 6.4%. In the summer of 2000, frog numbers were greatly reduced and some metamorphs were weak and dying during sampling. A ranavirus was isolated from some abnormal frogs collected at the Rachel Carson site. The Missisquoi site was monitored from 1997 through 2000. Abnormality rates for northern leopard frogs ranged from 2.4% to 8.1%. A standard FETAX; performed in 1997, showed that water and sediment samples from the Missisquoi site induced increased mortality and malformations in *Xenopus* embryos (Fort et al. 1999a).

In this study, we performed an evaluation of the potential impact of exposure to surface water and sediment from the three NWR sites on amphibian development using a

modified version of FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*). FETAX is currently the only standardized amphibian-based assay (ASTM 1998). Exposures encompassed the entire embryo-larval period, from blastula stage through metamorphosis. The prolonged exposure was used to evaluate the effects of site water and sediment on limb development and metamorphosis in *Xenopus*, endpoints which cannot be evaluated using standard FETAX. Limb development was a critical endpoint, as many of the abnormal native amphibians had various leg abnormalities. Several studies (Schuytema et al. 1991; Fort et al. 1999a; Gutleb et al. 1999) have successfully used longer-term modified FETAX assays to evaluate the effects of single chemicals, and contaminated water and sediment on important developmental processes, such as limb development and metamorphosis.

The objectives of this study were to assess the effects of long-term exposure to NWR site surface water and sediment samples on *Xenopus* larval development and growth, time and ability to complete metamorphosis, and size at metamorphosis. Although results of the *Xenopus* study cannot be directly extrapolated to native amphibians, we hope to be able to identify potential causative agents with the prolonged FETAX exposure and corresponding water quality and chemical analyses.

Materials and Methods

Sample Collection

Test water was collected as a series of grab samples from near-shore shallow water areas at each of the three NWR test sites by USFWS personnel. Samples were collected in 4- or 19-liter polyethylene cubitainers that had been rinsed three times with deionized water and three times with site water before collection. The first samples from Ferry Way Beaver Pond at Great Bay were collected on June 14, 2000 and the first samples from Brown Street Impoundment at Rachel Carson were collected on June 29, 2000. The grab samples were composited and thoroughly mixed. Grab samples were collected every two weeks and shipped overnight on ice to the testing laboratory for renewal. Multiple grab samples for the entire test period were collected from Black Creek in Missisquoi on October 18, 2000. These samples were composited, thoroughly mixed and shipped overnight on ice to the testing laboratory. All test water was filtered through a 250 μm stainless steel mesh to remove debris and indigenous organisms that could affect test results through predation, competition for food or by acting as vectors for parasites. Water was stored in the dark at 4 C during field collection, transport to the laboratory, and during the test.

USFWS personnel collected sediment as a series of grab samples from near-shore shallow water areas at the three test sites on the same dates as the initial water samples. At each NWR site, a stainless steel petite ponar (15 x 15 cm) grab sampler or stainless steel ladle was used to sample the top 2-5 cm of sediment. The sediment grab samples were composited, thoroughly homogenized, and stored in a 5-gallon (19-l) polyethylene bucket that had been rinsed three times each with deionized water and site water. All samples were shipped to the testing laboratory on ice the same day they were collected. At the laboratory, all test sediment was pressed through a 250 μm mesh stainless steel

sieve to remove debris and indigenous organisms. Sediment was maintained in the dark at 4 C from the time of collection and transport through storage in the laboratory prior to testing. After sieving, a 500 gram subsample was placed in chemically clean jars and sent to the U. S. Geological Survey, Organic Geochemistry Research Laboratory (Lawrence, KS) for analysis of organochlorine pesticides, organophosphorus pesticides, and herbicides by gas chromatography/mass spectrometry using the selected ion mode.

Test Procedure

University of Maryland - Wye Research and Education Center (UMD-WREC) non-chlorinated, deep well water was used as diluent and/or control water. An aqueous FETAX exposure served as a negative control, to compare the effects of well water and site water on *Xenopus* development, relative to FETAX solution. For the sediment exposures, UMD-WREC well water was used as diluent because FETAX solution appeared to ameliorate the effects of pond water and sediment extracts from Minnesota test sites (Burkhart et al. 1998).

Sediment collected from the James Pond area of the Magothy River, Maryland, was used as control sediment. James Pond sediment is used extensively by the UMD-WREC as a reference sediment for chronic toxicity tests with freshwater amphipods and has low concentrations of priority pollutants. For the Great Bay and Rachel Carson studies, UMD-WREC personnel collected control sediment as a series of grab samples from shallow water areas of James Pond on June 7, 2000. For the Missisquoi study, control sediment was collected on October 25, 2000. Control sediment was collected, sieved and stored in the same manner as NWR site sediment.

Xenopus adult care, breeding and embryo collection were performed as described in the American Society for Testing and Materials (ASTM) publication E1439-91 (ASTM 1998) for conducting FETAX. Four replicates of 20 blastula stage embryos for the Great Bay and Rachel Carson tests, and five replicates of 20 blastula stage embryos for the Missisquoi test were exposed to each of the following treatments for the prolonged FETAX exposures: 1) FETAX solution, 2) control water, 3) site water, 4) control sediment + control water, 5) control sediment + site water, 6) site sediment + control water and 7) site sediment + site water. At the start of each 140-d prolonged FETAX exposure, each replicate of 20 embryos was exposed to 1.2 L of test solution in the aqueous exposures, and 1.2 L of test solution overlying 600 g of sediment in the sediment exposures. Fifty percent water replacements were performed twice per week, and gentle aeration (1 bubble/second) was provided to each replicate to maintain adequate water quality. The test solution volume was increased incrementally to a maximum of 6 L as embryos grew, to prevent density-dependent effects. Embryos were fed crushed Tetramin® flake food twice daily. Debris, excess food and dead embryos were removed daily and observations of each replicate were also recorded daily. Dissolved oxygen and pH were measured daily in the overlying water. Conductivity, alkalinity, hardness, nitrates and ammonia were measured twice per week. Tests were conducted at 23.0±1 C with a 16h:8H light:dark cycle.

Organisms that completed metamorphosis were anesthetized with MS-222 and checked for malformations using a dissecting microscope. Snout-vent length (mm) and

wet weight (mg) were measured for each metamorph. Metamorphs were then fixed in 10% buffered formalin. Embryos which did not complete metamorphosis after 140 days of exposure were anesthetized, checked for malformations and preserved in 10% buffered formalin.

Survival, metamorphosis and growth data were analyzed using analysis of variance, followed by Dunnett's or Bonferroni's multiple comparison procedures. Results were considered significant at p 0.05. Site water exposures were compared with the control water exposure, while site sediment exposures were compared to the control sediment + control water exposure. Percentage data were arc-sine square root transformed before analysis. The ability of embryos to complete metamorphosis was analyzed both as the proportion of surviving embryos completing metamorphosis, as well as the proportion of original embryos completing metamorphosis.

Results

Great Bay NWR Study

Three replicate exposures were dropped from the study due to excess algal or fungal growth. Data from these replicates was not included in final statistical analyses. Embryo survival, metamorphosis and growth data for the Great Bay study are summarized in Table 1.

Table 1 - *Great Bay NWR Study: Embryo Survival, Growth and Metamorphosis Data*

Exposure	No. of Reps	Mean Survival (%)	Mean % Metamorphs/ Surviving Embryos	Mean % Metamorphs/ Original Embryos	Mean Duration of Metamorphic Period (d)	Mean Snout-Vent Length (mm)	Mean Wet Wt. (mg)
FETAX Solution	4	90.0	60.8	53.8	96.3	15.7	437.0
Control water	4	82.5	91.3	75.0	112.5	16.0	478.1
GB Water	4	5.0[a]	100.0	5.0[a]	101.3	15.3	444.0
Con. Sed.+ Con. Water	3	88.3	53.4	46.7	104.8	16.2	487.7
Con. Sed.+ GB	4	91.3	42.3	38.8	119.2[b]	15.8	458.9

Water							
GB Sed.+ Con. Water	3	76.7	6.4[b]	5.0[b]	123.3[b]	13.7[b]	299.3[b]
GB Sed.+ GB Water	3	70.0	20.4[b]	11.7[b]	120.8[b]	15.0	407.0

[a] Significantly different from control water exposure (p 0.05).
[b] Significantly different from control sediment/control water exposure (p 0.05).

Prolonged exposure to Great Bay water caused significant (p 0.05) *Xenopus* embryo mortality. There was 100% mortality in three of the four replicates, and only four embryos survived through metamorphosis in the remaining replicate. The mean period of metamorphosis was not affected by the Great Bay water exposure, and the four metamorphs were only slightly smaller than metamorphs from the control water exposure.

Mean survival was not affected by any of the Great Bay sediment or overlying water exposures, relative to the control sediment + control water exposure. The Great Bay sediment + control water and Great Bay sediment + Great Bay water exposures significantly inhibited or delayed metamorphosis in *Xenopus* embryos. Metamorphs in the Great Bay sediment + control water exposure were significantly smaller than metamorphs in the control sediment + control water exposure. The control sediment + Great Bay water exposure significantly delayed metamorphosis. Development was significantly inhibited or delayed in all Great Bay sediment or overlying exposures, as many of the embryos that did not complete metamorphosis in these exposures had not developed legs at test termination (140 d).

Rachel Carson NWR Study

Three replicates were dropped from the study due to excessive algal growth. Data from these replicates were excluded from statistical analyses. Embryo survival, metamorphosis and growth data for the Rachel Carson study are summarized in Table 2.

Table 2 - *Rachel Carson NWR Study: Embryo Survival, Growth and Metamorphosis Data*

Exposure	No. of Reps	Mean Survival (%)	Mean % Metamorphs/ Surviving Embryos	Mean % Metamorphs/ Original Embryos	Mean Duration of Metamorphic Period (d)	Mean Snout-Vent Length (mm)	Mean Wet Wt. (mg)
FETAX Solution	3	80.0	87.5	70.0	116.2	15.5	397.3

Control water	3	80.0	91.4	73.3	120.6	15.7	415.6
RC Water	4	67.5	92.5	62.5	100.6	15.5	401.3
Con. Sed.+ Con. Water	4	73.8	70.9	52.5	116.2	16.0	448.5
Con. Sed.+ RC Water	4	72.5	59.6	43.8	121.1	15.3	383.8
RC Sed.+ Con. Water	3	73.3	44.0	31.7	120.1	15.0	353.3
RC Sed.+ RC Water	4	73.8	58.7	42.5	110.0	16.2	476.7

Prolonged exposure to Rachel Carson sediment or water caused no statistically significant ($p > 0.05$) adverse developmental effects in *Xenopus* embryos. The Rachel Carson water exposure caused a slight reduction in embryo survival. The ability to complete metamorphosis and size at metamorphosis were slightly reduced in the Rachel Carson sediment and water exposures, relative to the control sediment + control water exposure.

Missisquoi NWR Study

Five replicates were dropped from the study due to excessive algal growth. Data from these replicates were excluded from final statistical analyses. Embryo survival, metamorphosis and growth data for the Missisquoi study are summarized in Table 3.

The Missisquoi water exposure caused significant (p 0.05) mortality, as only one out of 100 embryos survived to complete metamorphosis in the exposure. The lone survivor completed metamorphosis in a much shorter period of time, but weighed less than metamorphs from the control water exposure. Many of the embryos that died early in the Missisquoi water exposure had axial, gut or craniofacial malformations. An embryo that was missing an eye died on Day 39 of the Missisquoi water exposure.

Table 3 - *Missisquoi NWR Study: Embryo Survival, Growth and Metamorphosis Data*

Exposure	No.	Mean	Mean %	Mean %	Mean	Mean	Mean

	of Reps	Survival (%)	Metamorphs/ Surviving Embryos	Metamorphs/ Original Embryos	Duration of Metamorphic Period (d)	Snout-Vent Length (mm)	Wet Wt. (mg)
FETAX Solution	5	79.0	82.6	65.0	96.2	15.6	395.2
Control water	3	65.0	85.6	55.0	100.6	15.5	411.1
Miss. Water	5	1.0[a]	100	1.0[a]	69.0	15.0	315.0[a]
Con. Sed.+ Con. Water	4	76.3	60.7	43.8	117.4	14.4	302.1
Con. Sed.+ Miss.Water	4	25.0[b]	76.3	11.2[b]	116.9	14.0	302.4
Miss.Sed. + Con. Water	5	73.0	49.8	39.0	106.0	14.6	305.4
Miss.Sed. + Miss.Water	4	17.5[b]	28.6[b]	7.2[b]	115.5	15.3	379.0

[a] Significantly different from control water exposure (p 0.05).
[b] Significantly different from control sediment/control water exposure (p 0.05).

The control sediment + Missisquoi water and Missisquoi sediment + Missisquoi water exposures caused a significant reduction in mean survival of *Xenopus* embryos, and significantly inhibited the ability of embryos to complete metamorphosis. The sediment exposures with overlying Missisquoi water appeared to inhibit or delay development, as many of the embryos that did not complete metamorphosis had not developed rear legs at test termination. Many of the embryos that died early in these exposures also had axial, gut and craniofacial abnormalities. The period of metamorphosis or size of metamorphs was not affected by Missisquoi sediment or overlying water exposures. The Missisquoi sediment + control water exposure caused no adverse developmental effects in *Xenopus* embryos.

Water Quality and Pesticide Analyses

Mean water quality data for the three NWR sites are summarized in Table 4. Dissolved oxygen, pH, conductivity, alkalinity, hardness, ammonia and nitrate were monitored throughout each 140-d exposure. As shown in Table 4, pH values were consistently lower in water from all three NWR sites than in control water or FETAX solution. Conductivity and hardness were much lower in Great Bay and Missisquoi water, relative to control water or FETAX solution.

Table 4 - *General Water Quality for the Great Bay NWR, Rachel Carson NWR and Missisquoi NWR Studies (Mean values)*

Exposure	D.O. (mg/ L)	pH (range)	Conductivity (µmhos)	Alkalinity	Hardness	Ammonia
FETAX solution	7.7	6.91-7.81	1651	50	103	0.8
Control Water	7.6	7.15-8.18	344	97	96	0.6
Con Sed+ Con Water	7.2	7.15-8.29	1362	128	90	0.6
Great Bay Water	8.1	6.46-7.37	118	20	33	0.4
Con Sed+GB Water	7.1	6.25-7.69	355	45	50	0.5
GB Sed+ Con Water	7.2	6.85-7.48	1050	125	100	0.5
GB Sed+ GB Water	7.1	5.70-6.83	115	55	75	0.5
Rachel Carson Water	7.7	6.28-7.33	1560	23	183	0.5
Con Sed+ RC Water	7.3	6.07-7.81	1700	23	210	0.4
RC Sed+ Con Water	7.1	7.28-7.82	1050	118	90	0.5
RC Sed+ RC	7.3	5.19-	1750	20	215	0.5

Water		6.49				
Missisquoi Water	8.0	5.79-6.76	154	70	58	0.9
Con Sed+ MS Water	7.3	6.35-7.22	598	80	70	1.3
MS Sed+ Con Water	7.3	6.70-7.50	377	100	95	0.8
MS Sed+MS Water	7.3	6.23-7.15	218	75	70	1.2

Detectable concentrations of p,p -DDD (5.20-15.00 µg/kg dry wt.), p,p -DDE (3.57-16.00 µg/kg dry wt.), and p,p -DDT (0.49-1.81 µg/kg dry wt.) were measured in Great Bay sediment. Much lower concentrations of p,p -DDD (<0.2-0.55 µg/kg dry wt.) and p,p -DDE (<0.2-0.28 µg/kg dry wt.) were measured in Rachel Carson sediment. The herbicides alachlor (1.20 µg/kg dry wt.) and metolachlor (0.57 µg/kg dry wt.) were measured in Missisquoi sediment.

Discussion

All three of the NWR sites selected for the prolonged FETAX laboratory studies have a history of a greater than 5.0% prevalence of abnormal frogs, low numbers of metamorphs, delayed metamorphosis or "die-off" events (Eaton-Poole and Pinkney 2001; Eaton-Poole et al. 2002). Laboratory results indicate that long-term exposure to water and/or sediment samples from the Great Bay and Missisquoi sites caused significant embryo mortality and other significant adverse developmental effects in *Xenopus* embryos, whereas long-term exposure to samples from the Rachel Carson site caused no adverse developmental effects in *Xenopus* embryos, relative to control exposures. Results of the Great Bay and Missisquoi long-term FETAX studies are consistent with observations made by USFWS personnel during seasonal monitoring at these two sites (Eaton-Poole and Pinkney 2001), as well as standard and modified FETAX assays performed with Missisquoi water and sediment samples (Eaton-Poole and Pinkney 2001; Fort et al. 1999a). At the Great Bay site in 2000, metamorphosis occurred two to three weeks later than at an adjacent site and there was a lack of metamorphs despite the abundant presence of calling adult frogs, green frog egg masses and tadpoles earlier in the season (Eaton-Poole and Pinkney 2001). An increased prevalence of abnormal northern leopard frogs (*Rana pipiens*) has been reported at the Missisquoi site, and Missisquoi water and sediment samples have caused mortality and malformations in *Xenopus* embryos in standard and modified FETAX (Eaton-Poole and Pinkney 2001; Fort et al. 1999a). Field observations and diagnostics at the Rachel Carson site indicate that abnormalities and "die-off" events at the site may have been due to disease (ranavirus) (Eaton-Poole and Pinkney 2001).

Long-term exposure to Great Bay water and sediment samples significantly

increased mortality in *Xenopus* embryos, inhibited and delayed metamorphosis, and decreased size at metamorphosis, relative to control exposures. For the sediment exposures, the most significant negative developmental effects were caused by the Great Bay sediment + control water exposure. Metamorphosis was significantly inhibited or delayed, and metamorphs were significantly smaller in the Great Bay sediment + control water exposure. These results indicate the likely presence of a toxic agent(s) in Great Bay sediment. The Great Bay water exposure caused significant mortality in *Xenopus* embryos, while the Great Bay sediment + Great Bay water exposure caused only sublethal developmental effects in *Xenopus*. We suggest that agents present in the Great Bay water may have been initially bound by the sediment, and subsequently released slowly into the overlying water at sublethal concentrations.

Results indicate that Missisquoi water was responsible for the toxicity observed in *Xenopus* in the aqueous and sediment exposures. Long-term exposure to Missisquoi water caused significant mortality in *Xenopus* embryos. The control sediment + Missisquoi water and Missisquoi sediment + Missisquoi water exposures significantly inhibited the ability of *Xenopus* embryos to complete metamorphosis. The Missisquoi sediment + control water exposure caused no significant developmental effects.

None of the metamorphs from the Great Bay or Missisquoi exposures had significant limb deformities, such as missing or supernumerary limbs observed in native amphibians (Burkhart et al. 2000; Eaton-Poole and Pinkney 2001). This was not surprising, as very few laboratory exposures with *Xenopus* have induced the broad range of limb deformities observed in native amphibians in the field (Ankley et al. 1998). A few metamorphs in the Great Bay exposures had slightly reduced musculature of the rear legs, while four embryos had a moderate reduction of rear leg musculature in the Missisquoi exposures. Two of these metamorphs also had slightly reduced upper jaws. While very few metamorphs were malformed, many embryos that died early in the Great Bay and Missisquoi exposures were slightly to severely malformed. Similar malformations were observed in embryos from both studies, and included axial curvature, abnormal gut coiling and craniofacial abnormalities. Two embryos that died during the Missisquoi exposure had eye abnormalities, including one embryo that was completely missing an eye. Fort et al. (1999a, 1999b) observed similar types of malformations in *Xenopus* embryos exposed to sediment and water samples from affected sites in Minnesota and Vermont, including the Missisquoi site.

Several factors have been identified or speculated as contributing to declining amphibian populations and the increased frequency of abnormal frogs. These factors include climate changes (Laurance 1996), habitat destruction (Delis et al. 1996), endoparasite infestation and disease (Johnson et al. 1999), ultraviolet (UV) radiation (Licht and Grant 1997), mineral depletion (Luo et al. 1993), introduction of non-native predators (Kiesecker and Blaustein 1998) and natural and man-made chemical contaminants. There has been recent speculation that low concentrations of essential cations (Mg^{2+}, Ca^{2+}, Na^{2+}, K^{2+}) may influence the development of amphibians in aquatic environments, and compromise the validity of FETAX results in the laboratory (Tietge et al. 2000). Burkhart et al. (1998) and Fort et al. (1999a, 1999b) determined that although the low ionic concentrations of affected pond water was not the direct cause of developmental toxicity in *Xenopus* embryos, ion deficiencies may contribute to toxicity

by increasing stress to embryos or by modifying the response to contaminants in the water. Luo et al. (1993) and Morgan et al. (1996) demonstrated that the ionic matrix of water can enhance or decrease the toxicity to *Xenopus* embryos of some metals and pesticides, respectively. Although concentrations of anions and cations were not determined, general water chemistry measurements; especially conductivity and hardness, suggest that concentrations of important ions, primarily Ca^{2+}, may have been reduced in Great Bay and Missisquoi water samples, relative to control water and FETAX solution. The Great Bay and Missisquoi water exposures were dramatically more toxic than sediment exposures with overlying Great Bay and Missisquoi water. Water quality measurements from the aqueous and sediment exposures suggest that essential ions may have been released from sediment into overlying water, thereby potentially decreasing stress to *Xenopus* embryos in the sediment exposures. Water quality parameters from the Rachel Carson site were very similar to those of FETAX solution, indicating that the ionic matrix was sufficient to support survival and development of *Xenopus* embryos.

Burkhart et al. (1998) and Fort et al. (1999a, 1999b) speculated that a mixture of chemical contaminants were responsible for the adverse effects observed in *Xenopus* embryos in standard and modified FETAX performed with water and sediment samples from sites in Minnesota and Vermont, including the Missisquoi site. Land use surrounding the NWR sites from this study indicate potential contamination by agricultural chemicals. The Great Bay site was created from the former Pease Air Force Base. Previous monitoring of the site indicated the presence of DDT, DDD, DDE and metals. The Missisquoi site is located in a predominantly agricultural area of Vermont. The Rachel Carson site may be affected by herbicide use at an adjacent railroad right-of-way (Eaton-Poole and Pinkney 2001). Due to financial constraints, only pesticide analyses were performed on sediment samples from the three NWR sites. Analyses of other types of contaminants such as PCBs, PAHs and metals were not performed.

Elevated levels of p,p -DDD (5.20-15.00 µg/kg dry wt.), p,p -DDE (3.57-16.00 µg/kg dry wt.) and p,p -DDT (0.49-1.81 µg/kg dry wt.) were measured in Great Bay NWR sediment samples. Schuytema et al. (1991) observed that chlorinated pesticides in an aqueous exposure induced facial and skeletal abnormalities, and edema in *Xenopus* embryos. These types of malformations were observed in *Xenopus* embryos in standard FETAX and in embryos that died early in the prolonged FETAX exposure to Great Bay water and sediment samples.

Over the last decade, the presence of xenobiotic estrogens, which disrupt reproduction and developmental processes, have become a major environmental concern. Numerous chemicals, including DDT, have been identified as endocrine disruptors (Colborn et al. 1993). Endocrine disruptors have shown the ability to disrupt metabolism and storage of retinoids and thyroid hormone homeostasis in vertebrates (Gutleb et al. 1999). The interactions of thyroid hormones, retinoids and endocrine/thyroid receptors and disruptors are complex and crucial in embryonic development, pattern formation, growth and regulation of energy metabolism and ultimately, metamorphosis in amphibians. Decreased levels of thyroid hormones may result in prolonged metamorphosis, absence of metamorphosis or increased mortality of metamorphic larvae. The dramatic inhibition of metamorphosis in *Xenopus* embryos in the Great Bay exposures, and observations of delayed and inhibited metamorphosis in green frogs at the

Great Bay site, support the speculation that DDT and its metabolites could have contributed to the adverse developmental effects observed in the FETAX study. Since low external calcium concentrations have been shown to exacerbate the excitation effects of neurotoxic pesticides, like DDT, likely ion deficiencies in Great Bay water may actually enhance the toxic effects of DDT and its metabolites.

Elevated levels of the pre- and/or post-emergent herbicides alachlor (1.20 µg/kg dry wt.) and metolachlor (0.57 µg/kg dry wt.) were detected in Missisquoi sediment. Both herbicides are relatively stable, with alachlor being more water soluble, and are likely to be found in surface waters (Thurman et al. 1992). The Missisquoi sediment samples were collected in October, when concentrations of herbicides are generally at their lowest (Hall et al. 1999). Although surface water concentrations cannot be extrapolated from the concentrations measured in Missisquoi sediment samples, the fact that herbicides were detected four to five months after application suggests that herbicide concentrations were most likely higher earlier in the year when native amphibians were breeding. Because these herbicides are commonly mixed with other herbicides (e.g., atrazine, glyphosate, trifluralin, etc.) prior to application, other herbicides could potentially have been present during breeding and larval development of native amphibians at the Missisquoi site.

There are limited data available on the toxicity of herbicides to amphibian larvae. Howe et al. (1998) found that the 96-h LC50s for alachlor for early- and late-stage tadpoles of northern leopard frogs were 11.5 mg/L and 6.5 mg/L, respectively, and that 96-h LC50s for alachlor for early- and late-stage tadpoles of the American toad (*Bufo americanus*) were 3.9 mg/L and 3.3 mg/L, respectively. Howe et al. (1998) also demonstrated a significant chemical synergy interaction between a 50:50 mixture of alachlor and atrazine. Although there is no information available on the toxicity of metolachlor to amphibian larvae, it is very similar to alachlor in its toxicity to fish. Because it is common for many types of pesticides to be found in surface waters receiving agricultural runoff, and that commonly mixed herbicide formulations can have greater than additive toxicity, the threat to amphibian larvae and other aquatic organisms may be greater than expected at the Missisquoi site. Because Missisquoi water; and not sediment, caused negative adverse effects in *Xenopus* embryos, and concentrations of herbicides were not quantified in Missisquoi water, we cannot speculate on the contribution of herbicides to the toxicity observed in *Xenopus* embryos. Clearly, there is a need for further investigation of possible pesticide effects at this site.

Although there are considerable genetic differences between *Xenopus* and the *Rana* species observed in the field, the complex developmental pathways are highly conserved, and results of the *Xenopus* embryo-larval studies may provide valuable insight to the phenomenon of increasing amphibian abnormalities and decreasing amphibian populations. Rowe et al. (1998) demonstrated that tadpoles living in polluted sites had elevated maintenance costs associated with damage repair and elimination of pollutants, ultimately lowering the energy available for growth, development and metamorphosis. Pesticide contamination and ion deficiencies could have increased stress and elevated maintenance costs in *Xenopus* embryos and tadpoles, therefore contributing to the adverse developmental effects observed in the Great Bay and Missisquoi FETAX studies. Because amphibian larval performance strongly influences adult fitness (Smith 1987; Bridges 2000), the significant adverse developmental effects observed in the Great Bay

and Missisquoi FETAX studies would have potentially dramatic effects on natural frog populations. In addition to causing significant embryo mortality and some malformations, the exposures significantly inhibited and/or delayed metamorphosis, and in some cases, significantly decreased the size of metamorphs. In a natural environment, an inability to complete metamorphosis or delay in metamorphosis could reduce juvenile recruitment by an increased risk of predation or death due to dessication of ephemeral bodies of water (Howe et al. 1998). Delayed metamorphosis can also lead to delayed maturity, which can alter the demographic structure of a population, potentially resulting in a gradual decline in the size of the population (Bridges 2000). Decreased size at metamorphosis can lead to decreased overwintering success, delayed sexual maturation, decreased locomotor ability for predator evasion, and smaller adult size (Allran and Karasov 2000). Adult female frogs may lay fewer eggs due to smaller body size, and smaller male frogs would likely gain access to fewer numbers of females, leading to decreased reproductive success (Berven 1982).

Relatively low concentrations of p,p -DDE (0.44-0.55 µg/kg dry wt.) and p,p DDD (<0.2-0.28 µg/kg dry wt.) were measured in Rachel Carson sediment. Long-term exposure to Rachel Carson sediment and water caused no significant developmental effects in *Xenopus* embryos and metamorphs, and only slight to moderate malformations were observed in *Xenopus* in standard FETAX.

In summary, long-term exposure to Great Bay sediment and water, and Missisquoi water caused significant adverse developmental effects in *Xenopus*. We suggest that sediment contaminants, possibly including DDT and its metabolites, and ion deficiencies may contribute to the adverse developmental effects observed in *Xenopus* in the laboratory and possibly native amphibians at the Great Bay site. Because pesticide analyses were not performed on Missisquoi water, we cannot speculate on the contribution of pesticides to the adverse developmental effects in *Xenopus* embryos or native amphibians. However, possible ion deficiencies could be enhancing the effects of pesticides or other contaminants at the Missisquoi site. The contribution to toxicity by other factors such as UV light, endoparasites, viruses, or other contaminants like PAHs, PCBs and metals were not determined. Long-term exposure to Rachel Carson sediment and water samples caused no significant developmental effects in *Xenopus* embryos. Currently, expanded contaminant analyses and ion characterization are being included in prolonged FETAX studies with samples from the Great Bay and Missisquoi sites to further investigate potential causative agents. Although *Xenopus* is a non-native species, results of this study and previously performed long-term exposures with *Xenopus* embryos (Fort et al. 1999a, 1999b) coincide with negative developmental effects observed in native amphibian species at many sites. Future research will include laboratory exposures with both *Xenopus* and native amphibian embryos. These studies could further verify the use of *Xenopus* as a suitable, cost-effective surrogate species when investigating the effects of contaminants on amphibian embryo-larval development.

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Determination of Malathion Aerial Drift and Associated Effects to the Endangered Wyoming Toad (*Bufo baxteri*) Using Surrogate Woodhouse's Toads (*Bufo woodhousii*) at Mortenson and Hutton Lake National Wildlife Refuges and Potential Reintroduction Sites

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Abstract: The endangered Wyoming toad is confined to Mortenson National Wildlife Refuge in southeast Wyoming. Pesticide aerial drift from mosquito control activities adjacent to the refuge may be partially responsible for the toad's decline. Spray cards had detectable malathion concentrations at three sites but survival of surrogate Woodhouse's toads was 100% and mean ChE activities in brain and plasma samples did not differ significantly among sites. Righting trial times in surrogates among sites after spraying were not significantly different. Terrestrial invertebrate abundance results were inconclusive but differences in aquatic invertebrate relative abundance before or after spraying were not significant except at the reference site and Meeboer Lake. Our results indicate that, although some malathion drift is occurring, the toads were not exposed to malathion concentrations great enough to reduce adult survival, affect predator avoidance behavior, or reduce their food source. The data also provide needed information on ChE activities in amphibians.

Keywords: amphibians, cholinesterase, malathion, nontarget effects

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Introduction

Wyoming toads (*Bufo baxteri*), confined to wetlands and irrigated meadows in the Laramie Plains of Albany County, were abundant prior to the 1960s. By the 1970s, the population had declined drastically and was confined to Mortenson National Wildlife Refuge (NWR). In 1984, the species was listed as endangered under the Endangered Species Act and the last few remaining individuals were taken into captivity. Because of a successful captive breeding program, the endangered Wyoming toad was reintroduced to both Mortenson NWR and the nearby Hutton Lake NWR in June 1995.

Reasons for the Wyoming toad population decline are unknown, but pesticide use for mosquito control may have played a part. The last population of Wyoming toads was found adjacent to a ranch that did not spray for mosquitoes (Stone 1991). Initially mosquitoes were controlled in fields adjacent to Mortenson NWR and the Laramie River using widespread aerial applications of fenthion but this insecticide was replaced by malathion.

Malathion is aerially sprayed on private lands adjacent to Mortenson NWR, potential reintroduction sites, and along the Laramie River at 21 mg/m^2 (3 oz. active ingredient/acre) as an ultra low volume (ULV) spray with no carriers (97% malathion/3% impurities). Label requirements prohibit the application of malathion to open water and during unfavorable weather conditions because of its toxicity to aquatic and terrestrial invertebrates. Even so, one study indicates that ULV applications applied correctly can yield up to a 10-fold increase in off-target drift compared to solid stream nozzle applications that produce a coarser spray (Bird et al. 1996).

The application of malathion occurs during late June in the early morning. Malathion may be reapplied a second time in July if the mosquito outbreak is particularly severe. The application period is of particular concern because it coincides with the period when the toads are most at risk for exposure to this cholinesterase (ChE)-inhibiting insecticide. Absorption of malathion can result in death or cause indirect effects-intoxication, physical lethargy, or paralysis which increase the toads' risk of predation during this time of important growth and development (Mohanty-Hejmadi and Dutta 1981, Grue et al. 1991, Cowman and Mazanti 2000). Additionally during this early summer period, the toads are actively foraging for arthropods (Stone 1991) increasing their risk of pesticide exposure dermally and from ingestion.

The objectives of this study were to determine: 1) the extent of malathion entering Mortenson NWR or potential reintroduction sites by aerial drift; 2) if malathion is entering Mortenson NWR via water from the Laramie River; 3) the effect of malathion aerial drift on predator avoidance behavior in surrogate Woodhouse's toads (*B. woodhousii*); 4) the exposure of the Wyoming toad to malathion through aerial drift using percent survivability and cholinesterase activity in surrogate toads; and, 5) if malathion aerial drift is affecting the food source of Wyoming toads.

Methods and Materials

Study Sites

The Laramie Plains in Albany County, Wyoming (elevation 2135 to 2288 m) are semi-arid and consist of wetlands, ponds, seepage lakes, and irrigated meadows. Study sites included Mortenson Lake and Gibbs Pond on Mortenson NWR; Meeboer and Gelatt Lakes located on Wyoming State Land adjacent to Mortenson NWR; two sites near the Laramie River at 37 Mile Road and 7 mile Road; and private property (White's Place) bordering Mortenson NWR to the west (Figure 1). Water seeps from Mortenson NWR into Meeboer Lake via a small ditch making it possible for Wyoming toads to migrate to Meeboer Lake. Gelatt Lake is considered a potential reintroduction site for the Wyoming toad because of its similar wetland qualities to Mortenson Lake.

Mosquito control activities occur on private property surrounding Mortenson NWR, Meeboer Lake, and Gelatt Lakes. Mosquito control activities also occur along the Laramie River, which feeds these three lakes. Hutton Lake NWR served as the reference site because no mosquito control activities occur on or adjacent to this refuge.

Spray Application and Detection

The endangered status of the Wyoming toad requires that the local mosquito control district notify the U.S. Fish and Wildlife Service (USFWS) 48 hours prior to mosquito spraying. Pesticide indicator strips and spray cards were then placed at the study sites prior to spraying. Indicator strips, manufactured by Neogen Corporation, Lansing, Michigan, turn blue indicating that organophosphorus pesticides were not detected, or white indicating that such pesticides were present (detected in the part per billion range). Ten indicator strips were attached every 30 m to fence posts. Three or four pesticide spray cards (filter paper disks taped to index cards) were also attached to fence posts, alternating with pesticide strips. Strips and spray cards were placed near Mortenson Lake, Meeboer Lake, Gibbs Pond, Gelatt Lake, a canal at White's Place, Creighton Lake at Hutton Lake NWR, and along the Laramie River at 37 Mile Road and 7 Mile Road.

Spraying occurred on the morning of June 30, 1998, near Mortenson Lake. Mosquito control activities concluded the following morning near the Laramie River. Immediately after spraying, we retrieved the pesticide strips and spray cards from the fence posts. We dipped fresh pesticides strips in the water at each site to determine if drift entered the aquatic system. Pesticide strips were tested for indications of organophosphates. Filter paper disks were removed from the spray cards, preserved individually in glass jars with methylene chloride, and submitted to Patuxent Analytical Control Facility (PACF) in Laurel, Maryland, for analysis of malathion.

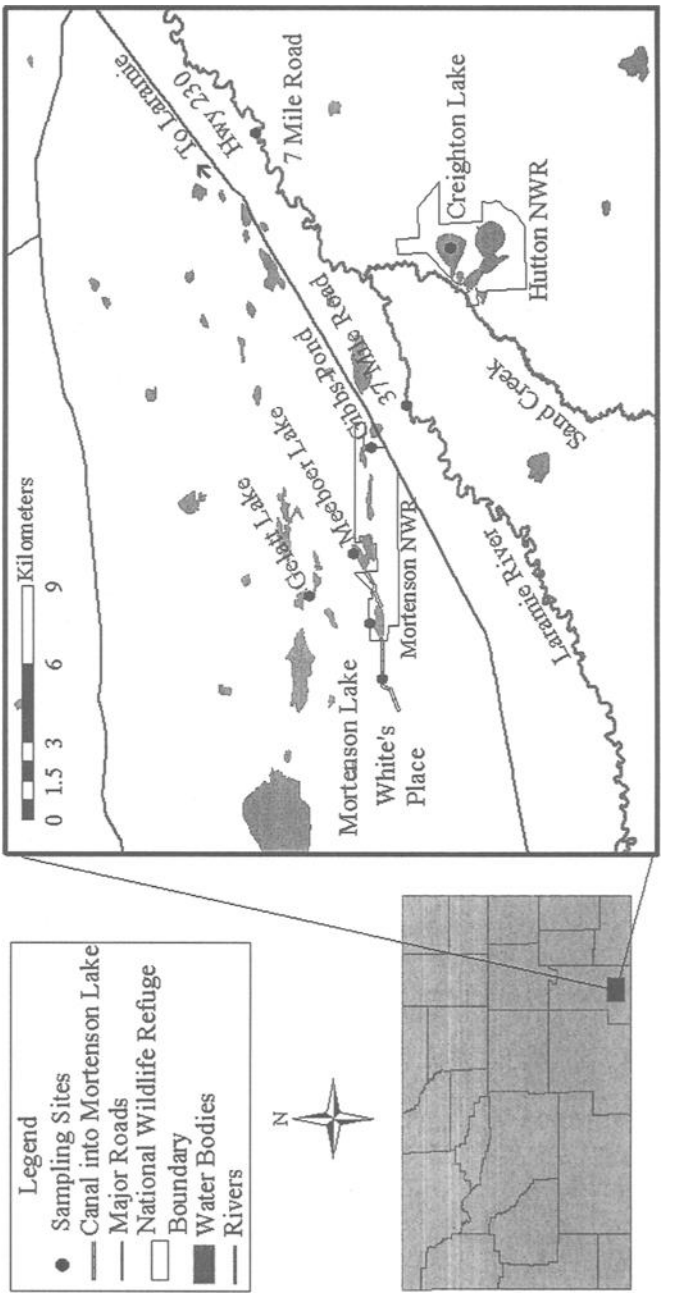

Figure 1 - *Sampling Sites in the Laramie Plains, Albany County, Wyoming.*

Surrogate Toad Exposures

Woodhouse's toads were used as surrogates to determine actual exposure to malathion at selected field sites. Surrogate animals were raised at the Wyoming Game and Fish Sybille Wildlife Research Unit. The Woodhouse's toads were one year of age to correlate with the age of the Wyoming toads at Mortenson and Hutton Lake NWRs.

Prior to spraying, righting trials were conducted on unexposed surrogate toads. Righting is the measure of time it takes a toad to right itself after being placed on its back and is used to determine if a pesticide affects the animal's behavior as demonstrated in a study of carbofuran effects to *B. valliceps* and *B. velatus* (Cowman and Mazanti 2000). Under normal conditions, toads will be able to right themselves within 10 seconds of being placed on their back (personal communication with Deborah Cowman, Texas A&M University, College Station, TX, January 1998).

Toads were then placed in stainless steel mesh (1.91 cm^2) enclosures (30.5 cm x 20.3 cm x 15.3 cm). Five toads were put in each enclosure and two enclosures were placed at each of the following sites: Gibbs Pond, Gelatt Lake, Meeboer Lake, a canal near White's Place, the Laramie River at 7 Mile Road, and Creighton Lake at Hutton Lake NWR. Enclosures were positioned next to the fence posts with pesticide strips and spray cards at the edge of the water at each site. Woodhouse's toads were not set inside Mortenson NWR to prevent potential disease transmittal to the Wyoming toads.

Immediately after spraying, we retrieved the surrogate toads. Some toads escaped from their cages during spraying (3 from White's Place, 5 from Meeboer Lake, and 2 each from Gibbs Pond, Gelatt Lake, and 7 Mile Road) and we were unable to recapture them. Righting trials were repeated on the remaining toads to determine if predator avoidance response was affected by potential pesticide exposure. Blood samples were drawn with heparinized 1-ml disposable syringes with 27½ gauge needles via heart puncture, placed in sterilized collection vials, and allowed to clot. The toads were placed in sterile plastic bags. Both the blood samples and toads were frozen in an alcohol / dry ice slurry and shipped overnight to Dr. Michael J. Hooper at Texas Tech University for analysis of acetylcholinesterase (AChE) and butyrylcholinesterase inhibition (BChE) in brain and plasma. AChE is the standard biomarker for indicating exposure to ChE-inhibiting pesticides, but BChE exhibits a higher percent of inhibition in lizards than AChE and was included for analysis (Bishop and Martinovic 2000). To remove any bias in the analysis, Dr. Hooper was not informed as to which samples came from the reference site.

Insect Collections

We used insect pitfall traps to collect terrestrial insects (ants and beetles) as described by Catangui et al. (1996). Ten pitfall traps were placed at each sampling site for two consecutive nights prior to and after the spray event. Insects were collected the morning following each trap night.

We collected aquatic invertebrates from the water column using a modified Gerking device as described by Kaminski and Murkin (1981) before and after the spray event. Species and quantity of both terrestrial and aquatic invertebrates were recorded for abundance estimates before and after spraying. Invertebrates were placed in chemically-cleaned glass vials and frozen immediately. Samples were submitted to PACF for malathion analysis. Study sites were observed for dead and dying terrestrial insects and aquatic invertebrates before and after the spray event.

ChE Analysis

Toads were thawed, skin and tissue of the neck removed, and the junction of the skull and spinal cord was exposed. A longitudinal cut through the skull was made following upward along each side of the spinal chord to the front of the cranium. The skull was then reflected forward to expose the brain. The brain was excised, cutting all neuronal connections consistently and severing the spinal cord at the base of the medulla oblongata where it exited the skull. Because of the extremely small size of the toads' brains and the importance of obtaining identical brain regions for each sample, every effort was made to ensure that the brain excision was consistent between all animals. However, we were unable to get an adequate brain sample from one toad at each of the following sites: Gibbs Pond, Meeboer Lake, Gelatt Lake, and 7 Mile Road.

Brain samples were weighed and homogenized (25-fold dilution, w/v, in 0.05 M tris buffer, pH 7.4) in a glass Wheaton homogenizing tube equipped with a Teflon-coated pestle attached to an overhead stirrer. Approximately 10 strokes were used to complete the homogenization. The samples were then diluted another 4-fold with the same buffer, giving a 100-fold overall dilution.

Blood samples were thawed and samples were carefully transferred to a refrigerated centrifuge (Heraeus Biofuge 15R), avoiding mixing of the clot and serum sample. Tubes were spun for 10 minutes at 3000 rpm and the resulting serum was removed, diluted five-fold in the 0.05 M tris buffer (pH 7.4), and assayed. One plasma sample was lost during preparation from Gelatt Lake and four plasma samples were lost during preparation from Gibbs Pond.

ChE activities were measured in brain and serum samples using the method of Ellman et al. (1961) as modified by Gard and Hooper (1993). The method was modified for use on a SPECTROmax 96-well spectrophotometric plate reader (Molecular Devices Corporation, Palo Alto, California), which was used in conjunction with a computer equipped with Softmax software (Molecular Devices Corporation). The spectrophotometer was set in a kinetic mode and measured absorption at 412 nm for three minutes with readings taken at thirteen second intervals with a zero second lag phase. AChE was differentiated from BChE by a five-minute pre-incubation with the specific BChE inhibitor, iso-tetraisopropyl pyrophosoramide (OMPA) (1×10^{-4} M final concentration [FC] in the assay). BChE activity was calculated as the difference between total ChE and AChE activity. Components of the assay were as follows: 0.05 M tris buffer (pH 8.0), 150 µmL; dithio[bis-2-nitrobenzoic acid] (DTNB), 20 µL, 3.23×10^{-4} M

FC; diluted enzyme source, 30 µmL; iso-OMPA (or buffer for total ChE), 20 µmL, 1 X 10^{-4} M FC; and AThCh, 30 µmL, 1 X 10^{-3} M FC. Final volume was 250 µL/well. In blank wells, buffer replaced enzyme volume. Samples were run in triplicate at 25°C. ChE activities were converted from optical density units/minute to µmoles AThCh hydrolyzed/minute (or "units") per ml plasma or g brain using the extinction coefficient, 13,600 cm^{-1} M^{-1}.

Diluted brain samples were divided into three 500 µL aliquots. One of the two aliquots was assayed immediately for absolute ChE activity and maintained on ice. The other two were used for 2-PAM (2-pyridinealdoxime methochloride) reactivation, which tests for the presence of cholinesterase-inhibited ChEs. One of the aliquots was spiked with 2-PAM (FC = 2 X 10^{-4} M) and the other with an equal volume of deionized water. These samples were incubated in a water bath at 25°C. Sub-samples were removed from the incubating material at 1 hour and assayed for ChE activity. An upper-tailed Student's t-test was then used to compare mean activities, from the values of samples run in triplicate, to determine if there was a significant activity increase in the 2-PAM incubated sample over the non-2-PAM incubated sample at any time point. Those samples found to have a significant increase in activity of at least 5% after 2-PAM incubation were considered to contain organophosphate-inhibited ChE and, thus, had most likely come from individuals exposed to an organophosphate.

Statistical Analysis

All statistical analyses were performed using Systat 8.0 statistical software (SPSS Corporation 1998) at alpha = 0.05 unless otherwise noted. Because of non-random sampling for pre-spray righting trial data, quantitative statistics could not be used to determine if there was a difference in right trial times of toads between pre-spray and post-spray trials. We were able to compare the righting trial post-spray times between the reference site and each study site using a Fisher's exact test.

AChE and BChE activity levels conformed to a normal distribution. We used general linear models to determine if there was significant interaction between the weight of the toads and the site because brain and plasma ChE means were lower at the reference site than at most of the sites adjacent to spraying activities. In the case of brain AChE and BChE, we also examined if there was a significant interaction between the brain weight of the toads and the site. If there was no significant interaction between the covariate (weight or brain weight) and the site, we used an analysis of covariance (ANCOVA) to determine if there were differences in ChE activities among sites adjusted for weight or brain weight. In some cases, the coefficient for the covariate was not significant. Therefore, we used a one-way analysis of variance (ANOVA) to determine if there were differences in ChE activities among sites to avoid the possibility that the ANCOVA may be taking away a degree of freedom without reducing the mean-square error (SPSS Corporation 1998).

Aquatic invertebrate abundance sample data were transformed by $\log_{10}$ to correct for skewness prior to statistical analysis. The pre- and post-spray means were compared

for each site using a paired t-test and are presented with 95% confidence intervals.

Results

Spray Application and Detection

Pesticide Strips - Pesticide strips tested positive for pesticides (with the number of strips testing positive/number of strips retrieved from the site in parentheses) at 37 Mile Road (6/9), Gelatt Lake (2/8), and 7 Mile Road (3/7). Pesticide strips tested negative for pesticides at Mortenson Lake, Gibbs Pond, Meeboer Lake, White's Place, and Hutton Lake NWR. Pesticide strips tested negative for malathion in water from all study sites.

Spray Cards - Malathion was detected on filter paper spray cards from three sites. Malathion concentrations in two of the three filter papers from Meeboer Lake were 0.298 and 0.354 μg/g wet weight (ww), although the pesticide strips tested negative. The three filter papers from spray cards at Gelatt Lake had malathion concentrations of 7.86, 21.4, and 5.97 μg/g ww. The four filter papers from 37 Mile Road had malathion concentrations of 28.8, 22.8, 16.3, and 0.595 μg/g ww. The filter papers indicate the amount of drift that may be entering the Laramie River, although pesticide strips used to detect the presence of organophosphate pesticides in the river water tested negative. At 7 Mile Road, filter papers (n=3) had no detectable malathion (<0.00200 μg/g ww) even though pesticide strips tested positive for pesticides. It is possible that the strips detected an organophosphate pesticide other than malathion. Malathion was below the detection limit (<0.00200 μg/g ww) for the remaining sites.

We extrapolated from the malathion concentrations measured on the cards for comparison to the label application rate of 21 mg/m^2. Concentrations were less than the label application rate indicating drift rather than direct spray at these sites.

Surrogate Toads

Righting Trials - The mean time for pre- and post-spray righting trials were all <10 s. There was no significant difference (Fisher's exact test; $p > 0.05$) between the post-spray ratios of those toads that could right in 10 seconds or less at the reference site and those toads at each site where nearby spraying occurred. This indicates that if the toads were exposed to malathion aerial drift, the concentration was not great enough to affect the toads' behavior when compared to the reference site. Meeboer Lake and 7 Mile Road were significantly different from the reference site (Fisher's exact test; $p = 0.095$ and $p = 0.069$, respectively) at $p < 0.10$.

Cholinesterase Inhibition - Mean brain AChE and BChE activities in surrogates were higher from spray sites (except at Gelatt Lake) than AChE and BChE activities in surrogates from the reference site (Table 1), although the differences among sites were not significant (AChE Anova p-value = 0.069; BChE Anova p-value = 0.363). None of the brain samples exhibited an increase in either AChE or BChE activity after the addition of 2-PAM.

Table 1 - *Mean (± 1 SE) Brain AChE and BChE Activities in Woodhouse's Toads Collected Following Malathion Spraying.*

Mean Brain Acetylcholinesterase µmol/min/g			Mean Brain Butyrylcholinesterase µmol/min/g		
Site	Reference Site	d / c % [1]	Site	Reference Site	d / c %
Gibbs Pond: 5.710 ± 0.583 n=8, (-14.3) [2]		114.3	Gibbs Pond: 0.462 ± 0.124 n=8, (-39.6)		139.6
Gelatt Lake: 4.957 ± 0.577 n=7, (0.78)	4.996 ± 0.329 (n=10)	99.22	Gelatt Lake: 0.330 ± 0.078 n=7, (0.31)	0.331 ± 0.053 (n=10)	99.69
7 Mile Road: 5.813 ± 0.294 n=7, (-16.4)		116.4	7 Mile Road: 0.389 ± 0.061 n=7, (-17.5)		117.5
Meeboer Lake: 6.200 ± 0.442 n=4, (-24.1)		124.1	Meeboer Lake: 0.510 ± 0.107 n=4, (-54.1)		154.1
White's Place: 6.567 ± 0.327 n=7, (-31.4)		131.4	White's Place: 0.557 ± 0.103 n=7, (-68.3)		168.3

[1] D / c % is ChE activity as % of control.

[2] Numbers in parentheses indicate % inhibition of dosed / controls (d / c).

Similar to the brain ChE results, mean plasma AChE and BChE activities in surrogates from spray sites were higher than from toads at the reference site (Table 2), but differences were not significant (AChE Ancova p-value = 0.311; BChE Ancova p-value = 0.308). None of the plasma samples exhibited an increase in either AChE or BChE activity after the addition of 2-PAM.

Table 2 - *Mean (± 1 SE) Plasma AChE and BChE in Woodhouse's Toads Collected Following Malathion Spraying.*

Mean Plasma Acetylcholinesterase µmol/min/ml			Mean Plasma Butyrylcholinesterase µmol/min/ml		
Site	Reference Site	d / c % [1]	Site	Reference Site	d / c %
Gibbs Pond: 0.067 ± 0.010 n=5, (-11.7)		111.7	Gibbs Pond: 0.232 ± 0.050 n=5, (-25.4)		125.4
Gelatt Lake: 0.070 ± 0.005 n=7, (-16.7)	0.060 ± 0.005 (n=10)	116.7	Gelatt Lake: 0.248 ± 0.090 n=7, (-34.1)	0.185 ± 0.029 (n=10)	134.1
7 Mile Road: 0.074 ± 0.006 n=8, (-23.3)		123.3	7 Mile Road: 0.192 ± 0.041 n=8, (-3.8)		103.8
Meeboer Lake: 0.084 ± 0.006 n=5, (-40.0)		140.0	Meeboer Lake: 0.192 ± 0.075 n=5, (-3.8)		103.8
White's Place: 0.069 ± 0.006 n=7, (-15.0)		115.0	White's Place: 0.199 ± 0.042 n=7, (-7.6)		107.6

[1] D / c % is ChE activity as % of control.

[2] Numbers in parentheses indicate % inhibition of dosed/controls (d / c).

Invertebrates

Terrestrial - Pitfall traps were not successful for collecting terrestrial invertebrate samples. The limited quantity of invertebrates collected was insufficient for malathion residue analysis or to make statistical comparisons before and after spraying occurred. No dead or dying terrestrial invertebrates were observed in the sampling areas after spraying occurred.

Aquatic - There were no differences in numbers of aquatic invertebrates collected before or after the spraying occurred except at Creighton Lake of Hutton Lake NWR ($p = 0.002$) and Meeboer Lake ($p = 0.017$). More aquatic invertebrates were collected prior to spraying than after spraying at the reference site but the opposite was true for the number of invertebrates collected at Meeboer Lake. Malathion concentrations in all invertebrates samples from all sites were below the detection limit (<0.05 μg/g).

Discussion

There was no evidence of cholinesterase inhibition in Woodhouse's toad at any of the study sites. However, the finding of lower cholinesterase activity at the reference site compared to the spray sites led to an investigation to determine if another factor such as brain weight or body weight was influencing cholinesterase activity. There was no significant interaction between the weight of the toads and the sites for either brain AChE ($p = 0.070$) or brain BChE ($p = 0.701$) activities based on a general linear model. Brain AChE, adjusted for weight using an ANCOVA, was not significantly different ($p = 0.111$) among sites. The coefficient for the covariate was also not significant ($F = 0.160$, $p = 0.692$), indicating that weight as a covariate is not correlated with AChE activities and was not helpful in explaining why AChE values at the reference site were lower than sites adjacent to spraying. Similarly, brain BChE, adjusted for weight using an ANCOVA, was not significantly different ($p = 0.407$) among sites and the coefficient for weight was also not significantly different ($F = 0.005$, $p = 0.945$).

Furthermore, there was no significant interaction between the brain weight of the toads and the sites for either brain AChE ($p = 0.775$) or brain BChE ($p = 0.746$) activities. Brain AChE, adjusted for brain weight using an ANCOVA, was not significantly different ($p = 0.076$) among sites. The ANCOVA, was not significantly different ($p = 0.407$) among sites and the coefficient for weight was also not significantly different ($F = 0.005$, $p = 0.945$). coefficient for the covariate was also not significant ($F = 0.238$, $p = 0.629$) indicating that using brain weight as a covariate was not helpful in explaining why AChE values at the reference site were lower than sites adjacent to spraying. Similarly, brain BChE, adjusted for brain weight using an ANCOVA, was not significantly different ($p = 0.374$) among sites and the coefficient for brain weight was also not significantly different ($F = 0.039$, $p = 0.845$).

We also examined the possibility that the weight of the toads may explain the differences in mean plasma AChE and BChE activities. There was no significant interaction between the toad body weight and the sites for either plasma AChE ($p = 0.595$) or plasma BChE ($p = 0.139$) activities. The coefficient for the covariate weight was significant ($F = 4.963$, $p = 0.032$) for plasma AChE indicating that weight was correlated with plasma AChE activities; but, mean plasma AChE adjusted for weight using an ANCOVA was not significantly different ($p = 0.311$) among sites. Similarly, the coefficient for weight was significant ($F = 27.511$, $p \leq 0.0005$) for plasma BChE; but, mean plasma BChE adjusted for weight using an ANCOVA was not significantly different ($p = 0.308$) among sites.

Physiological factors did not clarify the generally lower ChE activities at the reference site. Therefore, it is possible that airborne particles of malathion may have entered Hutton Lake NWR from mosquito spraying activities occurring near Sand Creek located approximately 1.6 kilometers southwest of the refuge. The wind direction in the Laramie Region is primarily from the southwest and southeast (National Weather Service Forecast Office, Cheyenne, Wyoming). One study conducted for the Boll Weevil Eradication Program at Penn State (1993), showed deposition of aerially applied ULV malathion up to 21.0, 11.5, 2.9, and 0.7% at 100, 200, 500, 1000 m downwind. Nevertheless, spray cards and aquatic invertebrates from Hutton Lake NWR had no detectable malathion residue.

Of the three sites where malathion was detected on the spray cards, only the mean brain ChE activities in toads from Gelatt Lake were lower than at the reference site, but differences were not significant. The behavior of the toads was not affected and malathion residues were not detected in aquatic invertebrates or water from Gelatt Lake. Although there is very limited information on ChE activities in adult amphibians, it is possible that the differences in ChE activities are due to natural variation rather than from malathion exposure. Typically, a 50% depression in ChE activities in birds from the reference mean is the threshold for indicating pesticide poisoning (Hill and Fleming 1982) though some laboratory studies suggest an inhibition of 20% below the control mean in a species is indicative of exposure (Grue et al. 1991). Analysis of malathion residues in body tissues of the Woodhouse's toads may have been helpful for indicating very recent exposure to malathion (Sparling 2001). It has been suggested that amphibians are resistant to some degree to organophosphorus insecticides and can bioaccumulate relatively high concentrations (Powell et al. 1982).

Moreover, we did not look at malathion spray drift concentrations that might affect Wyoming toad tadpoles because no natural reproduction at Mortenson NWR had occurred at the time of the study (personal communication with Mitch Bock, Wyoming Game & Fish, Laramie, WY, January 1997). Since the time of the study, natural reproduction has occurred. Further investigation into the possibility of Wyoming toad tadpole exposure to malathion is reasonable because very low concentrations of malathion in water are reported to adversely affect amphibian tadpoles (Sanders 1970; Mohanty-Hejmadi and Dutta 1981; Rosenbaum et al. 1988).

We were unable to evaluate the potential exposure of terrestrial invertebrates as dietary items of the Wyoming toad to malathion because of considerable uncertainties associated with the data. Setting up additional pitfall traps for more than two consecutive

nights may have allowed us to obtain the necessary information, while reducing variable factors such as temperature, meterological conditions, or episodic insect hatching events that influence invertebrate activity or numbers. Differences in aquatic invertebrate relative abundance before or after spraying were not significant among sites except for Meeboer Lake and the reference site. At Meeboer Lake, more aquatic invertebrates were collected after spraying than before, even though malathion residues were detected on spray cards. Conversely, fewer aquatic invertebrates were collected after spraying than before at the reference site. No malathion residue was detected in the tissue samples or spray cards indicating that the decrease in numbers was likely due to other factors.

Conclusions

The purpose of this study was to determine if the survival of the Wyoming toad is detrimentally affected by aerial drift of malathion through mortality, removal of food source, and reduced predatory avoidance. The data demonstrated that drift from mosquito control activities does not directly enter Mortenson NWR where the only population of the Wyoming toad exists. Drift does enter potential reintroduction sites but the malathion concentrations detected at these sites were much lower than the application concentration.

Our data, using surrogate Woodhouse's toads, indicate that any exposure to the pesticide was not great enough to affect predator avoidance behavior, ChE activities, or survival of adult Wyoming toads; but the data does provide some much needed information on ChE activities in amphibians. Tissue analysis for malathion residues may have helped to determine if the small differences in ChE activities observed were the result of malathion exposure or from natural variation. Exposure of the Wyoming toad to malathion drift concentrations greater than those we observed and repeated applications from drift or to direct spray, may be detrimental for the survival of the species. More research on the potential effects of spray drift on tadpole survivorship is needed.

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Synergistic Effects of a Combined Exposure to Herbicides and an Insecticide in *Hyla versicolor*

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Abstract: Combinations of the herbicides atrazine and metolachlor and the insecticide chlorpyrifos were tested under both laboratory and field conditions to determine their individual and combined effects on amphibian populations. In the lab *Hyla versicolor* tadpoles experienced 100% mortality when exposed to a high combination of the pesticides (2.0 mg/L atrazine, 2.54 mg/L metolachlor, 1.0 mg/L chlorpyrifos) whereas low concentrations of the pesticides (0.2 mg/L atrazine, 0.25 mg/L metolachlor, 0.1 mg/L chlorpyrifos) or high concentrations of either herbicides or insecticide alone caused lethargy, reduced growth and delayed metamorphosis but no significant mortality. In the field high herbicide, low insecticide and low herbicide, low insecticide mixtures significantly reduced amphibian populations compared to controls but in the low herbicide, low insecticide wetlands amphibian populations were able to recover through recruitment by the end of the season.

Keywords: chlorpyrifos, atrazine, metolachlor, *Hyla versicolor,* amphibians, pesticide interaction

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According to some estimates, approximately 50% of the wetlands within the United States have been drained within the past 200 years (Dahl 1990). In recognition that wetlands serve many important human-related functions, including water storage, improving water quality, and flood reduction, and that they are critical habitats for many species of wildlife, conservation agencies such as the Natural Resources Conservation Service, U.S. Army Corps of Engineers and U.S. Fish and Wildlife Service implemented wetland restoration programs several years ago. Through the use of set aside programs and other financial incentives, these agencies have encouraged private land owners to restore or reconstruct wetlands on lands that had been previously drained and cropped. While the primary purpose of many of these constructed wetlands is to improve water quality, man-made wetlands are open for colonization by plants and animals from nearby wetland habitats, and over time, may take on a similar biotic appearance as natural wetlands (Knight 1992, Hammer 1992, Galatowitsch and van der Valk 1994). For example, frogs use such wetlands for breeding and tadpoles may be found in constructed wetlands during the breeding seasons (Mazanti 1999). However, many of these restored wetlands are surrounded by agriculture and intercept surface water runoff from cropfields, thereby exposing tadpoles to multiple pesticides (Fairchild et al. 1994). Thus a legitimate question can be whether construction of wetlands in intensely agricultural areas is advantageous by providing habitat that would not otherwise exist or disadvantageous in that they attract wildlife to unsuitable areas.

There has been a fair amount of research conducted on the effects of agricultural contaminants on amphibians and other aquatic organisms but most of this research has focused on single contaminants, whether they are pesticides (reviewed by Cowman and Mazanti 2000), fertilizers (Hecnar 1996, Rouse et al. 1999, Schuytema and Nebeker 1999) or other factors. Whereas toxic effects have been found at environmentally realistic concentrations for both groups of contaminants -- as little as 0.1 ppb for atrazine (Hayes et al. 2002) and 0.01 ppm for chlorpyrifos (Marshall and Roberts 1978), little is known about possible effects when animals are exposed to multiple pesticides simultaneously as often happens under actual farming operations. A common scenario in the Mid Atlantic States, for example, is for farmers to apply herbicides such as atrazine and metolachlor in Spring before crops emerge to ward off weeds and then, after crops germinate, to apply insecticides such as chlorpyrifos to control insect pests. Because atrazine and metolachlor have half-lives of several weeks to months, runoff coming after the application of chlorpyrifos can carry both types of pesticides.

Abundant research has been conducted on the toxic effects of atrazine, metolachlor and chlorpyrifos on aquatic organisms under natural or semi-natural conditions. Eisler (2000) showed that atrazine is very common in agriculture runoff. Concentrations may reach 1.07 mg/L in runoff water (Forney 1980) and 69 µg/L in surface waters (DuPreez and van Vuren 1992). Chlorpyrifos is highly toxic but has a half life of about a week in water (Marshall and Roberts 1978, Eisler 2000) and few or no reliable estimates are available on anticipated environmental concentrations. In the Mid Atlantic region, Mazanti et al. (2002) calculated expected ranges of concentrations for atrazine and metolachlor were 0.2 ppm to 2.0 ppm, and 0.1 ppm to 1.0 ppm for chlorpyrifos.

The individual toxicities of these compounds have been studied (e.g. Marshall and Roberts 1978; Brockaway et al. 1984; Van Wijngaarden et al. 1996; Giddings et al.

1997), occasionally on frogs (e.g. Fairchild et al. 1994; Detenbeck et al. 1996; Calumpang et al. 1997; Britson and Threlkeld 1998; Allran and Karasov 2001). Detenbeck et al. (1996) found that atrazine concentrations between 15 ppb and 75 ppb had no observable adverse effects on *Rana pipiens* tadpoles in outdoor mesocosms. Howe et al. (1998) found larvae of *R. pipiens* and *Bufo americanus*, to be more sensitive to atrazine and alachlor than were trout, *Onchoryhnchus mykiss* or catfish, *Ictalurus punctatus*. They also found that older amphibian larvae were more sensitive than younger; the 96 hr median lethal concentration (LC_{50}) for Gosner (1960) stage 40 *R. pipiens* was 14.5 mg/L compared to 47.6 mg/L for stage 29 *R. pipiens*. It has been widely concluded that atrazine poses little widespread risk to aquatic ecosystems, including ponds, streams and wetlands (Klaassen and Kadoum 1979; Solomon et al. 1996). Allran and Karasov (2001) found concentration-dependent, positive relations between atrazine and deformities but concluded that lethal effects of the herbicide on *R. pipiens, R. sylvatica* and *B. americanus* occurred at concentrations higher than those expected under field conditions. Hayes et al. (2002) demonstrated significant sublethal effects of atrazine in *Xenopus laevis* at remarkably low concentrations.

Barron and Woodburn (1995), in a comprehensive review of the chlorpyrifos literature, found few quantitative studies documenting effects of chronic chlorpyrifos exposures on frogs or tadpoles, and reported that the LC_{50} values for *B. americanus* tadpoles and for *R. pipiens* tadpoles exposed to chlorpyrifos were 1 ppb and 3000 ppb, respectively. Under laboratory conditions, Britson and Threlkeld (1998) found that mixtures of atrazine, chlorpyrifos, and methyl mercury resulted in delayed metamorphosis, abnormal development and smaller body size at metamorphosis in *Hyla chrysoscelis* compared to controls. Inferring the significance of these findings to larval frogs under natural conditions is difficult due to confounding variables in the field and lack of realism in controlled experiments.

The objective of this study is to examine the interactive effects of atrazine, metolachlor and chlorpyrifos on the growth and survival of larval eastern gray treefrogs (*Hyla versicolor*). Two experiments were conducted to examine these effects. A laboratory study examined general toxicity, behavior and growth of wild-caught *Hyla versicolor* tadpoles exposed to different concentrations of the three compounds. In addition, the pesticides were applied to a set of outdoor naturalized macrocosms in a realistically simulated fashion to determine if similar effects could be observed under field conditions. To maintain environmental realism, test concentrations are based on runoff data generated in multiple agronomic experiments with atrazine, metolachlor and chlorpyrifos (White et al. 1967; Hall et al. 1972; Ritter et al. 1974; Wauchope 1978; Buttle 1990; Mickelson et al. 1998). Formulations of the pesticides (atrazine and metolachlor as trade name Bicep II and chlorpyrifos as trade name Lorsban 4E) were applied rather than reagent grade active ingredients. The calculation of test concentrations is more fully discussed in Mazanti et al. (2002). The overall null hypothesis was that simulated runoff of chlorpyrifos, atrazine and metolachlor, applied separately as a herbicide combination and a pesticide and in combination with one another will have no adverse impacts on the growth and survival of tadpoles. Together, the laboratory and field experiments increase the scope of inference of this study beyond that of previous bioassays or field investigations on the toxicity of these pesticides to

frogs.

Experimental Method

Laboratory Experiment

Early stage (< Gosner Stage 25) (Gosner 1960) eastern gray treefrogs tadpoles were collected by seine from a constructed, experimental-wetland complex at the Patuxent Wildlife Research Center, MD, USA. The tadpoles were acclimated to laboratory conditions for three days prior to the beginning of the experiment. They were fed daily with crumbled algae wafers. Tadpoles were selected for the laboratory experiments based on absence of gross abnormalities, lesions, or tail bites and on similar body sizes. They were separated into groups of 10 and an initial wet weight was obtained for each group after blotting up excess water. Each group was transferred into 75 L all glass aquaria filled with pond water and free of all aquatic predators. Water pH was between 6.5-6.8 and oxygen was above 8 ppm due to aeration.

Atrazine and metolachlor (formulation grade Bicep II (DowElantro)) and chlorpyrifos (formulation grade Lorsban 4E (Novartis)), formulated as aqueous phase suspensions/solutions were added to the aquaria at 7 different combinations of atrazine, metolachlor and chlorpyrifos: low herbicide (LH, 0.2 mg/L atrazine and 0.25 mg/L metolachlor); high herbicide (HH, 2.0 mg/L and 2.54 mg/L metolachlor); low insecticide (LI, 0.1 mg/L chlorpyrifos); high insecticide (HI, 1.0 mg/L chlorpyrifos); low herbicide + low insecticide combination (LHLI, 0.2 mg/L atrazine; 0.25 mg/L metolachlor; and 0.1 mg/L chlorpyrifos); high herbicide + high insecticide combination (HHHI, 2.0 mg/L atrazine; 2.5 mg/L metolachlor; and 1.0 mg/L chlorpyrifos).

Treatments were randomly assigned to aquaria, with seven treatments applied in replicates of three for a total of 21 experimental units. The pesticide treatments were applied once to each aquarium after the acclimation period. The treatments were static with no renewal of water and no movement of tadpoles to untreated aquaria. Tadpoles remained in their designated aquarium until metamorphosis, escape, death or release at the termination of study.

Four parameters were used as measures of the effects that chlorpyrifos, atrazine and metolachlor may have on tadpoles: a) weight change during larval period; b) weight of frogs at metamorphosis (Gosner stage 46); c) physical and behavioral abnormalities during larval period; and d) number of days to completion of metamorphosis. The tadpoles were examined daily for death, lassitude and physical abnormalities. Tadpoles also were prodded with a consistent pressure via glass stirring rod to determine behavioral responses. Response to the prod was recorded as movement or non-movement and by examining any resulting swimming motion. Movement in a generally straight line was considered 'normal' whereas tight circling movement was considered 'abnormal.' Five-minute group observations were made at different times of the day during the duration of the study during which the number of animals observed feeding were recorded. The wet weight of each group of tadpoles was measured on Day 0, Day 14, Day 21 and on Day 28

to determine the effects of the treatment regimes on tadpole weight during metamorphosis. At the completion of metamorphosis, juveniles were weighed individually. The number of days from the beginning of the experiment to completion of metamorphosis for each tadpole in each treatment was recorded.

Wetland Experiment

Twelve experimental wetlands ("macrocosms") located at the Patuxent Wildlife Research Center were used for the study. The macrocosms were constructed in 1989 as 80 cm deep pits with a mean surface area of 212 m^2 and lined with polyvinyl plastic sheeting. The sheeting was covered with 18 cm to 20 cm of sandy loam soil. The macrocosms were filled with water initially in May 1990. At the time of the experiment, the macrocosms supported about 40 volunteer species of hydrophytic plants with a collective cover of 25-80% of the water surface by late summer. The macrocosms were hydrologically isolated from surface water and groundwater, and were treated and drained independently of one another. Each macrocosm was enclosed by a double layer of 3 m high chain link fence which kept out many ground predators but allowed free access by frogs. Adjacent to these macrocosms were other larger and much older constructed wetlands characterized by open water interspersed with *Nuphar luteum* and *Nymphaea odorata*; a mixed deciduous hardwood forest also bordered the macrocosm complex.

Adult frogs and toads had unconstrained access to the macrocosm area from all directions, and six species were heard calling in adjacent wetlands. Previous studies (Sparling et al. 1995) and personal observations confirmed that adult frogs and toads jumped easily through the fencing of all the macrocosms. Larvae of at least seven species of frogs: *Rana catesbeiana, R. sphenocephala, R. clamitans, Bufo americanus, Pseudacris crepitans,* and *Hyla versicolor* had been captured by dip net and seine in the spring and summer for several years prior to the current study. Because of the limited number of wetlands for this experiment and a desire to avoid total mortality of amphibians in the macrocosms, the study design was randomized with respect to three treatments: Control (no pesticide); low herbicide + low insecticide combination (LHLI, 0.2 mg/L atrazine; 0.25 mg/L metolachor; 0.1 mg/L chlorpyrifos); and high herbicide + low insecticide (HHLI, 2.0 mg/L atrazine; 2.54 mg/L metolachlor; and 0.1 mg/L chlorpyrifos); four wetlands were used for each treatment. The herbicide was applied four weeks prior to the insecticide application to mimic the application sequence under typical field conditions. The pesticides were evenly applied with a hand-held spray boom that directed the spray outward in a 90° arc. The mix was applied from the shoreline by walking at a normal pace in one complete circle around the pond with the spray directed from shore out to the middle of the pond.

Each macrocosm was sampled for tadpoles once each month from the beginning of June through September 1998. Each wetland was seined five times during each sample period and the numbers of individuals per species were counted in each seine haul. The tadpoles were kept submerged in the water during the counts to minimize desiccation or asphyxiation while observations were made. Tadpoles were returned to the macrocosm gently to minimize any further stress or injury.

Tadpole counts and weight data were tested for the assumptions of ANOVA and

transformed as necessary — log transformation was used on data that were not normally distributed, square root on count data, and arcsine of the square root on frequency data. Statistical analyses included repeated measures and conventional ANOVA followed by Tukey's HSD and LSD *a posteriori* tests (SAS 1990).

Water was extensively collected and analyzed in both the aquaria and the outdoor experiments to determine degradation rates (Mazanti et al. 2002). Measured pesticide concentrations in the lab were: 1.7 ppm atrazine, 1.8 ppm metolachlor and 0.56 ppm chlorpyrifos at the high concentrations; and 0.19 ppm, 0.24 ppm and 0.053 ppm, respectively at the low concentrations. In the field herbicide concentrations were: 3.6 ppm and 4.4 ppm for the high atrazine and metolachlor treatments; 0.33 ppm and 0.35 ppm respectively for the low; and 0.2 ppm for chlorpyrifos . Whereas chlorpyrifos degraded rapidly under both test conditions, the herbicides remained constant throughout the lab study but degraded within a few days under field conditions.

Results

Laboratory Experiment

Differences occurred in feeding and fright responses of gray treefrog tadpoles across treatments (Figs. 1 and 2). For the duration of the experiment, all tadpoles in Control, LH, and HH treatments were observed grazing actively and swam rapidly away when prodded with a stirring rod. There was a dose related response in the other treatments. Those in the LI treatment were not seen feeding on the first day of the study but regained normal grazing behavior by Day 3. Those in the HI treatment reduced grazing through the eighth day of the study but fed normally on Day 10. Similarly, tadpoles in the LHHI treatment stopped feeding until Day 4 and by Day 6 had regained normal feeding activity. The difference in proportion of tadpoles feeding was significantly different among treatments ($p < 0.0001$) but there was no statistical difference found among days or in the treatment by day interaction. Eighty percent of the tadpoles in behavioral observations in treatments LI and HI exhibited normal resting postures and feeding behavior as controls, but six tadpoles in each treatment were lethargic for at least the first five days of experimentation. The proportion of tadpoles demonstrating normal flight response also differed among treatments ($p = 0.029$) but not among days or in the interaction between day and treatment. All tadpoles tested in the Control, LH and HH treatments moved quickly away from the stimulus throughout the study. Tadpoles in the LI were initially lethargic but recovered by Day 2. Those in the HI group were moribund on Day 1 but showed some recovery by Day 2 although full response was not recovered until Day 4. In the LHLI treatment tadpoles were lethargic through Day 4 but had recovered by Day 6. Within the first 8 days of the experiment, 26 tadpoles died in treatment HHHI; the survivors did not graze or swim off the tank bottoms, and all died by Day 11. No other mortality occurred.

There was significant difference among treatments in body weight ($p < 0.0001$) and in the interaction between treatment and time ($p < 0.0001$) (Fig. 3). Interpretations of changes in weight were complicated by the differences in time to metamorphosis and the

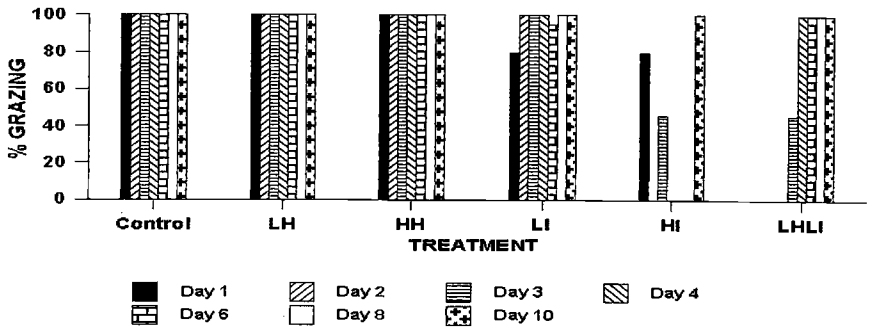

Figure 1 — Percentage of Gray Treefrog Tadpoles Displaying Normal Grazing Behavior by Treatment and Day of Test.

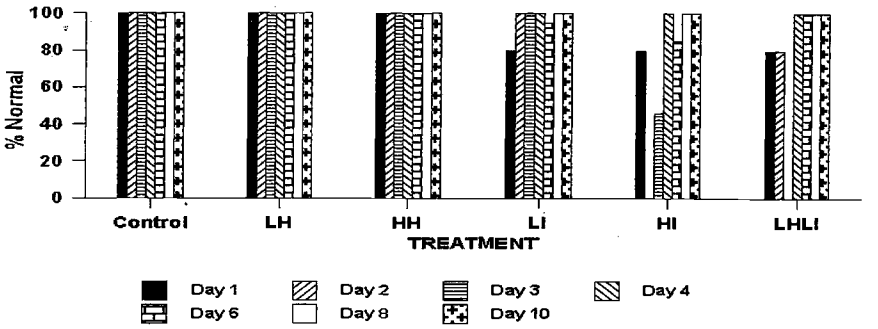

Figure 2 — Percentage of Gray Treefrog Tadpoles Showing Normal Fright Response by Treatment and Day of Test.

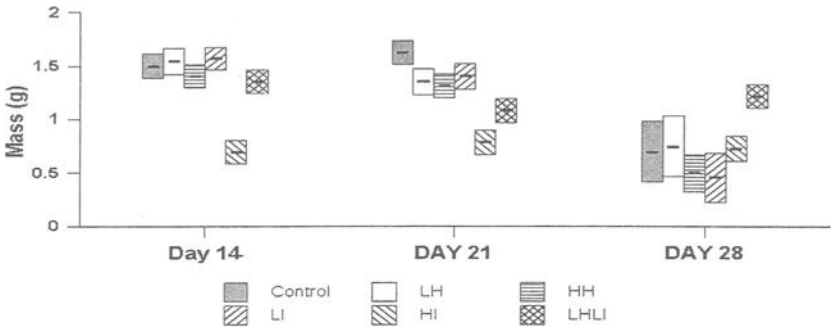

Figure 3 — Mean (± SD) Weight of Gray Treefrog Tadpoles by Treatment and Day of Exposure to Atrazine, Metolachlor and Chlorpyrifos.

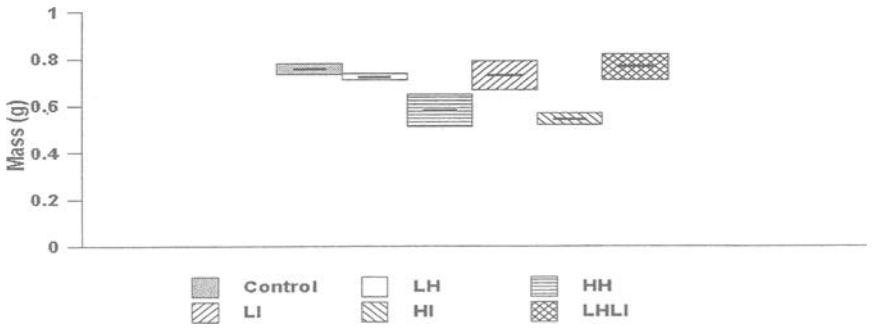

Figure 4 — Mean (± SD) Weight of Gray Treefrog Juveniles at Completion of Metamorphosis by Treatment.

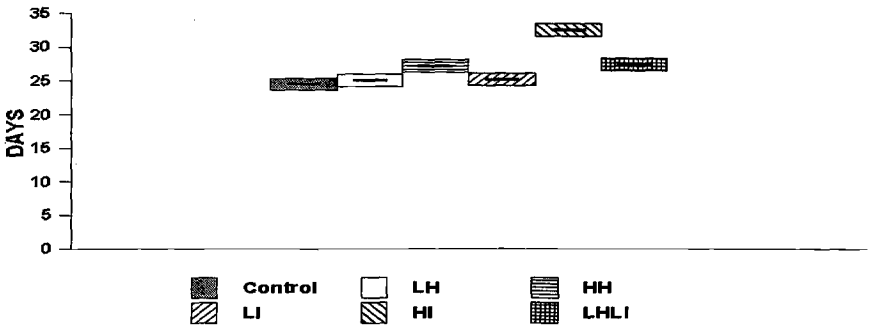

Figure 5 — Mean (± SD) Number of Days From Initiation of Experiment to Metamorphosis in Gray Treefrog Tadpoles Exposed to Atrazine, Metolachlor and Chlorpyrifos

weight loss that normally occurs during the climax stage of metamorphosis. Specifically, at Day 14 tadpoles in the HI treatment were lighter than those in all other treatments. At Day 21 tadpoles in the HI treatment were still lighter than those in all other treatments except LHLI which were lighter than controls. By Day 28 tadpoles in the LHLI treatment were heavier than those in other treatments because tadpoles in the other treatments had begun or completed the climax phase of metamorphosis.

Wetland Experiment

The mean weight of newly metamorphosed frogs differed among treatments ($p = 0.002$). In *a posteriori* comparisons mean juvenile weight was less in the HH and HI treatments than in the other groups (Fig. 4). There also was a significant difference in the mean number of days necessary to complete metamorphosis ($p < 0.0001$) (Fig. 5). Tadpoles in the Control and LI groups metamorphosed earlier than those in HH, HI and LHLI and HI took longer than any other group ($p < 0.05$).

The numbers and species of tadpoles caught varied considerably among sample periods, treatments and even among ponds within a treatment (Table 1). Tadpoles of three species, *Hyla versicolor, Rana clamitans, and R. catesbeiana*, were common at the time of application and were used in the repeated measures ANOVA. During the first period counting of seined tadpoles stopped after 100 individuals were counted in each species to reduce the stress placed on animals. All of the Control wetlands easily

exceeded that number.

Table 1 — *Mean (SD) Numbers of Tadpoles Seined From Experimental Wetlands by Sample Period.*

Period[a]	Treatment	*Rana clamitans*	*R. catesbeiana*	*Hyla versicolor*	*Acris crepitans.*
1	Control	> 100 [b]	> 100 [b]	> 100 [b]	0
	LHLI	22.2 (14.9)	0	15.0 (19.9)	0
	HHLI	4.00 (4.50)	0	0	0
2	Control	75.2 (22.2)	10.7 (20.8)	1.00 (1.15)	8.75 (6.70)
	LHLI	27.5 (29.3)	6.25 (11.8)	92.7 (107)	0.25 (0.50)
	HHLI	0.75 (0.96)	0	26.0 (40.3)	0
3	Control	94.5 (31.4)	0	0	9.00 (13.7)
	LHLI	185 (209)	0	94.2 (188)	0.75 (1.50)
	HHLI	105 (192)	0	0	0.25 (0.50)

[a] Period 1 = 2,1 days post herbicide spray, 3 days post insecticide spray (21/3); 2 = 54/36 days, 3 = 85/67 days post spray.
[b] Counts were stopped after 100 animals per species, thus no SD is presented.

In an ANOVA with captures within a pond treated as a repeated measures analysis, there was a significant difference ($p = 0.0053$) among treatments but no period or period x treatment difference for *Acris crepitans*. Control ponds had significantly more tadpoles than either of the other treatments. Counts of *Hyla versicolor* showed a significant interaction ($p = 0.024$) but no main effect. The interaction term was most closely related to the drop in numbers in the control group and a corresponding increase in the LHLI group after the first period. Bullfrogs had significant difference among periods, treatments and treatment x period interaction (all $p < 0.0001$). Control ponds had more tadpoles than the other treatments, Period 1 had a greater number than Periods 2 or 3, and the number of tadpoles declined with time in the Control group but remained uniformly low in the other groups. The mean numbers of tadpoles differed among treatments ($p = 0.018$) and the period by treatment interaction ($p = 0.0027$) but not among sample periods ($p = 0.339$). *R. clamitans* numbers differed among treatments ($p = 0.012$) but not periods or the interaction. In this species, Control had greater numbers than HHLI but LHLI did

not differ from either of the other groups. During the first period in the Control wetlands about half of the captured *R. catesbeiana* and *R. clamitans* were young of the year and half from the previous season but nearly 100% of the captures in Periods 2 and 3 were young of the year.

During the first period numerous dead fish, crayfish and tadpoles were observed in the littoral fringe, floating on the water surface, or on the bottom of the treatment wetlands in the HHLI and LHLI treatments but not the Controls. Crayfish and aquatic invertebrate populations remained visibly low or non-existent in the treated wetlands throughout the rest of the study. By the second period vegetation displayed signs of dying. Cattail stems (*Typha spp.)* turned brown and died by the third period and percent cover by submerged aquatics such as *Potamogeton spp.* was visibly much lower in the HHLI than in the LHLI or Control groups but this was not quantified.

Discussion

Laboratory Experiment

The herbicide alone treatments had some toxic effects on *Hyla versicolor* tadpoles and the response was essentially dose dependent. There were no apparent differences in response to stimulus, or frequency of grazing at the low or high herbicide concentration and no effect on rate of growth, weight at metamorphosis, or time to metamorphosis was observed in the low herbicide treatment compared to controls. However, the higher concentration of herbicide mixture did slow the rate of growth, reduced weight at metamorphosis and delayed metamorphosis compared to controls. Inhibition of growth with smaller body size at metamorphosis can be harmful to tadpoles if it makes them more susceptible to a larger range of predators (Relyea and Mills 2001). Smaller body size of frogs at metamorphosis affects survival by making juvenile frogs more susceptible to predation in a wetland's littoral fringe (Werner 1991; Britson and Threlkeld 1998). Small larval body size also may affect the survival of tadpoles in other ways. Werner and Anholt (1996) observed that when the growth of larval green frogs (*Rana clamitans*) was reduced below that of their main competitor, bullfrog (*R. catesbeiana*) tadpoles, green frogs suffered increased starvation and ultimately decreased survivorship. Delayed metamorphosis could be a disadvantage if ponds are drying in mid to late summer by catching animals before they are ready to leave a pond, or by exposing them to adverse water quality, increased concentrations of contaminants and greater predation pressure (Pechmann et al. 1989).

Similarly, chlorpyrifos alone had significant effects in several measures. Exposure to low (0.1 ppm) concentrations of chlorpyrifos inhibited grazing activity during the first day of exposure but animals quickly rebounded by Day 2. Likewise, tadpoles were lethargic on the first day of exposure but recovered quickly. The low chlorpyrifos treatment did not appear to inhibit growth, weight at metamorphosis, or time to metamorphosis compared to controls. Greater and more persistent effects on grazing and response to stimulus were observed with the higher (1.0 ppm) concentration of chlorpyrifos than at 0.1 ppm or controls. Moreover, 1.0 ppm chlorpyrifos reduced the rate of growth, weight at metamorphosis, and delayed metamorphosis compared to controls. Whereas the effects

on grazing and response appeared transitory in the low concentration of chlorpyrifos, they were more long lasting at 1.0 ppm. Tadpoles that are slow to respond to physical threats or unable to swim swiftly are much more prone to predators, such as fish and adult frogs, than normal tadpoles (Werner 1991). Weaker tadpoles are also more prone to competitive exclusion from prime feeding or loafing habitat by larger tadpoles of the same or other species (Griffiths 1991), which only exacerbates their potential for reduced survival.

The recovery in both treatments, however, was probably related to the short half-life of chlorpyrifos under the laboratory conditions. Mazanti et al. (2002) reported that the half rate of this pesticide in this study was less than one day. In contrast, no degradation was observed for the atrazine or metolachlor during the entire study. We did not observe any mortality through metamorphosis in either of the single pesticide treatments. Thus we do not know how the sensitivity of *Hyla versicolor* might compare to that of other amphibians reported by Barron and Woodburn (1995).

There appeared to be substantial interaction between the herbicide mixture and insecticide when used in combination. The LHLI treatment, for example, significantly reduced grazing activity and response to stimulus compared to low concentrations of either pesticide alone. For instance, neither grazing nor response were affected by the low concentration of herbicide and the low insecticide treatment only affected these measures during the first day. In the LHLI treatment both grazing and response were markedly reduced through the third day of exposure. Also, except for Day 28, body mass was lower for tadpoles in the LHLI treatment than in the LH or LI groups and the greater mass on this day was due to fewer individuals entering the climax stage of metamorphosis. Moreover, mass at metamorphosis was lower and time to metamorphosis was longer in the combined group than in the low insecticide or low herbicide groups. Synergism was most clearly observed, however, in the HHHI treatment group. Whereas no mortality was observed in HH or HI, all of the tadpoles died by Day 11 in the high combined treatment group and the few that survived over a week exhibited severe symptoms, including lethargy, inability to maintain balance, abnormal swimming and cessation of feeding. No further comparisons could be made because of this mortality.

Fairchild et al. (1994) hypothesized that ecological synergism may be a reason for some forms of interaction between contaminants if they act at different levels of community organization, thereby triggering increased effects of an herbicide and insecticide beyond those normally predicted to occur. For example, when a mixture of pentachlorophenol and simazine was applied to experimental ponds, primary production was adversely affected by the simazine, which killed entire stands of *Naja* spp and *Chara* spp (Robinson-Wilson et al. 1983). This apparently increased the bioavailability of the pentachlorophenol, and largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*) survival was adversely impacted. Fairchild et al. (1994) found that when atrazine applications were combined with esfenvalerate, a pyrethroid insecticide, ecological synergism did not occur, as the plant community compensated for the loss of sensitive macrophytes with increased production of non-impacted species. The functional redundancy, manifested by a shift in species composition from *Naja* to *Chara*, served to prevent adverse effects of esfenvalerate to the zooplankton or fish. However, because the tadpoles in the laboratory study were fed every day, it is unlikely that a similar food-chain explanation exists for the results we obtained. Instead, the study raised the possibility that

the high atrazine-metolachlor and chlorpyrifos combination caused a synergistic toxic response, and not food chain disruption.

Using *Chrionomus tentans* as a test species, Belden and Lydy (2000) showed a synergistic effect between atrazine and the insecticides chlorpyrifos, methyl parathion and diazinon. Of these, the interaction was strongest between atrazine and chlorpyrifos. Atrazine concentrations as low as 40 µg/L significantly increased the toxicity of chlorpyrifos. Using radio-labeled chlorpyrifos, they determined that the presence of atrazine increased chlorpyrifos uptake. They also showed that 2 mg/L atrazine significantly increased microsomal enzyme activity which could accelerate the conversion of chlorpyrifos into its more toxic oxon form. Larson et al. (1998) found that atrazine increased the concentrations of plasma thyroxine in *Ambystoma tigrinum*, and suggested that this could increase metabolic rate and chlorpyrifos conversion. Despite the higher thyroxine concentrations, salamander larvae exposed to atrazine took longer to metamorphose than control animals, which may have been due to a concomitant depression in corticosterone levels. Hayes et al. (2002) presented startling evidence that very low concentrations of atrazine can cause ovitestes in *Xenopus laevis*. Thus atrazine has many sublethal physiological effects, which could influence insecticide toxicity.

Because the concentration of the herbicides did not decrease significantly during the course of the experiment (Mazanti et al. 2002), the tadpoles were continually exposed to high concentrations of atrazine and metolachlor for the duration of their larval period. However, their exposure to chlorpyrifos was more acute. Short term effects consistent with organophosphorus insecticide toxicity were seen in the grazing and response to stimulus measures which lasted for 1 to 3 days in all treatments where an effect was observed except HHHI. In this treatment the pesticide combination led to 100% mortality. Longer term effects such as inhibition of growth rate, delayed metamorphosis and lower weight at metamorphosis were related to a persistent effect from either of the pesticide mixtures but appeared to be accentuated by the LHLI combination.

Wetland Experiment

The wetland experiment, which was meant to more closely reflect what might occur under actual agricultural operations, had some confounding factors. Some of the amphibians were breeding during the time of application, others were finished breeding, and still others such as the previous-year's green frogs were probably metamorphosing and leaving the ponds during the sampling periods. However, comparison of treatments during the first sampling period strongly suggested that there was a toxic effect in both the LHLI and HHLI treatments. Not only were numbers of larval amphibians significantly less than in the controls, but there appeared to be almost total mortality among the tadpoles and several other aquatic species. Also, incidental observations of dead invertebrates, sunfish and amphibians were confined to the HHLI treatment. Most young-of-the-year *R. clamitans* and *R. catesbeiana* were completely eliminated, and only a few second year tadpoles and larval gray treefrogs survived.

By the second sampling event, 54/36 days post-herbicide and insecticide sprays, the wetlands were becoming naturally recolonized by free-ranging frogs especially gray treefrogs, although the numbers recovered could have been due to the efforts of single egg

masses in each wetland for this highly fecund species. Other observations indicated that the species richness and individual abundance of the insect community remained depressed, with virtually no recolonization observed by the second sampling event. At this point, concentrations of all three pesticides had dropped to very low levels, particularly in the LHLI treatment (Mazanti et al. 2002).

The dissipation of the three pesticides in the outdoor wetland macrocosms declined more rapidly than in the laboratory investigation (Mazanti et al. 2002). This was particularly true with the herbicides because the half-life of chlorpyrifos was less than a day in either scenario. Thus exposure to atrazine, metolachlor and chlorpyrifos that occurred in the wetlands was similar to the high initial pulse exposures that occur in restored wetlands when spring rains cause runoff events coincident with the application of herbicides and insecticides. Previous studies (e.g. Larson et al. 1998; Britson and Threlkeld 1998; Allran and Karasov 2001) concluded that because the higher pesticide levels were transitory, realistic experimental exposures should mimic the lower, chronic pesticide levels measured in field studies, not the higher levels present in concentrated storm runoff. This choice generally produced experimental results showing few to no adverse effects on anuran tadpoles, leading to conclusions that runoff of single pesticides was not likely to have harmful effects on aquatic organisms residing in receiving waters. In contrast, this study suggests that even realistic exposures to combined herbicide and insecticides may cause significant mortality and other adverse effects to the larvae of anurans.

Management Considerations

Can created wetlands in agricultural landscapes serve dual environmental purposes of protecting water quality and providing much needed freshwater habitat for anurans and other wildlife? Is there any way to reduce the episodic high pulses of pesticide residues that enter created wetlands without interfering with the primary water quality mission for which they were designed? Potential solutions will most likely include the application of cropland management techniques that essentially pre-treat the runoff. For example, land management practices ascribed to fallow field areas buffering cropland influence the quantity of herbicides moving beyond them and into surface waters. Two papers investigated the effects of incorporation of herbicides and the location of vegetative buffer strips on the reduction of herbicide flowing from the cropland. Arora et al. (1996) evaluated the retention of metolachlor and atrazine, applied to adjacent corn fields at rates of 2.8 kg/ha and 2.12 kg/ha, respectively, in vegetative buffer strips approximately 1.5 m in width and 21.1 m in length under natural rainfall conditions. Over the course of the two-year study, 15 runoff events occurred. One that occurred within two days of herbicide application resulted in runoff concentrations of 730 and 580 ug/L metolachlor and atrazine, respectively. The buffer strip reduced these values on average between 70 and 190 ug/L. Duration of rainfall and pre-existing soil moisture were shown to greatly affect retention capacity of the buffers, giving highly variable retention levels over the course of the study. For metolachlor, retention rates ranged between 16 and 100%, and for atrazine rates ranged from 11 to 100%.

Mickelson et al. (1998), looked at the efficacy of non-cropped setback areas situated

adjacent to crop fields on the transport of metolachlor and atrazine from cornfields. The herbicides were applied at standard rates (2.8 kg/ha metolachlor and 2.1 kg/ha atrazine). On the whole, the runoff concentrations were not significantly different for setback versus non-setback conditions, and soil incorporation did to a certain degree reduce herbicide levels. For example, the first rainfall event of 1993 resulted in 52 ug/L metolachlor entering the setback plot, and then 87 ug/L metolachlor discharging from the setback plot; meanwhile, the inflow and outflow concentrations of metolachlor from the non-setback areas were 146 and 90 ug/L. Concentrations in subsequent rain events were much reduced, but still no large difference in the two setback treatments was observed. These studies clearly show that the variation in soil type, soil moisture and chemical characteristics seen at different sites makes it difficult to project what percent reductions in runoff could result for any given treatment system.

Nonetheless, restored or reconstructed wetlands can provide a freshwater habitat that is extremely limited in agricultural environments and they will be used by frogs and toads. Given the concern over declining amphibian populations, it would therefore be prudent to use available methods of cropland management, including vegetated buffers, banded pesticide applications and variable timing of pesticide applications, to reduce the higher edge-of-field concentrations of pesticides that may periodically discharge into nearby wetlands.

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Ammonium Perchlorate Disruption of Thyroid Function in Natural Amphibian Populations: Assessment and Potential Impact

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Abstract: We examined indices of thyroid development in tadpoles from ammonium perchlorate (AP)-exposed sites. Bullfrog (*Rana catesbeiana*) tadpoles collected from a reference site exhibited normal developmental features, with many completing metamorphoses. In contrast, tadpoles collected from the AP contaminated site exhibited a 5-fold lower hindlimb/snout-vent length ratio than tadpoles from the reference site. The volume of the thyroid gland was 2.5-fold larger in the tadpoles from the reference site, presumably because they had progressed to late prometamorphosis and early metamorphic climax. Premetamorphic western chorus frog tadpoles (*Pseudacris triseriata*) inhabiting an ephemeral pond contaminated with AP exhibited gross morphological abnormalities of the thyroid including colloid depletion and follicle cell hypertrophy. We conclude that tadpoles exposed to AP-contaminated pond water early in larval life exhibit delayed development of thyroid-hormone sensitive structures. Additionally, there are abnormalities in the developing thyroid gland that seem to depend upon the window of AP exposure. The potential impact of thyroid disruption on development and reproduction in amphibian populations will be discussed.

Keywords: perchlorate, thyroid, metamorphosis, amphibian

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Amphibian metamorphosis is the end result of a complex set of biochemical, morphological, and behavioral changes that prepare an aquatic larval form to survive in a semi-terrestrial environment. Initiation and coordination of these events is determined in part by the onset of thyroid hormone (TH) secretion (LeLoup and Buscaglia 1977, Suzuki and Suzuki 1981, Norman et al. 1987), the timing of thyroid hormone receptor gene expression (Yaoita and Brown 1990, Kawahara et al. 1991), and tissue-specific expression of genes encoding iodothyronine deiodinase enzymes that ensure adequate conversion of tetriodothyronine (T_4) to triiodothyronine (T_3) in target tissues (Becker et al. 1997). For example, the hindlimbs respond to TH relatively early in development whereas other TH-dependent events, such as programmed cell death in tail structures, do not occur until the final stages of metamorphosis. The increase in TH secretion during metamorphic climax is generally accompanied by corresponding changes in the size and appearance of the thyroid gland, and, in some instances, an increase in the height of the thyroid follicular epithelium (Norman et al. 1987, Goleman et al. 2002b).

Reported worldwide declines in frogs species (Houlahan et al. 2000, Alford et al. 2001, Pounds 2001, Kiesecker et al. 2001) have focused attention on environmental factors that may interfere with amphibian development and reproduction. One environmental contaminant of particular concern is ammonium perchlorate (AP), a compound widely used in the aerospace industry and by the military as an oxidizer in rocket fuels. Perchlorate salts have been used for decades to experimentally inhibit amphibian development and metamorphosis (Miranda et al. 1996, Brown 1997, Rollins-Smith et al. 1997). Recently, perchlorate has been detected in surface and ground waters at various places in the Western US at levels ranging from 8 μg/L to 3.7 g/L (Urbansky 1998). Perchlorate levels as high as 31.2 $\pm$ 0.21 mg/L were recently found in surface waters at Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas (Smith et al. 2001). These concentrations fall within a wide range of sublethal perchlorate concentrations that inhibit amphibian metamorphosis as evidenced by concentration-dependent reductions in forelimb emergence, tail resorption, and hindlimb growth (Goleman et al. 2002a, 2002b). These effects are accompanied by an increase in thyroid follicle cell height and reduced whole-body thyroid hormone content in tadpoles exposed to perchlorate (Goleman et al. 2002b).

Although environmentally relevant concentrations of perchlorate inhibit metamorphosis in *Xenopus laevis*, these frogs are not native to the US and the effects of AP on native species are largely unknown. In this report we provide evidence of thyroid disruption in American bullfrog (*Rana catesbeiana*) and western chorus frog (*Pseudacris triseriata*) tadpoles collected from AP-contaminated sites at the LHAAP in East Texas.

Methods

Field Collections

All animals were collected from ponds located on site at the LHAAP in Karnack, TX. The LHAAP is located in the watershed of Caddo Lake, the largest natural lake in Texas. In order to decommission this military base, groundwater on site has been treated

to precipitate metals and with air stripping and carbon polishing to remove volatile organics. Until the spring of 2001, perchlorate in the ground water was not removed. For a more complete description of site characteristics refer to Smith et al. (2001).

Sampling of each site took place for approximately 1-2 hr by a minimum of two personnel. Tadpoles were dip-netted from around the bank around the entire circumference of each pond. Tadpoles were rapidly euthanized in MS-222 and then stored in 10% neutral-buffered formalin for subsequent histological analysis. Bullfrog (*Rana catesbeiana*) tadpoles were collected in April 2000 from one control (site A) and one AP-exposed site (site B). Tadpoles from both sites were of the same age class based on the fact that they were identical in snout-vent length and were collected in early spring, indicating that they had over-wintered at least one season. Western chorus frog (*Pseudacris triseriata*) tadpoles were collected in April 2001 from one control (site C) and one exposed (site D) site. Species identification was aided using the key developed by Altig (1970). Bullfrog developmental stages were identified using the methodology of Taylor and Kollros (TK, 1946). Gosner staging (1960) was used for chorus frog tadpoles. All animals were collected under Texas Parks and Wildlife scientific permit no. SPR1098-984. All procedures were approved by the Texas Tech University Animal Care and Use Committee.

Field sites were selected based on their history of perchlorate contamination (Smith et al. 2001). Exposed and control sites were selected based on their close proximity to one another to control for differences in rainfall and temperature that may affect development. Control sites had no detectable perchlorate at the time of animal collection. At each visit to the field site, we attempted to measure various compositional and physical parameters, including: dissolved oxygen, air and water temperature, conductivity, salinity, and pH. Perchlorate content of field water samples was determined as previously described (Smith et al. 2001). Detection and quantitation limits for perchlorate were 1 μg/L and 2.5 μg/L, respectively.

Histological Assessment of Thyroid Activity

Tadpole heads were dehydrated in a graded series of alcohols and processed for routine paraffin embedding. Serial transverse sections (10 μm) through the head were mounted on glass slides and stained using Harris' progressive hematoxylin and eosin procedure and coverslips mounted. For bullfrog tadpoles, follicle epithelial cell height and right thyroid gland volume were measured as described previously (Goleman et al. 2002b) using an Olympus BH-2 compound microscope equipped with a Sony CCD/RGB video camera and monitor and a CompuAdd 450DX2 computer with Image Pro (Media Cybernetics, Silver Spring, MD, USA) imaging software. The software was calibrated using a Bausch & Lomb calibration slide prior to recording. The calibration was saved to the computer hard drive to ensure consistent measurements over time. Epithelial cell height measurements were taken from 5 arbitrarily chosen cells in 5 sections of the right thyroid gland for each specimen (n = 25 observations/animal). A single mean value for epithelial cell height was then calculated for each animal. The cross-sectional area of all serial sections through the right thyroid gland for each animal was measured and

summed. Total cross-sectional area (mm^2) x section thickness (10 μm) was used to calculate the right thyroid gland volume for each animal.

Although quantitative image analysis yielded useful data, the method proved too inefficient to serve as a high throughput means for assessing thyroid disruption in large numbers of samples. For analysis of thyroid disruption in chorus frog tadpoles we employed a semi-quantitative approach to determine colloid depletion, follicle cell hypertrophy, and follicle cell hyperplasia using methods based on Hardisty and Boorman (1990) as modified by Hooth et al. (2001) and by the US EPA Pathology Working Group of the Effects of Ammonium Perchlorate on Thyroids (Mann 2000). Serial sections through each animal were scanned and colloid depletion, follicle cell hypertrophy, and follicle cell hyperplasia determined for all follicles in one section each from the rostral, middle, and caudal region of the thyroid gland. Values were averaged for each section and a mean calculated for all three combined sections per animal. Colloid depletion, follicle cell hypertrophy, and follicle cell hyperplasia were scored by a naïve rater on a scale of 0, 1, or, 2 in order of increasing severity as shown below:

Colloid Depletion

0 No reduction
1 Pale, lacy, vacuolated, or granular appearance; slight to moderate reduction
2 Large reduction or absence

Follicle Cell Hypertrophy

0 No hypertrophy – follicles lined by squamous to cuboidal epithelium
1 Follicles lined by tall cuboidal to columnar epithelium; cytoplasm: nucleus ratio increased
2 Follicular epithelial cells distinctly larger than normal; follicular lumen severely decreased or obliterated

Follicle Cell Hyperplasia

0 No hyperplasia – follicles lined by a single layer of normal appearing, squamous to short cuboidal epithelium
1 Two or more follicles exhibiting stratification of follicular epithelium, usually 2-3 cells thick, protruding into lumen
(Note: follicles exhibiting stratification near gland periphery are not counted)
2 Greater number of affected follicles exhibiting stratification of follicular epithelium, usually more than 3 layers thick, protruding into lumen; areas of hyperplasia may also have microfollicular formation within them

Data Analysis

Mean differences were compared by two-tailed Student's t-test.

Results

Perchlorate contamination of surface waters at LHAAP in 2000 and 2001 was principally confined to two areas associated with the manufacture of AP and the demilitarization of munitions in accordance with the Intermediate-range Nuclear Forces (INF) treaty; the INF pond as described by Smith et al. (2001) (site B in the present study) and temporary ponds in the vicinity of Building 25C (site D in the present study). Two cases of apparent thyroid disruption in animals collected from these AP-contaminated sites are described below.

Comparison of Bullfrog Tadpoles from Site A and Site B

Water Quality Parameters- Water quality parameters at sites A and B are shown in Table 1. Site B tended to be slightly more alkaline than site A. Perchlorate concentrations at site B have historically reached levels as high as 31 ppm (November 1999, Smith et al. 2001). At the time that bullfrog tadpoles were collected for analysis perchlorate levels at site B were close to 2 ppm but were non-detectable at the reference (site A).

Developmental Endpoints- The animals collected from the reference site (site A) were in the later stages of prometamorphosis or early metamorphic climax (TK stages XVII-XXXI). In contrast, tadpoles collected from the contaminated site had only reached premetamorphosis and early prometamorphosis (TK stages IX-XIII). Snout-vent lengths were identical between tadpoles from sites A and B (Table 2), suggesting that these animals were roughly the same age, as age and body length are generally correlated in ranids (Sagor et al. 1998, Miaud et al. 1999). In contrast, hindlimb length was 5.2x smaller in the animals from the contaminated site compared to the reference site (Table 1). To directly compare relative hindlimb lengths in the size-matched animals, we calculated the hindlimb length to snout-vent length ratio. This ratio was 5.3-fold lower in the animals collected from the contaminated site compared to animals from the reference site. This is not an insignificant finding as hindlimb length/snout-vent length ratio is an accurate predictor of perchlorate exposure in laboratory studies on developing *X. laevis* (Figure 1). Approximately 93% of the variation in hindlimb length/snout-vent length ratio in *Xenopus laevis* can be explained by perchlorate exposure (Figure 1) when exposure begins in early embryonic life and continues for at least 70 d post-hatching.

*Thyroid Histopathology-*The quantitative assessment of thyroid gland volume and follicle cell height is presented in Table 3. There were no differences in follicle cell height between the collection sites. In contrast, thyroid gland volume was 2.5-fold larger in tadpoles from site A compared to tadpoles from site B.

Comparison of Chorus Frog Tadpoles from Site C and Site D

These sites were small temporary ponds that were too shallow to record from using our analytical water quality probe. Perchlorate in the water at site C was not detectable but was close to 10 ppm at site D at the time tadpoles were collected in April 2001. We

Table 1- *Site Characteristics*

Parameter	Site A	Site B
Source of water	Rainfall	Treatment plant effluent; rainfall
Conductivity[1]	70-560 µS/cm	80-2,052 µS/cm
pH[1]	6.2-7.1	7.8-9.3
Dissolved oxygen[1]	2.6-7.8 mg/L	7.3 mg/L
Temperature range[1]	16.9-21.1 °C	15.8-31.4 °C
Perchlorate[2]	ND	1,970 µg perchlorate/L

[1]Range of values between April 2000 and July 2001.
[2]Measured in water samples collected on the day that the tadpole specimens were collected in April, 2000.
ND, Not detectable.

Table 2- *Length Measurements in Bullfrog Tadpoles*

Site	Tail Length (mm)	SVL[1] (mm)	HLL[2] (mm)	HLL/SVL
A	48.3 ± 3.52	24.3 ± 1.75	26.2 ± 2.36	1.12 ± 0.09
B	41.2 ± 1.74	24.9 ± 0.75	5.02 ± 0.60[3]	0.21 ± 0.02[3]

[1]SVL, Snout-vent length.
[2]HLL, Hindlimb length.
[3]Statistically different from control site (site A) based on Student's *t*-test, $P < 0.05$.
Values are mean ± standard error of 11-13 animals per group.

Table 3- *Thyroid Histology in Bullfrog Tadpoles*

Site	Cell Height[1] (µM)	Thyroid Gland Volume[2] (mm^3)
A	4.53 ± 0.33 (n=11)	0.035 ± 0.004 (n=10)
B	4.53 ± 0.32 (n=13)	0.014 ± 0.002[3] (n=13)

[1]Mean ± standard error of measurements from 25-30 cells per animal from the right thyroid gland.
[2]Mean ± standard error of measurements from consecutive serial sections through the right thyroid gland.
[3]Statistically different from control site (site A) based on Student's *t*-test, $P < 0.05$.

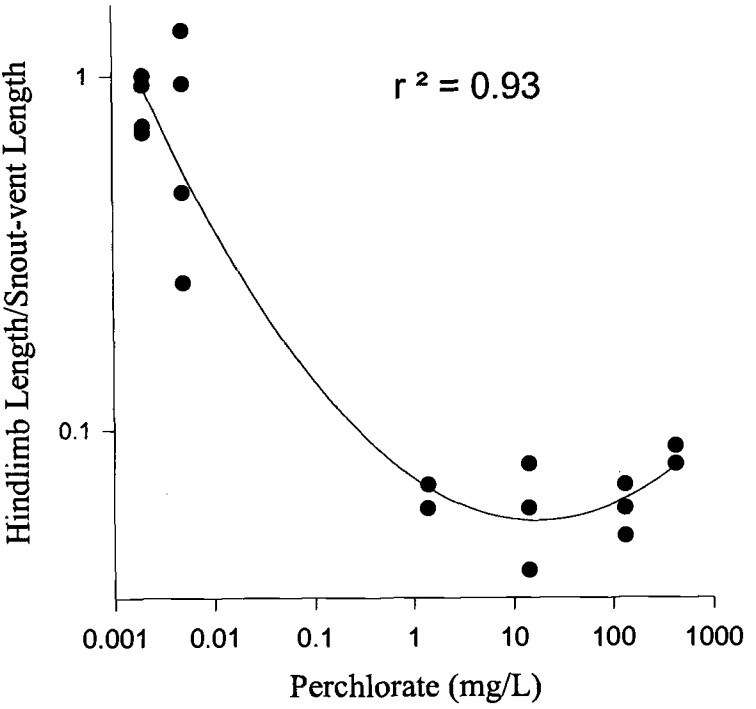

Figure 1- *Perchlorate concentration explains greater than 90% of the variation in hindlimb length/snout-vent length ratio in developing* X. laevis. *Measurements made after a 70-d exposure to AP beginning < 24 h after fertilization. Each point represents the mean of four replicates pooled from two independent trials. Sample size per replicate was 25-50. Data are re-graphed from Goleman et al. 2002a.*

Table 4- *Length Measurements in Chorus Frog Tadpoles*

Site	Tail Length (mm)	SVL (mm)	HLL (mm)	HLL/SVL
C	14.6 ± 0.41	8.72 ± 0.22	1.30 ± 0.08	0.15 ± 0.01
D	15.2 ± 0.27	9.80 ± 0.14	1.50 ± 0.06	0.15 ± 0.01

[1]SVL, Snout-vent length.
[2]HLL, Hindlimb length.
ND, Not detectable.

Table 5- *Thyroid Histology in Chorus Frog Tadpoles*

Site	Perchlorate (μg/L)	Gosner Stage[1]	Colloid Depletion[2]	Hypertrophy[2]
C	ND	33-34	0.02 ± 0.02 (n=8)	0.00 ± 0.00 (n=8)
D	9,802	33-34	0.71 ± 0.22[3] (n=15)	0.79 ± 0.20[3] (n=15)

[1]After Gosner (1960).
[2]Mean ± standard error of scores made from all follicles present in three sections from the rostral, middle, and caudal regions of the thyroid gland.
[3]Statistically different from reference site (site C) based on Student's *t*-test, $P < 0.05$.
ND, Not detectable.

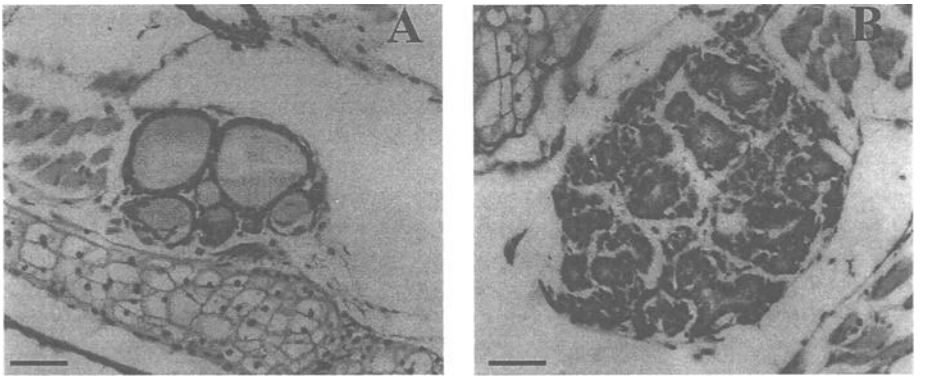

Figure 2 - *Photomicrographs of thyroid tissue from western chorus frog tadpoles (Gosner stages 33-34) collected from a reference site (Figure 2A) or an AP-contaminated site (Figure 2B). Figure 2A. Note the squamous shape of the follicle cells and the abundance of colloid. Figure 2B. Note the marked hypertrophy of the follicle cells and the reduction in follicle lumen and absence of colloid. Also note the apparent increase in size of the thyroid gland from the AP-exposed animal (Figure 2B, compare to Figure 2A). Both photomicrographs were taken at the same magnification. Bar = 50 μm.*

immediately noticed distinct hypertrophy and colloid depletion in thyroids from the animals collected at D (Figure 2). Semi-quantitative assessment of the thyroids from these animals revealed statistically significant colloid depletion and follicle cell hypertrophy compared to stage-matched animals from site C, where perchlorate was not detected. Follicle cell hyperplasia was not detected in tadpoles collected from either site.

Discussion

Between April 2000 and July 2001 we examined approximately 97 adult and larval frogs (*Hyla cinerea*, *R. catesbeiana*, and *P. triseriata*) collected at various perchlorate-free reference sites at LHAAP and prepared as described above. There was no evidence of thyroid disruption in any of the animals from the perchlorate-free sites. In contrast, our data suggest that bullfrog tadpoles inhabiting a perchlorate-contaminated pond at LHAAP (site B) exhibited signs of thyroid disruption as evidenced by reduced hindlimb growth relative to body growth and delayed metamorphosis relative to size-matched tadpoles from a nearby reference site. The tadpoles from the control and reference sites had over-wintered at least one season and had identical snout-vent lengths, suggesting that they belonged to the same age class (Sagor et al. 1998, Miaud et al. 1999). The volume of the thyroid gland was more than two times larger in the tadpoles from the reference pond, presumably because these animals were entering the later stages of prometamorphosis and metamorphic climax, which begins at TK stage XX. Whether animals from site B had been exposed to perchlorate during their entire larval period, which can last up to 2-3 years, isn't known, although perchlorate levels at the time of capture at site B were close to 2 ppm. Certainly one factor that can determine the extent to which developing frogs can be affected by perchlorate exposure is the variability with which perchlorate is present within surface waters. This in turn can be affected by several factors including leaching of perchlorate from soil, movement of perchlorate from ground water into surface water, and runoff of perchlorate from surface sediments. Data that have been collected from LHAAP suggest that the presence of perchlorate at site B can vary significantly from month to month. Perchlorate levels at site B the November preceding capture were close to 31 ppm (Smith et al. 2001) while in the months following capture, perchlorate levels ranged from 1700 μg/L in May to a range of 0-257 μg/L in June of 2000. At site B we would expect perchlorate levels to vary with the discharge of treated groundwater into the site. During dry periods treated groundwater was historically pumped to site B, but during the wetter months the discharge was diverted to nearby bayous that emptied into Caddo Lake (Smith et al. 2001). Thus it is likely that developing bullfrog tadpoles at site B were exposed to intermittently high levels of perchlorate. The fact that the effects of perchlorate on metamorphosis are largely reversible (Goleman et al. 2002b) suggests that developing frogs that are exposed intermittently over the course of a long larval period may have low- or non-exposure recovery periods, which may explain the fact that the bullfrog tadpoles at site B had progressed to early prometamorphosis and exhibited evidence of hindlimb growth, albeit significantly less than the hindlimb growth observed in tadpoles from the reference site. We (Carr and Goleman) know from ongoing studies in our laboratory that the $t_{1/2}$ for whole-body

elimination of perchlorate anions in bullfrog tadpoles is approximately 48 hr (unpublished data). Thus it is likely that over the course of their development tadpoles from site B had non- or low-exposure recovery periods from perchlorate.

The chorus frog tadpoles collected at site D exhibited dramatic disruption of thyroid function at the histological level compared to stage-matched tadpoles from a nearby reference site (site C). To our knowledge this is the first report of thyroid disruption in an amphibian inhabiting an AP-contaminated site. Given the relatively early developmental stage (Gosner stages 33-34) of the animals we examined, we would not have expected to see measurable differences in hindlimb growth in the animals from the contaminated site. However, the thyroid histopathology data clearly indicate thyroid disruption consistent with perchlorate exposure in the animals from site D. Exposure of frogs (Goleman et al. 2002b) or rodents (Siglin et al. 2000) to perchlorate under laboratory conditions causes a specific set of histopathological changes in the thyroid gland that include follicle cell hypertrophy, colloid depletion, and, in some instances, follicle cell hyperplasia. These changes result from over secretion of TSH due to lack of negative feedback that results from reduced TH synthesis (Norris 1997). The fact that the amphibian thyroid is functionally identically to that of mammals suggests that histopathological characteristics developed for assessment in mammals are equally useful in assessing perchlorate exposure in amphibians.

Collectively, results of the present study and previous work performed in *X. laevis* (Goleman et al. 2002b) suggest that thyroid histology is a useful indicator of perchlorate disruption at earlier stages of development, prior to metamorphic climax. However, because thyroid histology changes dramatically during metamorphic climax, other endpoints, such as hindlimb length relative to snout-vent length and forelimb emergence, are useful for detecting perchlorate exposure in late developmental stage tadpoles.

The potential impact of perchlorate on amphibian development goes beyond the predictable effects on thyroid-sensitive aspects of development and metamorphosis. Disruption of thyroid function interferes with normal gonadal development in some frog species (Hayes 1997; Goleman et al. 2002b). Whether perchlorate disrupts gonadal development under field exposure scenarios remains an open question, as we did not examine species that possess fully differentiated gonads at the time of capture. However, it is an important endpoint to keep in mind for future studies, as disruption of gonadal differentiation could clearly impact reproductive fitness at the population level.

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Interaction Between Perchlorate and Iodine in the Metamorphosis of *Hyla versicolor*

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Abstract: Perchlorate (ClO_4-) is a water-soluble, inorganic anion that is often combined with ammonium, potassium or other cations for use in industry and agriculture. Ammonium perchlorate, for example, is a potent oxidizer and is used in various military applications including rocket fuel. It has also been found in an historically widely used fertilizer, Chilean nitrate and in other fertilizers. It has been found in ground and surface waters of over 30 states and is considered a human health risk. Because of its similar atomic radius and volume, perchlorate competes with iodide for thyroid uptake and storage and thereby inhibits production of thyroid hormones. Amphibians may be particularly affected by perchlorate because they rely on the thyroid for metamorphosis. This study exposed early larval *Hyla versicolor* to concentrations of perchlorate ranging from 2.2 to 50 ppm to determine the effects of perchlorate on a native amphibian. In addition, three controls, 0 perchlorate, 0 perchlorate with 0.10 ppm iodide (C + I) and 50 ppm perchlorate + 0.10 ppm iodide (50 + I) were tested. Mortality (< 11% with all treatments) and growth appeared to be unaffected by perchlorate. Inhibition of development started with 2.2 ppm perchlorate and little or no development occurred at 22.9 ppm and above. This inhibition was particularly apparent at the latter stages of development including hindlimb formation and metamorphosis. The estimated EC50 for total inhibition of metamorphosis at 70 days of treatment was 3.63 ppm. There was no evidence of inhibition of development with the 50 + I, C + I, or controls, indicating that the presence of small concentrations of iodide could counter the effects of perchlorate. When tadpoles that had been inhibited by perchlorate were subsequently treated with iodide, development through prometamorphosis progressed but mortality was very high.

Keywords: perchlorate, iodide, *Hyla versicolor*, metamorphosis, amphibians, thyroid

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Introduction

Perchlorate anion (ClO_4-) can be a contaminant in surface and groundwaters located near testing and fueling areas for aircraft and missiles. When combined with various cations, especially ammonium, it is a potent oxidizer to solid rocket fuels and pyrotechnics (Von Burg 1995). It is also present in fertilizers, especially Chilean nitrate that was used extensively on rye , cotton, citrus, and tobacco crops several decades ago (Susarla et al. 1999, Ellington et al. 2000). Although this fertilizer is not used to any extent now in the United States, perchlorate is persistent and has been found in other currently applied fertilizers at concentrations up to 1.9% (Susarla et al. 1999). The acute toxicity of perchlorate is fairly low in mammals and varies upon the specific salt. For example, the LD50 for ammonium perchlorate ranges from 750-4200 ppm in rabbits and rats, respectively (Von Burg 1995). In aquatic animals potassium perchlorate had an LC50 of 500 ppm in *Rana temporaria* and *Xenopus laevis* and ammonium perchlorate had an LC50 of 400 ppm for a 96 hr test in rainbow trout (*Salmo gairdinerii*) (Von Burg 1995, Goleman et al. 2002).

The primary environmental risk from perchlorate is that it is highly soluble and can be a potent competitor with iodide for storage in the thyroid and thus interferes with thyroid hormone production (Carrasco 1993, Wolff 1998). The anion has a similar volume (44.5 ml/mol at 25°C compared to 36.7 ml/mol for iodide) and diameter (2.43 Å versus 2.16 Å). Iodide is essential for the production of both thyroxine (T4) and the hormonally more active triiodothyronine (T3) analog. Because of its ability to inhibit the thyroid, potassium perchlorate and other salts were used therapeutically to diagnose and treat thyroid disorders (Von Burg 1995, Wolff 1998). Perchlorate contamination of ground and surface waters has been found in at least 30 states. Contamination in the West has been associated primarily with aerospace and military activities and their supporting industries (Urbansky and Schock 1999, Smith et al. 2001). Groundwater concentrations have been low (< 50 ppb) but cause concern for human health because groundwater is a source of drinking water and there is some worry that perchlorate may adversely affect children (Brechner et al. 2000). The U.S. Environmental Protection Agency is proposing a safe drinking water standard of 1 ppb (http://www.epa.gov/ncea/) and several methods of cleaning up groundwater have been proposed (Urbansky and Schock 1999).

Ecological concerns focus on aquatic animals that may inhabit perchlorate-contaminated waters. Surface water concentrations have been measured as high as 31 ppm (Smith et al. 2001). In addition, perchlorate can concentrate in aquatic organisms and has been measured as high as 5,500 ppm in vegetation and 2 ppm in invertebrates. Amphibians especially may be affected by perchlorate in water (Bantle et al. 1999). The hypophyseal-thyroid axis is the primary initiator and regulator for metamorphosis as mediated by thyroid hormones (Buscaglia et al. 1985, Etkin 1968). Perchlorate concentrations of 340 ppm inhibited metamorphosis in *Bufo arenarum* (Miranda et al., 1992) but this was the lower of only two dose concentrations used and is approximately an order of magnitude higher than concentrations detected in the field. The greatest concern for inhibitory effects of perchlorate is in areas with low concentrations of natural iodine in soil or water. Historically, these lie in the so-called 'goiter belts' of the Great Lakes Basin, North Dakota, Montana, Idaho and Utah (Drever 1997). The objectives of

this study are to determine at what concentrations perchlorate inhibits metamorphosis in a native amphibian, the gray treefrog (*Hyla versicolor*), and to assess the effects of exogenous iodide on perchlorate inhibition.

Experimental Method

The study consisted of two continuous phases or sub-experiments. The first phase determined the effective concentration of perchlorate anion necessary to inhibit metamorphosis in the gray treefrog, *Hyla versicolor,* and whether the presence of iodide could counter an inhibitory effect. The second phase examined if the addition of iodide to water could induce metamorphosis in tadpoles that had been previously inhibited by perchlorate.

Early larval (Gosner stage 24 or 25, Gosner 1960) *Hyla versicolor* tadpoles were captured with dip nets from constructed wetlands located at Patuxent National Research Refuge, Laurel, MD, USA in mid July 2001 and moved to an environmental chamber where they were allowed to acclimate for three days before treatment. Photoperiod was set at 14 hrs L/10 hrs D and temperature at 23 $\pm$ 1°C for the duration of the study. Lighting was subdued and provided by fluorescent fixtures. There were four replicates per treatment and 6 replicates for controls. Seven tadpoles were placed in each 8 L, all glass aquarium and air was provided through glass bubblers connected to standard aquarium pumps. Test water came from wells located on the premises and filtered through ion exchange columns, producing deionized water. Following ASTM E729-88 guidelines (ASTM 1988) reagent grade salts were added to create hard water. The tests were static renewal with complete replacement of water twice each week.

Tadpoles were examined daily for mortality and abnormal behavior and were fed romaine lettuce that had been boiled for 30 minutes ad libitum. Every 10 days they were removed from the tanks with a net, gently shaken to remove excess water and weighed as a group. Each individual was also measured for snout to vent length with calipers and staged using the following categories: pre limb (denoted by absence of hind limbs to the unaided eye, corresponding to Gosner stages 24-26), limb bud (hind limb visible to naked eye but no clear joint formed - Gosner stages 27-34), hind limb (knee joint apparent - Gosner stages 35 to 40), and metamorph (at least one forelimb present - Gosner stages 41 to 46). At the first appearance of a forelimb animals were transferred from group tanks to individual tanks with sloped bottoms that allowed metamorphs water and dry surfaces until they completed metamorphosis as determined by total absorption of the tail. After complete metamorphosis they were euthanized with MS-222 and frozen at -80°C for residue analysis.

Treatments in the first phase of the study were at 0 (control), 2.2, 4.8, 10.5, 22.9, 33.8 and 50 ppm. Potassium perchlorate was used to make a perchlorate stock solution. The potassium rather than ammonium salt was used because in an earlier study it was determined that toxicity to embryos and early larvae from ammonium perchlorate was attributable the more toxic ammonium ion. In addition, two other treatments of 0 (C + I) and 50 ppm perchlorate (50 + I) with 0.10 ppm iodide as KI were added to test the effects of iodide on perchlorate inhibition and as a control with iodide.

In the second phase of the study, aquaria that still had tadpoles after 70 days of

exposure were split into two groups. Two aquaria at each concentration were used as "controls" whereas two other aquaria were dosed with the equivalent concentration of perchlorate but had either 0.05 or 0.10 ppm iodide as KI added (one tank each, 4 to 7 tadpoles per tank). Twelve days after the last tadpole that had been exposed to iodide in this phase had metamorphosed, the remaining "control" aquaria were again divided: one continued with perchlorate as before and one was given the same concentration of perchlorate and 0.01 ppm iodide. The animals and aquaria in this phase were not biologically or statistically independent of the first phase but the results could be used to estimate the effects of low concentrations of iodide on animals that had been previously inhibited by perchlorate.

Composite whole body samples of four to five metamorphs were freeze-dried, homogenized, extracted with deionized water, centrifuged and filtered. Perchlorate was analyzed on an ion chromatograph and its detection limit in tissues was 12 ppm. Stock solutions of perchlorate and iodide were analyzed to verify nominal concentrations. Measured perchlorate concentrations in water averaged 12.2 percent higher than nominal values. Over the four days of exposure between total changes of water perchlorate concentrations declined by an average of 4.7 percent. Measured iodide was within 5% of the nominal concentration.

Data were examined for normality and heterogeneity of variance and most variables had to be transformed to meet the assumptions of analysis of variance. Frequency data were transformed with the arcsine of the square root of the value, count data with the square root of the value + 0.5 and continuous data (e.g., weights, snout vent lengths) with log transformation. Statistical tests included COANOVA with 10 day interval as the covariate for measures that changed through time (snout vent length, body mass, proportion of tadpoles at specific stages) or ANOVA for endpoint information such as time to metamorphosis. Proc GLM (SAS 1990) was used for these analyses. Proc Probit was also used to derive a median effective concentration (EC_{50}) of metamorphosis inhibition at 70 days of testing.

Results

First Phase: Effects of Perchlorate and Iodide on Inhibition of Metamorphosis

Larval Mortality — Survival during the period of pre-metamorphosis was high. During the entire 120 day experiment survivorship of tadpoles ranged from 89% at 2.2 ppm perchlorate to 100% at 50 ppm and 50 + I treatments. Control survival was 95%.

Effects on Growth and Development — Body mass steadily increased during the 70 days of this phase of the experiment. The period covariate was highly significant ($p < 0.0001$) but the treatment effect and treatment by period interaction were not statistically significant (Fig. 1). Similarly, snout vent length showed a significant period covariate effect ($p < 0.0001$) but neither the treatment or the interaction was significant (Fig. 2). However, when body mass and snout vent length were compared across treatments by individual periods, significant differences were observed from the third 10 day period through the end of the test as tadpoles entered the climax phase and subsequently lost weight and shrunk.

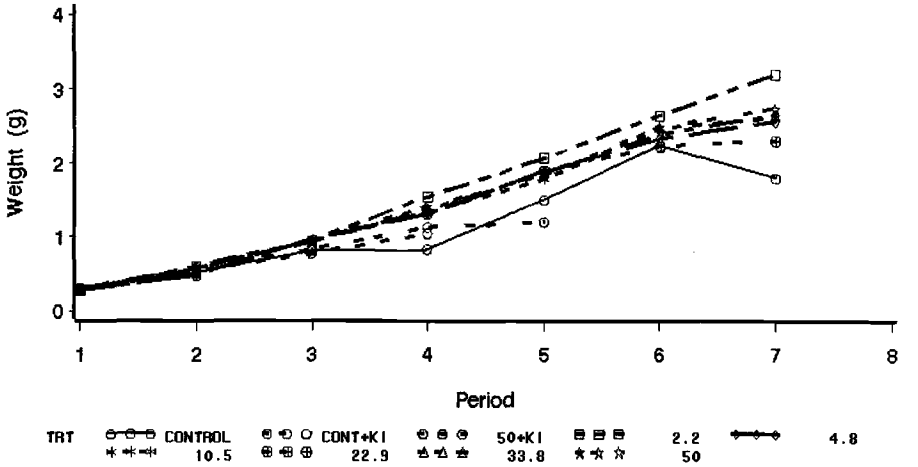


Figure 1 — Change in body weight of tadpoles exposed to different concentrations of perchlorate and iodide through 70 days of testing.

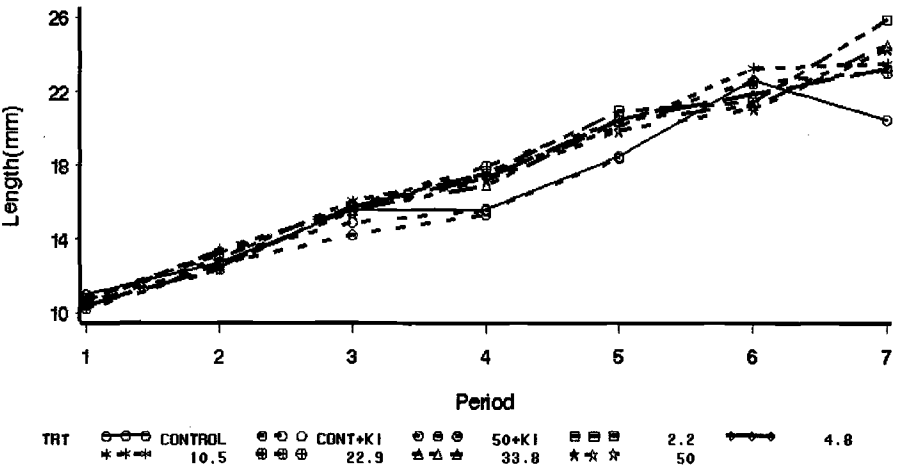


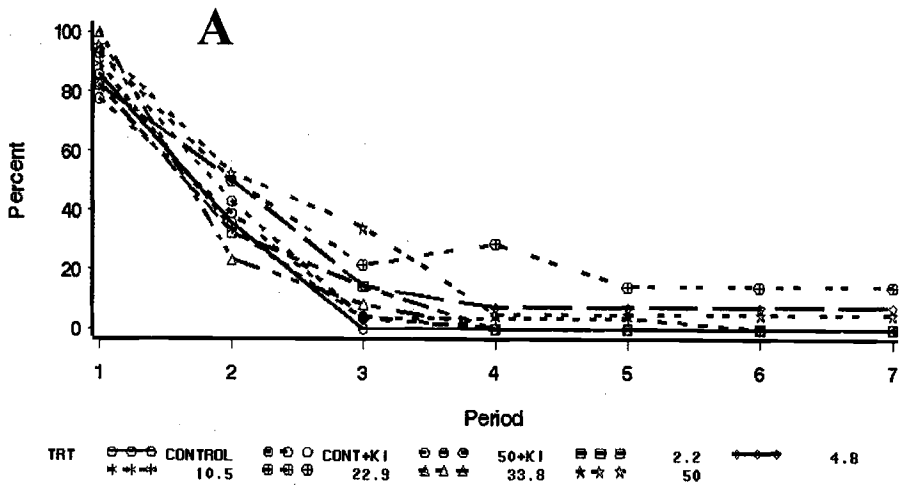
Figure 2 — Growth in snout-vent length of tadpoles exposed to different concentrations of perchlorate and iodide through 70 days of testing.

Developmental progress, as determined by the percent of the test animals in different stages, was significantly affected by treatment. The earliest of the stages, pre-limb, differed with the period covariate ($p < 0.0001$) but not with treatment or treatment by period interaction (Fig. 3a). The limb bud stage showed a significant interaction between period and treatment ($p < 0.0001$) but neither the period effect ($p=0.080$) nor treatment ($p= 0.83$) alone was significant. The significance in the interaction term was due primarily to an initial increase in frequency in all treatments due to animals developing into this stage from the pre-limb phase by the 20th day of testing, but a much more rapid decline in frequency in the control, C + I and 50 + I treatments as tadpoles continued development into the next stage (Fig. 3b). The hindlimb stage had significant period and period by treatment effects (both $p < 0.0001$) and the treatment effect was approaching significance ($p=0.063$). The slopes for the Control, C + I and 50 + I treatments could not be distinguished from each other but all of the other slopes differed from these. In these three treatments most tadpoles developed hindlimbs by the end of 30 days and then progressed into the metamorph stage. For the other treatments, tadpoles generally developed hindlimbs but development beyond that stage depended on perchlorate concentration (Fig. 3c). These trends were reflected in the dynamics of the metamorph stage (Fig. 3d) that also showed significant period and period by treatment effects ($p < 0.0001$) but no treatment effect.

Metamorphosis - One hundred thirty tadpoles entered the climax stage of metamorphosis. Of these, 125 (96.1%) had no visible problems or abnormalities. Two (1.5%) had malformations, one with a bifurcated tail with visible segments of a spinal column in each piece and one with a truncated foot and leg. Two metamorphs demonstrated signs of edema and one was found dead and possibly had drowned. Of the 118 juvenile frogs with clearly documented fate, 103 (87.3%) appeared normal and 15 (12.7%) died during the climax stage from unknown causes. There was no apparent relationship between dose and fate (alive or dead).

There was an obvious and significant difference ($p < 0.0001$) among treatments in the frequency of tadpoles metamorphosing during the 70 days of the first phase. All of the tadpoles in the C + I and 50 + I and all but one in the Control treatment that survived to the beginning of the climax stage metamorphosed (Fig. 4). The remaining Control tadpole eventually metamorphosed after 100 days. In an a posteriori test with Tukey's HSD there were no differences in frequency of metamorphosis among 50 + I, C + I and controls but these differed from all other treatments. No tadpoles metamorphosed at 22.9 or 33.8 ppm perchlorate and only 1 metamorphosed at 50 ppm. An approximate EC_{50} of 3.63 ppm was calculated through probit analysis but the distribution of data would not permit the calculation of 95% confidence limits or an accurate estimate of a lowest observed effects level (LOEL).

Based on a repeated measures analysis of variance for only those tadpoles that entered the climax stage, there was no significant ($p =0.40$) difference in the number of days to metamorphosis among treatments (Table 1). However, there was a significant difference in the number of days required to complete metamorphosis once a forelimb had emerged ($p = 0.01$). No significant a posteriori differences could be identified but tadpoles that were exposed to perchlorate alone took from 0.5 to 4 days longer to complete metamorphosis than those on Control, C + I or 50 + I treatments.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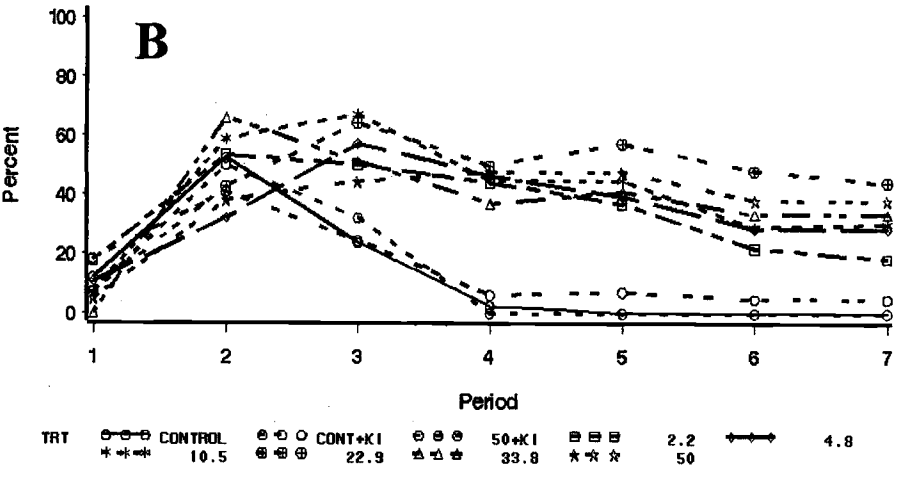
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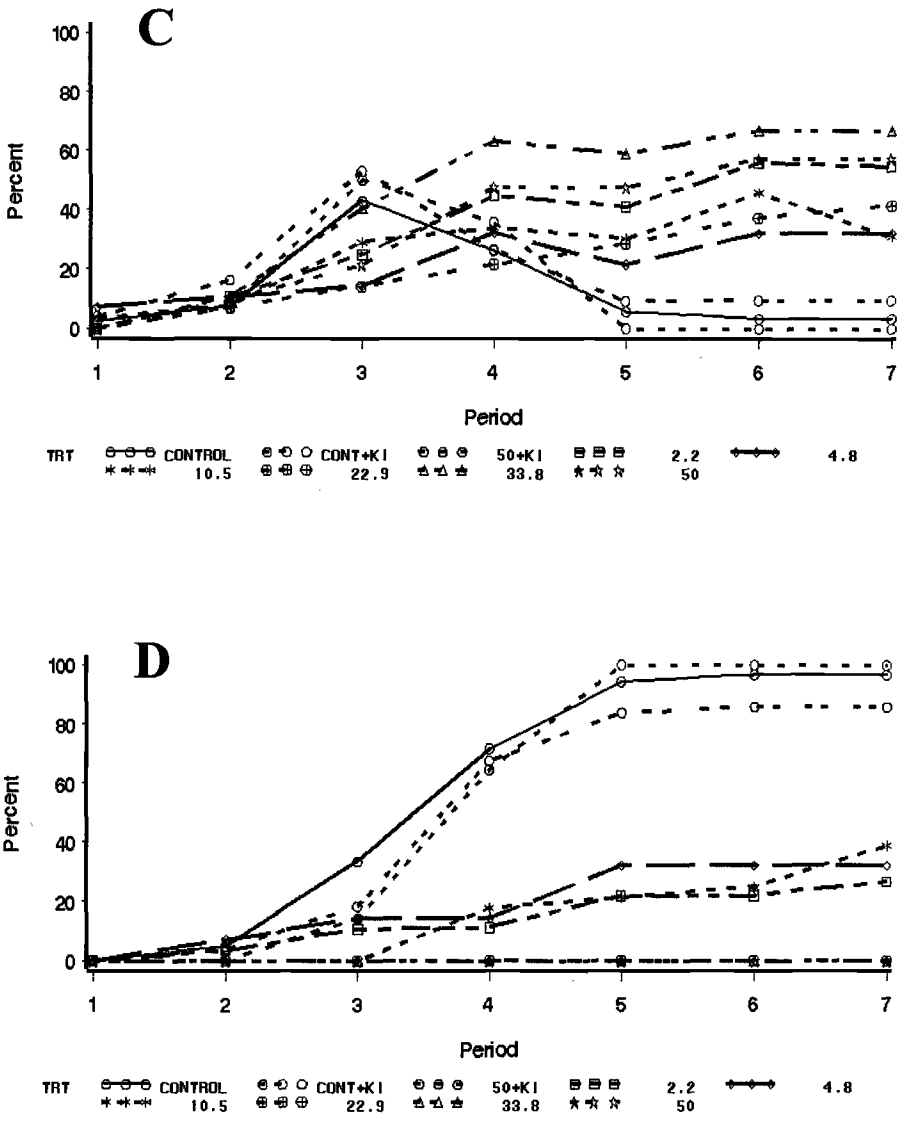

Figure 3 — Changes if frequency of different developmental stages by perchlorate and iodide treatment. A) Pre limb bud stage; B) limb bud stage; C) hindlimb stage; D) metamorph stage. See text for definitions of these stages.

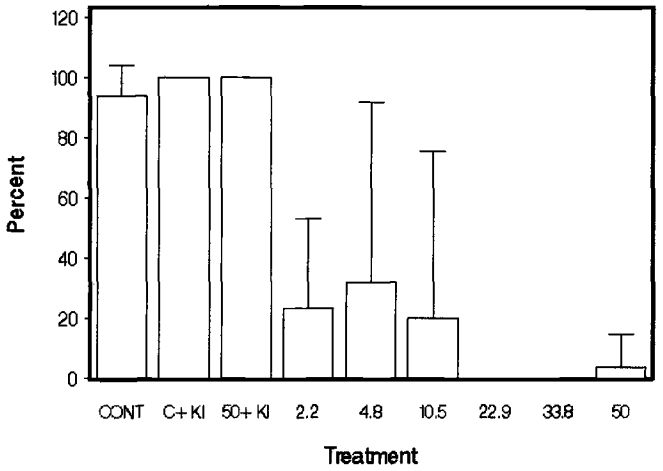

Figure 4 — Percent of Hyla versicolor tadpoles metamorphosing during exposure to perchlorate and iodide treatments.

Table 1 — Characteristics of Hyla versicolor exposed to perchlorate and iodide at metamorphosis

Treatment (ppm)	Body Weight (g)	Snout vent length (mm)	Days to Climax Stage	Days for completion of metamorphosis
Control	0.60 ± 0.18	15.8 ± 1.5	30.4 ± 10.0	3.2 ± 1.8
C + I	0.61 ± 0.14	15.5 ± 1.6	26.5 ± 7.6	2.6 ± 1.1
50 + I	0.62 ± 0.15	16.3 ± 1.6	29.6 ± 6.8	2.8 ± 1.5
2.2	0.96 ± 0.43	17.4 ± 2.8	35.0 ± 16.9	4.8 ± 2.5
4.8	0.62 + 0.26	15.9 ± 2.5	26.8 ± 12.6	5.7 ± 3.9
10.5	0.55 ± 0.13	15.7 ± 1.5	29.6 ± 10.6	6.6 ± 9.0
22.9	NA	NA	NA	NA
33.8	NA	NA	NA	NA
50	0.36	13.5	23	4

Anion Concentrations — Perchlorate was difficult to measure accurately in whole bodies of tadpoles which accounted for the relatively high detection limit of 12 ppm. Ten composite samples were analyzed for perchlorate. Four control samples, one group exposed to 2.2 ppm, and one to 4.8 ppm perchlorate had body concentrations below the detection limit. A second sample at 4.8 ppm had a body residue of 67 ppm. A single

composite sample exposed to 10.5 ppm had a body residue of 19 ppm and two samples at 50 ppm had 94 and 109 ppm, respectively. Four samples that had been exposed to 50 + I or C + I were measured for iodide and had concentrations varying from below detection to 59 ppm with no interpretable difference among the samples.

Second Phase: Effects of Exogenous Iodide After Inhibition

This phase of the study consisted of two sub-phases and tested if addition of iodide would interact with the prior inhibition of metamorphosis. The first sub-phase of this test exposed animals to 0, 0.10 and 0.05 ppm iodide. When that was completed, remaining tadpoles in the second sub-phase were exposed to 0 or 0.01 ppm iodide. Overall, there was a significant difference in the proportion of tadpoles entering climax stage due to the concentration of iodide ($p=0.0008$) but not due to the concentration of perchlorate ($p=0.28$) or the interaction of the two chemicals ($p=0.24$). During the 60 days of this phase two individuals metamorphosed without added iodide, one each at 4.8 and 33.8 ppm perchlorate. During the first sub-phase, 90-100% of the tadpoles exposed to iodide entered the climax stage regardless of perchlorate concentration. With 0.01 ppm iodide, 75% of the tadpoles entered the climax stage at 2.2 ppm perchlorate, 82% at 4.8 ppm, and 70% at 22.9 ppm. However, none of the tadpoles at 10.5, 33.8 or 50 ppm perchlorate advanced from the hindlimb stage at this concentration of iodide (Fig. 5).

The number of days to the beginning of the climax stage differed significantly among concentrations of perchlorate ($p < 0.0001$), iodide ($p < 0.0001$), and in the interaction of the two anions ($p=0.0007$). The mean time required to enter metamorphosis varied from 3.4 days at 2.2 ppm perchlorate to 4.5 days at 50 ppm (Fig. 6). Tadpoles exposed to 50 ppm perchlorate took longer to metamorphose than those at all other concentrations except 22.9 ppm. Similarly, tadpoles exposed to 0.05 or 0.10 ppm iodide entered climax stage more quickly than those at 0.01 or 0.0 ppm iodide. The significant interaction between iodide and perchlorate was due to: a) at the lower perchlorate concentrations time to metamorphosis increased as the concentration of iodide decreased; and b) at 33.8 or 50 ppm perchlorate 0.01 ppm iodide did not stimulate metamorphosis. Snout vent length at the beginning of the climax stage varied with iodide concentration ($p=.0.037$) but not perchlorate concentration ($p=0.421$). A posteriori tests with Tukey's HSD did not distinguish group means but there was a general trend for snout vent lengths to increase as iodide concentrations decreased. Growth was continuous through the entire study and the mean snout vent length was 24.8 ± 3.3 mm for all tadpoles entering climax at the second half of this phase (0.01 ppm iodide), 21.6 ± 2.3 mm at the first half of the phase (0.15 and 0.075 ppm iodide) and, for comparison, 15.8 ± 1.7 mm at the comparable stage in the first phase (ANOVA across the three phases, $p=0.008$, a posteriori tests distinguished each phase). Considering only the second phase, body mass at the beginning of the climax stage differed across iodide concentrations ($p=0.004$) but not perchlorate ($p=0.289$). A posteriori contrasts for iodide showed that tadpoles at the second half were heavier than those in the first half ($p=0.05$) but this could be explained by the longer growing period. As with snout vent length, body mass increased through the study with significant differences among both phases ($p=0.001$) and each phase was significantly different from the others in a posteriori comparisons. For the second phase

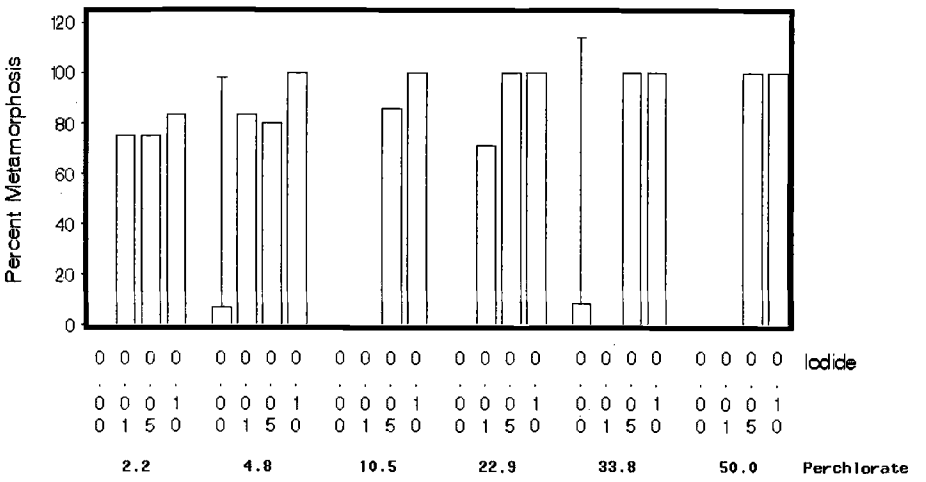

Figure 5 — Percent of previously developmentally inhibited Hyla versicolor tadpoles metamorphosing at 0.0, 0.01, 0.05, and 0.1 ppm iodide at perchlorate concentrations ranging from 2.2 to 50 ppm.

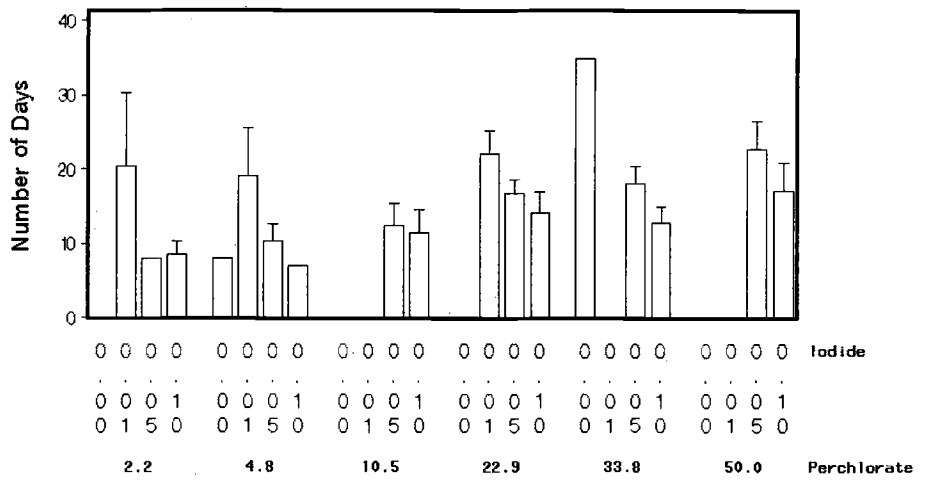

Figure 6 — Number of days required to initiate the climax stage in Hyla versicolor tadpoles that had been developmentally inhibited by perchlorate and then exposed to iodide.

animals in the first half weighed 2.41 ± 0.51 g and those in the first half 1.81 ± 0.40 g. In contrast, tadpoles at climax stage in the first phase of the study averaged 0.62 ± 0.19 g.

Whereas most of the animals in the first phase of the study entered climax stage and metamorphosed in good health, the majority of animals in the second phase were not healthy as they entered this stage. Thirty-seven percent (30) of those entering climax did not display any overt signs of poor health. However, another 37% were found dead during daily checks, often with only one forelimb emerged. The remaining metamorphs (26%) displayed obvious signs of edema that could have reflected problems in maintaining water and ion balance. Fully 96% of those entering metamorphosis died before they could complete development into juvenile frogs, 75% of those that died during climax were edemic and many displayed signs of stress including hyperventilation and lack of coordination.

Discussion

The primary effect of perchlorate in the first phase of this study was an inhibition of metamorphosis. Mortality at all treatments was less than 4% in this phase. There were no differences between controls and treated animals in snout vent length or weight gain during the 70 days of the study. Even at 50 ppm perchlorate, growth appeared normal compared to controls.

However, perchlorate did affect the onset of metamorphosis at environmentally realistic concentrations. In a study of a U.S. Army ammunition manufacturing site, water concentrations of perchlorate ranged from 4 ppb to 31 ppm (Smith et al. 2001). Our lowest test concentration of 2.2 ppm perchlorate was sufficient to inhibit metamorphosis in *Hyla versicolor* tadpoles and the estimated EC50 was 3.63 ppm. This estimate is substantially lower than the previously published concentration known to inhibit metamorphosis in an amphibian of 340 ppm determined for *Bufo arenarum* (Miranda et al. 1992) and has important consequences for risk assessments. Because of inconsistent rates of metamorphosis among treatment levels this is only an estimate and a lowest observed effects level could not be determined. However, concentrations lower than 2.2 ppm would be expected to inhibit metamorphosis in some animals because some inhibition was noted at this concentration. Delayed metamorphosis can be detrimental to amphibians if they are unable to leave wetlands that subsequently dry up (Pehek 1995, Warner et al. 1993). In addition, longer developmental times may make tadpoles more susceptible to aquatic predators, although the significance of this is difficult to assess since frogs also are vulnerable to terrestrial predators.

Because control animals without the addition of iodide in the water were able to metamorphosis at the same rate as those given 0.1 ppm iodide in water and because iodide is required for metamorphosis (Buscaglia et al. 1985), tadpoles must have been able to have access to iodide in some way. The only sources would have been naturally occurring iodide acquired while animals were still free-ranging and very immature or from their food. Although thyroid glands in stage 25 tadpoles only exist as primordial organs, iodide is stored in salivary glands, gastric mucosa, and choroid plexus in mammals (Wolff 1998) and amphibians may have secondary storage depots as well. The sediments at Patuxent had once been inundated with sea water and likely have abundant

iodide (Oliver 1997). Although romaine lettuce is not considered a high source of iodine, it may have had adequate amounts for thyroxine production.

The transport and storage of iodide into the thyroid are complex physiological processes involving a Na+/I- symporter (see Carrasco 1993, Wolff 1998). In mammals perchlorate and other monovalent anions are transported into the thyroid more readily than iodide and can inhibit iodide uptake or accelerate its loss. Without iodide, thyroxine cannot be produced. Thus in the presence of both perchlorate and iodide, perchlorate should overcome iodide uptake and become an effective inhibitor of thyroid function. This study contradicts this premise in that some animals metamorphosed even when perchlorate concentrations were as high as 10.5 ppm. Moreover, animals given 0.1 ppm iodide and 50 ppm perchlorate, a ratio of 1:200, were able to complete metamorphosis as quickly as controls and with very low mortality. It appears, therefore, that *Hyla versicolor* do not preferentially transport or store perchlorate compared to iodide.

From a hazard assessment perspective, the results of the first phase of the study suggest that the presence of biologically available iodide, either in solution or in food such as some algae, may reduce or prevent the negative effects of perchlorate. There may be substantial variation among wetlands in aqueous iodide concentrations and the element does not appear to be regularly measured by any federal monitoring program but Drever (1997) indicated that stream concentrations are around 7 ppm which should be adequate to mitigate most perchlorate problems. The availability of iodine in soils varies considerably through the United States and some regions of the Midwest, North Central States and Southwest have very low levels of natural iodine. Amphibians may be at greater risk there than in areas with higher concentrations of iodine. Other factors including thiocyanates and nitrates also may interfere with the iodide symporter (Carrasco 1993, Eskandari et al. 1997) and interact with perchlorate to affect iodide uptake by the thyroid. Our study suggests, although with caution, that application of iodine either as an iodide salt or iodate might be useful in protecting a federally or state listed amphibian species that is breeding in a pond receiving perchlorate contamination and low iodide concentrations. Further research on the effects of iodine applications on the ecology of wetlands would have to be conducted before such treatment could be considered efficacious and safe.

Carr et al. (this volume) provided evidence that bullfrog (*Rana catesbeiana*) and western chorus frog (*Pseudacris triseriata*) tadpoles in contaminated wetlands experienced signs of thyroid disorder. Signs included reduced hindlimb length relative to snout vent length and histological abnormalities. The wetlands were located on an U.S. Army ammunition site and were subjected to episodic exposure of high (up to 31 ppm) perchlorate concentrations so dose/response could not be evaluated. The reduced hindlimb length compared to body size found by Carr et al. is consistent with the reduced developmental rate and continuous growth seen in this study.

The current study found whole body concentrations of perchlorate ranging from below detection limits to 109 ppm, depending in part on exposure concentration. The wide within-dose range could be explained in that perchlorate can be evacuated from the thyroid within 48 hrs in mammals (Wolff 1998) and presumably in amphibians. Juvenile frogs often spent substantial and variable amounts of time out of water as their tails were absorbed and perchlorate could have been depurated. In comparison, bullfrogs collected

from the U.S. Army ammunition site had 1.1 to 2.6 ppm perchlorate in their tissues (Smith et al. 2001).

Wolff (1998) stated that the effects of perchlorate inhibition of iodide uptake and storage are reversible with the removal of perchlorate treatments. Some bacteria may decompose perchlorate (Herman and Frankenberger 1999) but total removal from contaminated sites may be difficult. The second phase of this study showed that iodide concentrations at least as low as 0.01 ppm can be effective in counteracting perchlorate inhibition up to concentrations of 22.9 ppm. However, during this phase mortality was nearly 100%. Most of the tadpoles entering or progressing through the climax phase were edemic, suggesting problems in water regulation; others showed signs of respiratory stress. The cause of death is unclear but these physiological problems and resulting mortality may have been due to the rapid onset of the climax phase following treatment with iodide. It is also possible that the tadpoles had passed a critical window for normal metamorphosis.

Acknowledgements.

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Christine M. Bridges and Edward E. Little

Using Semipermeable Membrane Devices (SPMDs) to Assess the Toxicity and Teratogenicity of Aquatic Amphibian Habitats

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Abstract: Environmental contamination has been suspected of being partially responsible for recent declines in amphibian populations. It is often not feasible to identify all of the compounds in an environment, nor the concentrations in which they are present. SPMDs are passive sampling devices that uptake lipophilic compounds from the environment in a manner similar to aquatic organisms. The extracts from the SPMDs, therefore, contain a composite sample of the compounds that are present in the environment. In this paper, we outline the methods from studies in which we have used extracts from SPMDs in toxicity tests on amphibian larvae. Using SPMD extracts makes it possible to establish potential links between amphibian deformities and declines and environmental contamination by lipophilic compounds.

Keywords: amphibian declines, amphibian deformity, environmental contamination, SPMD, tadpoles, UV-B radiation

Introduction

Reports of amphibian declines and deformities have increased in recent years. Many causes have been proposed (Ouellet 2000, Lannoo 2000), including contaminants (Ouellet et al. 1997, Bonin et al. 1997), ultraviolet (UV) radiation (Blaustein et al. 1997), and parasites (Johnson et al. 1999), either singly or in combinations. Analysis of water and sediment samples have revealed that many types of contaminants are often present in amphibian habitats and have the potential to trigger developmental abnormalities (Fort et al. 1999a, 1999b). However, the identity of specific contaminants present at a site and their concentrations are often uncertain, which makes it difficult to determine the influence of contaminants on the populations of organisms inhabiting the area.

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Semipermeable membrane devices (SPMDs) are integrative samplers containing lipid that accumulates lipophilic organic compounds from the environment in a manner that is similar to aquatic organisms (Huckins et al. 2002). The uptake rates of fat-soluble compounds by SPMDs can be used to define the approximate daily exposure to lipophilic compounds by aquatic organisms (Huckins et al. 2002). Thus, exposure of organisms to the range of waterborne environmental contaminants occurring in amphibian habitats can be reproduced by using SPMD extracts as toxicant solutions in bioassays (Huckins et al. 2002, Petty et al. 2000) based on the duration of deployment in the environment. Tests with extracts obtained from SPMDs offer a convenient means of screening the toxicity of lipophilic contaminants in aquatic habitats (Petty et al. 2000).

It is also possible to use SPMDs to examine the interaction between lipophilic contaminants and abiotic factors (e.g., pH, temperature, and ultraviolet (UV) radiation). For example, exposure to UV-B radiation at larval and egg stages has been suspected of negatively impacting some amphibian species directly by reducing hatching success and increasing rates of embryonic deformities (Lizana and Pedraza 1998, Blaustein et al. 1998, Broomhall et al. 2000) as well as generating adult deformities (Ankley et al. 2000). UV radiation is also known to increase the toxicity of various compounds to amphibians in aquatic habitats (Little et al. 2000). Therefore, examining the effects of SPMD extracts in tandem with UV radiation, for example, may be more environmentally realistic than studying the effects of either stressor alone. Furthermore, the size the of SPMD membrane is small enough (10 Å) to prohibit the passage of parasitic trematode cercariae and viruses (both of which have been thought to cause amphibian decline and/or deformities), so the effects generated by SPMD extracts can be attributed solely to the chemical compounds in the extract.

We have exposed tadpoles of various amphibian species native to the United States to SPMD extracts from paired contaminated and reference sites to determine whether the chemicals potentially present in either of these sites are acutely toxic. Because most toxicity and teratogenicity occurs throughout development, we also conducted chronic studies. Tadpoles were exposed to environmentally realistic concentrations of SPMD extracts throughout development until metamorphosis. In this chapter, we outline some recommended methods for using SPMDs to determine toxicity and teratogenicity of site water to amphibians using our own experiments as a guideline. We will also discuss some of the endpoints we examined as well as statistical methods used to analyze our data.

Materials and Methods

Test organisms

If possible, it is advisable to use species native to the region in which the SPMDs are deployed. For acute tests, any species can be used. For chronic studies, it is recommended that amphibians with shorter larval periods be tested (e.g., bufonids and hylids). For instance, in our studies with Pacific treefrogs (*Hyla regilla*), all tadpoles had metamorphosed within 40 d (unpub. data). Leopard frogs (*Rana pipiens*) had significantly longer larval periods (100+ days) which extended beyond the amount of extract available.

SPMD exposure and extraction

We have deployed SPMDs at several paired sites. Contaminated sites in each region were characterized by a decline in habitat quality or the presence of amphibian decline or deformity as recorded by local authorities (e.g., Minnesota Pollution Control Agency, U.S. Fish and Wildlife Agents, U.S. Forest Service, U.S. Geological Survey employees). Reference sites were similarly characterized by good habitat quality and little or no indication of amphibian decline or deformity. When possible, contaminated sites and reference sites were paired.

For each site, we placed 5 standard SPMDs (described elsewhere, [Huckins et al. 2002]) in each of three replicate stainless-steel canisters (total SPMDs per site = 15) attached to a steel cable anchored to the shore. The SPMDs were submerged at a depth of at least 1 m for approximately 30 d. We recorded water temperature at the time of deployment and retrieval because the uptake of contaminants from the water can be influenced by temperature. Similarly, notice was taken of the degree of fouling (e.g., silt, sediment, or algae gathered on the stainless-steel canisters) upon retrieval as this can hamper the degree to which site water can flow freely over the surface of the SPMDs within.

At the time of deployment a metal can containing SPMDs (hereafter, field blanks) was opened and exposed to the air at each site. These provided a control for any airborne contaminants present while the SPMDs were exposed to the air (i.e., before being placed into the water). Once the SPMDs were in the water, the cans containing the field blanks were sealed and stored at 0-4°C for the 30 d between deployment and retrieval to halt contaminant uptake (from the site air that had filled the can). During retrieval, field blanks were once again exposed to the air at the site for as long as it took to remove the SPMDs from the water and seal them in cans. After retrieval, both SPMDs and field blanks were shipped on ice and remained frozen until they were processed at the Columbia Environmental Research Center (CERC, Columbia, MO) using techniques outlined in Petty et al. (2000).

SPMDs in our studies were deployed within 1 m of one another, so extracts from each site were pooled into a single composite sample. Field exposure and control extracts were dissolved into sterile dimethylsulfoxide (DMSO) by solvent exchange from high purity hexane to a total volume of 0.5 mL for acute tests. SPMD extracts for the chronic test were diluted with sterile DMSO to obtain the approximate equivalent of a 1-d exposure (i.e., represents the amount of bioavailable residues extracted from site water by a standard SPMD in one days' time) when added to 1 L of water.

Assuming lentic conditions, an ambient temperature between 10 and 18° C, and minimal biofouling, 1-g equivalent of a standard 1-mL triolein SPMD will extract hydrophobic organic contaminants (e.g., PAHs, PCBs, organochlorines, pyrethroids) from about 0.01 to 2.0 L/g of water daily (Huckins et al. 2002). Although much greater variability exists in the uptake rate constants of aquatic organisms for the same chemicals, the values for invertebrates and fishes generally range from 0.03 to 8.0 L/d/g (Mackay et al. 1992a, 1992b, 1997). Thus, it is reasonable to expect that aliquots of SPMD extracts, which represent the daily volume of water extracted by a whole standard

SPMD, are representative of the amounts of chemicals to which aquatic organisms (e.g., tadpoles) are exposed daily.

Acute toxicity

We designed acute experiments to determine the acute toxicity of the chemicals extracted by the SPMD from each site. These were static tests conducted over 10 d using native tadpoles (collected as eggs from at least three egg masses) that had reached stage 25 (Gosner, 1960), which is prior to hindlimb development.

Test solutions were created by filling 1-L glass beakers with 1 L of well water (pH 7.8; hardness 286 mg/L $CaCO_3$; alkalinity 258 mg/L $CaCO_3$), and adding 0.5 mL of the appropriate SPMD extract treatment (hereafter, SPMD treatment) or control treatment (see below). Tadpoles were exposed to one of five SPMD concentrations, based on daily exposure equivalents of 30, 15, 7.5, 3.25, and 1.75 d (i.e., a tadpole in the 30-d treatment would be exposed in a single dose approximating a 30-d exposure). A total of five controls were used: 1) a procedural blank which controlled for any contamination occurring during laboratory assembly of the SPMDs; 2) a field blank from each site, which controlled for potential contamination during deployment; 3) reagent blanks which controlled for any solvent-related contamination that would occur during processing of samples, and consisted of all the solvents used during these processes; 4) a sterile DMSO control which controlled for the carrier solvent used in toxicity testing; and 5) a well water control.

We used UV radiation as an additional stressor in each experiment. UV radiation intensity was measured at each site using either an Optronics scanning spectroradiometer or a Macam Photometrics broadband UV meter (see Little et al. [2000] for details on recording measurements). Each experimental chamber was wrapped and covered with various combinations of plastic to achieve high (values measured at approximately 10 cm below the water's surface) and low (near zero) UV-B intensities. All tests were conducted in a solar simulator, as described by Little and Fabacher (1996). Prior to testing, the irradiance level of each experimental UV treatment was measured with a spectroradiometer at a 10 cm depth.

Groups of ten tadpoles were placed in the 1-L beakers containing the test solution. Beakers were randomly arranged under a solar simulator in a 17° C flow-through waterbath. The simulator was programmed on a 16L:8D photoperiod. UV-B lights were activated five h into the light cycle for five h to simulate midday solar intensity. Solutions were not renewed and tadpoles were not fed during the tests. Mortality was checked daily and dead animals removed. We initially constructed our tests to be conducted over 96 h, however when there was no mortality at 96 h, the tests were carried out to up to 10 d. Had we seen mortality, single daily equivalent dose as concentration, LC50s and 95% confidence intervals would have been calculated using either the Spearman-Karber method or nonlinear interpolation (Stephan 1977).

Chronic toxicity

Our experiments were each designed as a 2 × 5 complete factorial: tadpoles were exposed to one of two UV light treatments (as described above) and to one of five SPMD

treatments (i.e., the SPMD extracts from the two sites, the extracts from the field blanks at each site, and a water control) and replicated at least three times. It is also possible that non-lipophilic compounds of concern detected at a site (e.g., nitrogen-based fertilizers, pesticides, metals), may be added to the SPMD extracts. This would increase the number of treatments, but more closely reflect the exposures taking place in the environment. While it may be impossible to predict or detect the types of contaminants present in a habitat, any steps taken to increase the realism of exposures will clarify the role of the lipophilic compound in the environment relative to other contaminants.

In our studies, eggs from all pairs of frogs were pooled and 30 eggs were placed into 1-L glass beakers containing 500 mL of test solution. On day 3, hatching success and number of deformed embryos (exhibiting characteristics such as stunted growth, lordosis, and abnormal facial features) were counted and healthy viable embryos were placed back into the beakers with new test solution. On day 6, ten viable tadpoles were haphazardly selected and placed back into beakers with new test solution. On day 12, seven tadpoles were weighed (wet weight, nearest 0.1 mg) and the remaining three tadpoles were placed back into the beaker with new test solution. On day 21, tadpoles were transferred into 6-L glass chambers with 2 L of test solution. From day 0, test solutions were changed every third day. After reaching stage 25 (i.e., prior to hindlimb development, approximately day 6; [Gosner 1960]) tadpoles were fed ground fish flakes (Tetra-Min® brand) *ad libitum* at each test water change until the end of exposure. Occasionally, the supply of SPMD extract was exhausted before the larval period ended. In these instances, water changes and feeding continued as described above, and development was allowed to progress until metamorphosis.

Potential endpoints

When conducting chronic studies with tadpoles, many types of data can be collected. If exposure begins in the egg stage, hatching success can be recorded. To account for egg and embryo mortality, the number of eggs in the initial exposure should be higher than the number of individuals that will be reared through metamorphosis. No more than five individuals should be reared in a chamber containing 2 L of water, as crowded growing conditions lead to longer larval periods and unsynchronous development. When determining hatching success we began with as many as 30 eggs. At the same time as hatching success is recorded, incidence of embryonic deformities may also be observed. Most developmental abnormalities can be observed without the aid of a dissecting scope and can include things like stunted growth, edema, and spine curvature. It may also be desirable to examine each embryo more closely; however care must be taken to examine each animal so that each individual is exposed to the same degree of stress.

Growth and development are also important endpoints that can be documented in a chronic exposure. Amphibians balance between growth and development to optimize size at and time to metamorphosis. There are many advantages to a larger mass at metamorphosis and reduced larval period including greater overwintering success, increased fecundity, and shorter exposure to aquatic predators (Heyer et al. 1975, Berven 1982, Smith 1987, Semlitsch et al. 1988). Growth and development are recorded by noting the date at metamorphosis (defined as the emergence of at least one forelimb (stage 42; [Gosner 1960]), and the mass of the metamorphosed individual upon tail

resorption. Upon metamorphosis the presence of deformities (e.g., bony triangles, skin webbing, missing or additional limbs) can be also be recorded.

It is also possible to observe behavioral changes during exposure to SPMD extracts. An endpoint such as activity, which has been shown to be a good indicator of tadpole toxicity (Bridges 1997, 1999), can be measured directly in the testing vessel. Other observations can be made using the extra animals that have been removed from the experiment. Behavioral endpoints such as swimming performance and stamina, and predator avoidance can be included. It would also be possible to determine whether recovery from behavioral alterations occurs.

Statistical Analyses

Mean values derived from individuals within each replicate chamber should be used in all analyses. Analyses of variance (ANOVA) can be used to determine the effects of SPMD treatments and additional stressors on the endpoints measured. When analyzing mass at metamorphosis and the duration of the larval period, is it necessary to implement analysis of covariance (ANCOVA) using mass at metamorphosis as the covariate in the analysis for time to metamorphosis, and vice versa. This is important because these two factors are often correlated; individuals that metamorphose later will likely be larger because they have had a greater amount of time to grow. It is also important to use survival as a covariate if there is differential survival in the experimental chambers because uneven numbers of amphibians in chambers could lead to differences in growth rates. If the covariate in each model is not significant it can be removed from the model, but should always be included in initial analyses. When necessary, significant pairwise differences can be determined using a post-hoc multiple comparison test (i.e., Bonferoni, LSD, Dunnett's). Data should be transformed appropriately (e.g., arcsin square-root transformation for proportional data, log transformation for mass), after which a Shapiro-Wilk test should show the data are normally distributed. If transformations do not normalize the data, non-parametric statistical options should be explored.

Discussion

Determining whether contaminants detected at a site can elicit direct mortality is often a sisyphean task, especially in sites contaminated with complex mixtures of chemicals. SPMDs offer a unique opportunity to sample lipophilic compounds present in the environment, whether natural or anthropogenic, without necessarily having to establish their identity. Furthermore, inclusion of other abiotic factors (e.g., UV radiation) in studies offers the opportunity to determine the extent of the impact when multiple stressors are present, and increases the degree of ecological relevance.
For example, we have found higher embryonic deformity rates at our Minnesota reference site in our experiment in the absence of UV light (Fig 1), which suggests the presence of teratogenic compounds that are broken down by UV radiation. This may explain the near absence of deformities in the field at this site (Helgen et al. 2000), which is likely continuously exposed to UV radiation. Conversely, the teratogenicity of the

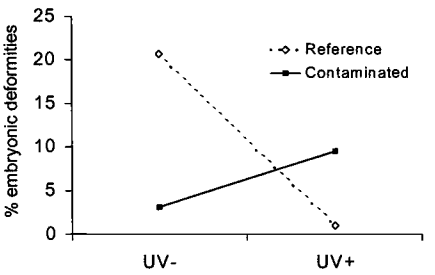

Figure 1, *Reaction norm depicting percent embryonic deformities in the reference and contaminated sites under low (UV-) and high (UV+) ultraviolet-B intensities.*

compounds at the contaminated site was enhanced by UV light, which could lead to a concomitant increase in embryonic deformities. The possibility that sunlight breaks down contaminants found in the reference site may explain why the amphibian population at this site is larger and contains fewer deformed frogs than at the contaminated site (Helgen et al. 2000). Based on our field measurements, the high UV treatment in our experiment was roughly equivalent to that which developing leopard frog embryos would be exposed in the field. In order for the contaminants at our contaminated site to not cause embryonic deformities, UV radiation would have to be absent, a situation unlikely in a natural environment.

The physical structure of the SPMDs (i.e., the size of the transient transport corridors in the membrane are ≤ 10 Å), and the chemical processes involved in processing the extracts precludes the possibility that adverse effects observed are a result of a pathogen or parasite. Using SPMD extracts allows us to examine how the potency of the compounds sequestered by SPMDs can be affected by abiotic factors such as UV radiation. Therefore, SPMD extracts can be useful in screening for the presence of unidentified toxic and teratogenic lipophilic chemicals in environments in which frog deformities are common. By employing a toxicity identification evaluation (TIE) process (i.e., fractionation of SPMD extracts and toxicity assessment of isolated compounds) it would be possible to identify the causal agent or agents responsible for the observed teratogenic effects. Such a procedure would be analytically and exposure intensive, but may ultimately provide data to determine the potential linkage between hydrophobic contaminants present in amphibian habitats and observed deformities.

In conclusion, field concentrations of contaminants are typically considerably lower than those generating direct mortality, making it important to examine the effects of sublethal levels of contaminants on endpoints such as juvenile deformities or reductions in growth and development. Determining whether contaminants detected at a site can generate amphibian mortality is often a sisyphean task, especially in sites contaminated with complex mixtures of chemicals. SPMDs provide a unique means of obtaining a composite extract of lipophilic compounds (natural and anthropogenic) present in amphibian habitats for use in toxicological evaluations without having to identify specific compounds. Furthermore, including other environmental variables (e.g., UV radiation) in the study designs offers the opportunity to determine the extent to which toxicity is

influenced by multiple stressors, and increases the degree of ecological relevance. SPMDs can thus serve as a tool to determine whether contamination is a causal factor in habitats having deformed or declining amphibian populations. Once a link between chemicals and injury to amphibians has been established, investigations can identify chemicals or mechanisms responsible.

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Methods for Testing the Combined Effects of Contamination and Hibernation on Terrestrial Amphibians

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Abstract: Much work needs to be done to improve and develop techniques for contaminant studies with post-metamorphic, terrestrial amphibians. Evaluations also should be made of the impacts of multiple stressors, because single-factor studies may underestimate environmental stress. Habitat contamination is one of the proposed causes of the global decline of amphibians. Hibernation is an annual period of natural stress when mortality can be very high. This paper describes methods for testing the combined effects of contamination and hibernation on terrestrial amphibians, and includes model species selection, culture practices, hibernation conditions, soil exposures, and food chain exposures. The guidelines provided are based on what works well with American toads (*Bufo americanus*), but should be useful for other amphibian species.

Keywords: amphibian, contaminant, hibernation, oral uptake, soil toxicity

Introduction

Many amphibian populations and species have declined or disappeared around the globe in the last several decades (Wake 1998, Houlahan et al. 2000). Important advances have been made toward understanding the effects of specific environmental variables on amphibians (Wyman 1988, Kiesecker and Blaustein 1995, deMaynadier and Hunter 1998) and in some instances the ultimate cause of population loss appears to have been determined (Berger et al. 1998, Morell 1999, Knapp and Matthews 2000). Yet, even when ultimate causality is known, there often remains the question of whether other factors or stressors are acting to make amphibians more susceptible. The combined effects of two or more stressors can be more harmful to amphibians than single stressors acting alone (Kiesecker and Blaustein 1995, Long et al. 1995). This, and the recognition that organisms are simultaneously regulated by a multitude of physical, biological, and chemical factors, should stimulate and justify a multiple stressor approach.

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Chemical contamination of the environment due to human activities could be contributing to amphibian declines (Davidson et al. 2001, Sparling et al. 2001, Hayes et al. 2002). Most amphibian ecotoxicological research has focused on the effects of aquatic pollutants on the egg and larval stages (see Sparling et al. 2000). This is likely because early lifestages are easy to obtain and work with, and may be more sensitive to contaminants (Schuytema et al. 1991). However, several cases of amphibian decline have been attributed to the loss of adults (Morell 1999, Carey et al. 2001) and older lifestage vital rates are likely disproportionately influential on population dynamics for some amphibian species (Biek et al. 2002). There are few published studies that assess the effects of environmentally realistic exposure regimes on juveniles and adults (but see Baker 1985, Oldham et al. 1997, Linder et al. 1998, Johnson et al. 1999, Laposata and Dunson 2000). Research on post-metamorphic lifestages has largely focused on physiological responses using artificial exposure routes (e.g., injections; Hilmy et al. 1986, Pramoda and Saidapur 1986). However, some suborganismal-level responses are of unknown importance to individuals and natural populations. Whole-body endpoints such as behavior, growth, survival, and reproduction should be incorporated into ecotoxicological studies because they affect fitness and are a more direct measure of contaminant impacts on populations.

Because of the overwhelming focus on young, aquatic lifestages, the published literature is also lacking in research evaluating the effects of terrestrial contamination on amphibians. Many amphibian species spend the vast majority of their lives on land and use aquatic habitats only for breeding. Terrestrial habitats are important sources of food and shelter and individuals may move hundreds or thousands of meters across landscapes during seasonal migrations. Amphibians can be exposed to terrestrial contaminants by any one of three main routes: oral, dermal, and pulmonary. The relative importance of each route will vary over space and time, and also by contaminant and species. Oral uptake has been explored in a few studies in which amphibians were fed contaminated prey items and monitored for bioaccumulation and survival (Hall and Swineford 1979, Nebeker et al. 1995, Linder et al. 1998, Johnson et al. 1999, Huang and Karasov 2000). Dermal exposure (Baker 1985, Oldham et al. 1997, Johnson et al. 1999, Laposata and Dunson 2000, Hatch et al. 2001, Marco et al. 2001), including the avoidance of contaminated substrate (Laposata and Dunson 2000, Hatch et al. 2001, Marco et al. 2001), has received some attention. The effect of inhaled contaminants (pulmonary pathway) on amphibian populations is unknown.

While contaminant stress can often be attributed to human activities, hibernation stress is a natural and annual occurrence experienced by amphibians. During hibernation, individuals rely on a limited energy supply while occupying a severe environment. Besides the risk of starvation, amphibians may face temperatures near the low end of their tolerance range, oxygen depletion, predation, and desiccation. Natural mortality can be as high as 100% in some populations, but there is insufficient data to suggest a normal death rate (but see Resetarits 1986, van Gelder et al. 1986, Sinsch 1988). Organisms simultaneously stressed by other environmental factors (e.g., contaminants) may be at greater risk of winter mortality or lower body condition at emergence. Because many populations appear to be declining due to a loss of adults, the role of hibernation in conjunction with other stressors should be explored. Unfortunately, there is a paucity of references that include laboratory hibernation procedures (but see Brenner 1969, National

Research Council 1974, Jorgensen 1984). I am unaware of any published studies that have assessed the combined effects of contaminant and hibernation stress on amphibians.

General Guidelines for Research on Terrestrial Amphibians

Standardized and accepted protocols for amphibian toxicity testing are limited to the ASTM Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians (E 729-96), the ASTM Standard Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians (E 1192-97), the ASTM Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (Fetax) (E 1439-98), the ASTM Standard Guide for Behavioral Testing in Aquatic Toxicology (E 1604-94), and the USEPA Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians (EPA-660/3-75-009), all of which are for aqueous exposures. Protocols for dermal (e.g., ASTM Standard Test Method for Determining Subchronic Dermal Toxicity (E 1103-96)) and oral (e.g., ASTM Standard Test Method for Chronic Oral Toxicity Study in Rats (E 1619-95)) uptake are few in number and of limited applicability to amphibians. What follows are some guidelines for conducting toxicity tests with post-metamorphic anurans and salamanders, including information on how to hibernate terrestrial species during or following exposure. Of the three main routes of uptake, only the dermal and oral routes are discussed. These guidelines are based on research I have conducted on the effects of dermal (2 studies) and oral (1 study) cadmium exposure on hibernating American toads (*Bufo americanus*), the results of which are presently being prepared for journal publication.

Model Species Selection

Species differences in life history characteristics, physiology, and behavior can affect the experimental design and research results. Hence, the species selected should be appropriate for the questions being addressed. For example, researchers interested in investigating the uptake of contaminants from soil should choose fossorial species such as toads, leopard frogs, and spadefoots. A soil exposure with aquatic species such as bullfrogs (*Rana catesbeiana*) and green frogs (*Rana clamitans*) or arboreal species such as gray treefrogs (*Hyla versicolor*) and green treefrogs (*Hyla cinerea*) may be less realistic and valuable. When possible, more than one species should be tested simultaneously because of the very real possibility of species differences and also to broaden the applicability of the results. Although *Xenopus laevis* has been advocated as a good model species for egg and larval studies (e.g., Fort et al. 1999), their restriction to aquatic habitats as adults makes them inappropriate for terrestrial toxicology studies.

It is also important to select a species that does well under laboratory conditions and is easy to handle and manipulate. Many amphibians thrive in aquaria with moist sphagnum moss; moist sand or soil, and refuges such as inverted clay pots, may also be added. The ability of the investigator to obtain food that the species will eat is certainly crucial; fortunately, amphibians eat most live insect prey that are smaller than their gape size. Juveniles and adults readily consume fruit flies (*Drosophila* sp.), small crickets (*Acheta* sp.), mealworms (*Tenebrio* sp.), pill bugs (*Armadillidium* sp.), and lepidoptera larvae.

The diet should be periodically sprinkled with a powder containing calcium and vitamin D when working with metamorphs or when keeping amphibians in the laboratory for more than a few weeks. Exposure to ultraviolet light may be necessary for normal vitamin synthesis and calcium metabolism. Trembling can be an indication of malnourishment and weak bones.

Culture Practices for Study Organisms

Post-metamorphic amphibians may be collected in the field, bought from biological suppliers, or reared by the investigator from tadpoles or eggs. Each has its advantages and disadvantages and the investigator must decide which option is most feasible. Rearing animals from an early lifestage is likely the best route because the age and history of the organisms is known, chemical contamination and other rearing conditions can be controlled, and large numbers of organisms can be produced.

Eggs should be transported to a laboratory and added to plastic or glass containers and allowed to hatch. Organisms should be gradually acclimated to the rearing water if it differs notably from the collection site water in variables such as pH and hardness. Once larvae are free-swimming, they can be reared indoors or outdoors at a density ≤ 1 tadpole/L. Ranges of natural densities for many species can be found in the published literature. Amphibians raised outdoors tend to metamorphose at a larger size, perhaps in part due to a better diet and warmer temperatures. Polyethylene cattle tanks that hold 1000-2000 L of water can be manipulated to simulate natural ponds and have been very successful in producing metamorphs (see Wilbur 1997). Tanks should be placed on grass in an open area with little shading to minimize temperature differences. Several weeks before larvae will be added, the tanks should be filled with water from an uncontaminated source. If a nearby pond is used, the water should be filtered to prevent the introduction of predators. After the tanks are filled, 1 kg dry leaf litter or grass/1000 L water should be added and allowed to settle. Once most of the leaves are at the bottom, aliquots of plankton and periphyton should be added on several different days. The establishment of zooplankton is crucial for preventing bacterial fouling of the water and for rearing carnivorous species such as salamanders. A fiberglass screen lid can be placed on top of each tank to deter predators and competitors. Metamorphs can be placed in plastic shoeboxes or glass aquaria containing a moist substrate such as paper towels, sand, soil, or sphagnum moss. The substrate should be changed out regularly to prevent fouling and rearing density should be low enough that individuals do not cause harm to each other.

Hibernation Conditions

The selection of an appropriate hibernation temperature is important for survival and metabolism. Amphibians vary in supercooling temperature and only a few North American species tolerate freezing through the production of cyroprotectants (e.g., *Rana sylvatica, Hyla versicolor, Pseudacris crucifer*; Schmid 1982, Storey and Storey 1986). American toads will not survive at temperatures below $-2^{\circ}C$ (Storey and Storey 1986). If the hibernation temperature is too warm, metabolism is higher and energy consumption subsequently increases, thus enhancing the possibility of starvation. Most temperate species should probably be hibernated between 0 and $5^{\circ}C$. Standard hibernation

temperatures should be established to enhance consistency among studies and because contaminant toxicity and metabolism may vary with temperature (Janssen and Bergema 1991). The temperature can be kept constant or on a fluctuating cycle. If the investigator simply wants to keep the animals cold, a constant temperature is adequate. One potential disadvantage of fluctuating temperatures is periodic emergence by individuals to the surface in response to relatively warmer temperatures. Such movement could result in unnaturally high energy-use and reduced burial time.

Before placing amphibians into hibernation, they should be gradually acclimated to a temperature between 17 and 21°C. To initiate hibernation, the study organisms should be transferred to an environmental chamber set at approximately 7°C. Amphibians tolerate this rapid drop in temperature very well and American toads will burrow into the soil in a matter of minutes or hours to escape the cold air. The experimental units and the study organisms should be the same temperature when hibernation begins so that there is a temperature difference between the substrate and the air that induces burrowing. From 7°C, the temperature can be dropped by 1°C every 2-3 days until the target temperature is reached. When the investigator is ready to end hibernation, this process is simply reversed. American toads generally take less than 48 hours to emerge from the soil once the temperature is increased just a few degrees above the hibernation temperature.

A few weeks before hibernation begins, feeding rate should gradually decrease. Feeding should be discontinued at least five days prior to hibernation to allow gut clearance and avoid the possibility of death due to intestinal infection. While some species, lifestages, or individuals defecate every few days, others may take over a week.

The role of photoperiod in hibernation initiation and emergence is unclear but may be of some importance. Terrestrial hibernation studies should take place in complete darkness to simulate natural underground conditions and avoid premature emergence by the study organisms. This is particularly important when some individuals fail to burrow. A headlamp or other light source may be used to monitor the amphibians during hibernation and does not appear to be disturbing (personal observation).

The following suggestions are based on what I have found to work well for the American toad. Investigators working with other species or substrates should conduct pilot studies to determine whether different strategies are needed. Terrestrial hibernators should be overwintered in soil or traction sand. Sphagnum moss may be mixed in to create more structure and reduce bacterial problems. Amphibians will not burrow if the substrate is too compacted, coarse (i.e., chunky), or wet. The substrate should be moist (75-175% of water holding capacity, which is the amount of water held by soil at 1/3 bar pressure) to prevent desiccation and maintained at a constant mass throughout hibernation by misting periodically with a spray bottle of deionized water. The frequency with which this needs to be done depends on the type of experimental unit and relative humidity of the hibernation chamber. This main substrate layer may be placed over drainage material such as clean gravel or coarse sand. However, a drainage layer may not be necessary; I have never observed water at the bottom of hibernation chambers, and American toad survival was similar among those hibernated with and without gravel. A top layer of sphagnum moss or leaves may also be added for species that naturally hibernate directly under leaf litter. Researchers investigating the effects of soil contamination might want to minimize the number of non-soil layers in order to better account for and control contaminant partitioning. The substrate that the amphibian is

expected to hibernate in (e.g., soil) should be at least twice as deep as that required for complete burial. Eight to 10 cm is adequate for anurans that weigh less than 25 g. A depth of 2-3 cm should be sufficient for the gravel and litter layers. The diameter of the experimental unit should be at least twice the snout-vent length (SVL) of the organism.

Amphibians should be hibernated in individual containers to prevent possible negative effects from fouling if an organism dies. Despite the cool temperatures, toads in hibernation will decompose quickly and fungi can grow not only on the toads but also throughout the soil layer. Weekly visual observations should be made to determine the location (i.e., buried, partially buried, or unburied) and condition of the organisms, and more frequent checks may be necessary if amphibians are hibernated in groups.

One-liter plastic freezer containers work well as the experimental unit, but larger or smaller containers can be used depending on the size of the study species. The lid of the experimental unit should have air holes or consist of a screen. A solid lid with holes reduces the need for misting, but moisture accumulates on the container sides and fungi may become a problem if conditions are too wet. A screen lid allows misting and observation without disturbance of the experimental unit, but evaporation increases significantly and the substrate will need to be misted several times weekly.

The timing and duration of hibernation for free-ranging amphibians varies by species, geographic location, age, and gender (Kelleher and Tester 1969, Resetarits 1986). Amphibians undergo many physiological and behavioral changes before hibernation, which appear to be endogenous and driven at least in part by hormones (Pinder et al. 1992). These changes include the reduction or cessation of feeding, alteration of enzyme activity, and adjustments to cellular proteins and membranes (Pinder et al. 1992). Hence, the normal response to and readiness for hibernation may be altered if hibernation does not take place when it would naturally. Amphibians obtained from known field sites should be hibernated when the natural populations are observed to overwinter. An abnormally short or long hibernation duration may bias results.

Soil Exposures

Free-ranging amphibians may be dermally exposed to contaminants whenever they are in contact with a contaminated medium (e.g., vegetation, soil, water). Soil exposure can occur in terrestrial species throughout the year, but is likely to be the most prolonged and consistent during winter when amphibians are sedentary. Individuals that actually burrow into the ground may be in close contact with contaminants because their entire body is surrounded by soil. Dermal permeation appears to be determined by the physiochemical properties of permeants and lipophilic compounds may penetrate more easily and quickly than polar structures (Hostýnek 2001). Short-term soil exposures with active study organisms have been conducted (Baker 1985, Oldham et al. 1997, Johnson et al. 1999, Laposata and Dunson 2000, Hatch et al. 2001), but what follows are guidelines for hibernation exposures using spiked soils.

Investigators may use artificial, formulated soil or natural soil from a field site. The soil should be characterized for at least the following: particle size distribution, pH, organic carbon, cation exchange capacity, and water holding capacity. These properties can affect the absorption of compounds to soil and toxicant bioavailability (Alloway 1995). Sieving dried, pulverized soil will make the soil more uniform and enhance the

spatial distribution of the contaminant if the soil is to be spiked. However, care should be taken to minimize the disturbance of soil integrity (i.e., microenvironments, gradients, biological and physicochemical processes) and soil should not be sieved too finely (use of sieves ≥1 mm recommended). Few guidelines exist on issues such as spiking, mixing, and equilibration time. A dry-spike method followed by periodic mixing with an electric stainless steel augur (at least 4 times, 3-5 minutes each time) and an equilibration period of at least two weeks has produced satisfactory results regarding homogeneity of distribution, and is likely adequate for at least fast-state equilibrium of metals. However, a longer equilibration time is recommended to reduce the chances of overestimated bioavailability. Time to equilibrium will vary by chemical and soil type. Unfortunately, there is little information on equilibration times and they have not been standardized. Study animals can be placed on the soil surface just before hibernation initiation and allowed to burrow themselves. However, a tunnel may have to be made for some species of salamanders and anurans that only use pre-existing animal burrows. Investigators should refer to ASTM Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests with the Lumbricid Earthworm *Eisenia fetida* (E 1676-97) and ASTM Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing (E 1391-02) for additional guidance on methodology.

Possible endpoints for dermal exposure hibernation studies include survival, change in mass, body lipid levels, percent of time spent burrowed, time to initiate burrowing once hibernation begins, contaminant uptake, and post-hibernation locomotory and feeding performance. Hibernating amphibians should lose mass because they are utilizing existing energy stores, and body fat may be a sensitive measure of metabolic costs and body condition. If soil contaminants are detected and perceived as a threat, individuals may reduce their exposure by spending less time buried or delaying burrowing when initially placed in hibernation. Contaminant uptake during hibernation may affect the ability of amphibians to move and feed, behaviors that are important for growth and survival. I have found that whole body cadmium burdens in juvenile American toads significantly increase with soil cadmium concentration at environmentally relevant levels (unpublished data). Several published studies may be referenced for assessing body lipids (Scott and Fore 1995, Álvarez and Nicieza 2002) and locomotory (Goater et al. 1993, Tejedo et al. 2000, Álvarez and Nicieza 2002) and feeding (Goater 1994, Scott and Fore 1995, Hatch et al. 2001) performance.

Food Chain Exposures

The prey species selected should be readily available and one that the amphibians are accustomed to eating. Amphibians should be fed either on an *ad libitum* or percent body weight basis to minimize confounding factors. They may be fed by hand if trained to do so, or allowed to self-feed. In the case of self-feeding, the substrate in the experimental unit should not allow the opportunity for the prey to escape or avoid detection.

Investigators may use prey items that they contaminate themselves or obtain prey from contaminated field sites. The former is advantageous because there is some control of the quantity and type of contamination the prey receives, while the latter may be more realistic for site-specific studies. Two main options exist for controlled dosing of prey: 1)

injection of prey, 2) uptake through the prey's diet. Once the investigator becomes proficient with a needle, injections can be a rapid way to contaminate prey (Garside et al. 2000, Kostaropoulos et al. 2001). Organisms as small as fruit flies have been successfully injected, so size is not necessarily a limitation as long as the techniques exist. However, the exoskeleton or dermis of some species is not always conducive to injections and contaminant loss may result from leakage (Huang and Karasov 2000). Exposing prey through the diet is a more hands-off approach, but heterogeneity of contamination among prey may be a problem due to selective feeding or shedding of the exoskeleton. There will also be variation among species and individuals in how long it takes for the desired whole body contaminant level to be reached. Pilot studies should be conducted to determine this and the appropriate concentrations at which to dose the prey diet. Hall and Swineford (1979), Nebeker et al. (1995), Linder et al. (1998), and Johnson et al. (1999) describe techniques for dosing a variety of diets.

The following suggestions for contaminating prey via the diet are based on what works well for mealworms (*T. molitor*). This framework may be useful for other species that have similar feeding habits (e.g., crickets). Plastic or glass cups should be prepared that contain a homogenous, dry mixture of the prey's diet; cornmeal, bran, and cricket food are all good possibilities. The diet can be sieved to remove coarse materials and enhance the distribution of the contaminant. Once the diet is dry-spiked, it should be well-mixed (for samples of less than 200 g this is easily done by hand with a small plastic spatula) and allowed to dry. A 1:2 ratio by weight of dosing solution:diet works well. The spiked diet should be subsampled to determine contaminant distribution. A known mass of prey can then be added and allowed to feed for either a set amount of time or until the desired whole body concentration has been achieved.

Amphibians used for the feeding trials should be kept individually in glass or plastic containers with air holes and a moist substrate. American toads do well in 1.5 L plastic containers with a small tray of wet sand. Feedings should occur at least 2-3 times per week and the amount of uneaten prey recorded. If a radiolabeled contaminant is used, prey for each individual should be weighed and counted for radiation before being fed to the amphibians so that the exact amount of contaminant consumed is known. Fecal pellets can also be collected and analyzed, but should be considered a conservative measure of excretion. Feeding duration may range from a single feeding to several months, depending on the research objectives and realistic exposure regimes.

Feeding study endpoints may include survival, change in mass, SVL, and tibia, body lipid levels, contaminant uptake, contaminant excretion, avoidance of prey, and locomotory performance. If the prey are considered noxious, amphibians may refuse food or reduce consumption. Growth during the feeding study, mass loss during hibernation, survival, and contaminant body burden may be affected by the amount of contamination in the diet. Normal locomotion may be altered by a contaminant body burden or treatment effects on growth. I have found that the whole body cadmium burdens of American toads fed contaminated mealworms increased significantly with diet treatment level at environmentally relevant concentrations (unpublished data). Hibernation survival was notably reduced in the cadmium treatments relative to the controls (unpublished data).

Considerations and Discussion

One problem that can arise in contaminated soil hibernation studies is the failure of organisms to remain completely buried throughout the study duration. I have observed both control and treatment toads to unbury themselves shortly after hibernation commencement and remain partially buried for the remainder of the study. These individuals likely do not receive the full nominal exposure and uptake may be less than it would be in natural hibernation conditions. The ventral pelvic area is the primary region for environmental water absorption for some species (McClanahan and Baldwin 1969, Shoemaker et al. 1992), so it is likely very important that the pelvis be in direct contact with the soil when attempting to simulate a natural exposure. If a subterranean hibernator is used, the whole body should be buried throughout hibernation so there is the opportunity for uptake across the entire dermal surface. This would likely require the elimination of any space between the surface of the soil and the lid of the hibernation container. Two possible ways to achieve this are: 1) fill the container partially with soil, add the amphibian and allow natural burial, then fill the container to the top, 2) bury the amphibian and fill the container. However, the impacts of these methods on amphibians need to be tested. A third and more sophisticated option would be to create a thermal gradient within each container that would induce the toads to remain buried. Free-ranging toads stay below the frost line (Breckenridge and Tester 1961, Tester and Breckenridge 1964), so a surface temperature near 0°C and a subsurface temperature a few degrees warmer should be adequate.

The maintenance of soil moisture is another problem of soil exposure studies. Mesh lids facilitate the observation of amphibians and allow air circulation. However, soil moisture loss is greater than it would be if a solid lid with holes was used. In order to compensate for this loss, regular mistings can be performed in which the soil surface becomes very wet. The effects of this on the dynamics of contaminants in the soil should be investigated, particularly if it results in a loss of homogeneity and vertical stratification. A top layer of sphagnum moss to maintain soil moisture and minimize the direct impact of spraying on the soil may be used. However, the moss is very quick to dry out if there is much airflow. For this reason, and the possibility that organisms would hibernate in the moss or the contaminant would partition into the moss, I would not recommend using any type of surface cover above the soil for subterranean hibernators.

Food chain studies should incorporate radiolabelled chemicals and frequent sampling to track contaminant dynamics and uptake. I selected mealworms because they are easy to obtain and handle, their food is easily dosed, and American toads eat them readily. Unfortunately, the periodic loss of the contaminant by shedding the skin reduces total uptake and contributes to the heterogeneity of whole body concentrations (S. James, unpublished data). A species with less frequent molts may be a better choice and possible options include lepidoptera larvae, worms, and crickets. However, crickets are difficult to work with because of their propensity to hop. Feeding studies should also be of realistic duration using prey with environmentally relevant contaminant levels. Studies that involve single feedings or extraordinarily high concentrations are of questionable use to managers and scientists assessing the hazards of oral uptake. Gradual consumption of contaminants over time is a more likely scenario. The amount, duration,

and medium (e.g., prey species) of exposure could affect contaminant distribution and toxicity in amphibians (Harrison and Klaverkamp 1989, Huang and Karasov 2000).

The amphibian ecotoxicology literature is notably lacking in studies that test terrestrial lifestages and multiple stressors. Additional research is needed to fill this large gap and ensure that older amphibians are adequately protected. The development of standards for terrestrial amphibian testing would be instrumental in promoting such research and reducing inter-laboratory variability in methodology. Assessments that do not include data on both aquatic and terrestrial amphibian lifestages are incomplete and may underestimate exposure, fitness costs, and changes in population dynamics. However, much more experimentation needs to be done before aqueous and terrestrial data can be weighted. Any available information on the combined effects of contaminants and other relevant environmental stressors should be included in assessments. A multiple stressor approach is more realistic and may produce very different results than the more typical and traditional toxicity tests. Although terrestrial, multi-factor studies require more space and resources, and it can be difficult to obtain post-metamorphs, such studies are not too difficult to perform if appropriate model systems are selected. Testing should be conducted on a multitude of contaminants, soil types, and species of amphibians and their prey, and the methods described here can be duplicated and modified for such research. While components of my methods may be useful guidelines, much work remains to be done to improve and develop techniques.

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Greg Linder[1]

Integrated Field and Laboratory Tests to Evaluate Effects of Metals-Impacted Wetlands on Amphibians: A Case Study from Montana

Reference: Linder, G., **"Integrated Field and Laboratory Tests to Evaluate Effects of Metals-Impacted Wetlands on Amphibians: A Case Study from Montana,"** *Multiple Stressor Effects in Relation to Declining Amphibian Populations, ASTM STP 1443,* G. Linder, S. Krest, D. Sparling, and E. Little, Eds., ASTM International, West Conshohocken, PA, 2003.

Abstract: Mining activities frequently impact wildlife habitats, and a wide range of habitats may require evaluations of the linkages between wildlife and environmental stressors common to mining activities (e.g., physical alteration of habitat, releases of chemicals such as metals and other inorganic constituents as part of the mining operation). Wetlands, for example, are frequently impacted by mining activities. Within an ecological assessment for a wetland, toxicity evaluations for representative species may be advantageous to the site evaluation, since these species could be exposed to complex chemical mixtures potentially released from the site. Amphibian species common to these transition zones between terrestrial and aquatic habitats are one key biological indicator of exposure, and integrated approaches which involve both field and laboratory methods focused on amphibians are critical to the assessment process. The laboratory and field evaluations of a wetland in western Montana illustrates the integrated approach to risk assessment and causal analysis. Here, amphibians were used to evaluate the potential toxicity associated with heavy metal-laden sediments deposited in a reservoir. Field and laboratory methods were applied to a toxicity assessment for metals characteristic of mine tailings to reduce potential "lab to field" extrapolation errors and provide adaptive management programs with critical site-specific information targeted on remediation.

Keywords: amphibians, wetlands, mining impacts, metals, in situ toxicity tests

Introduction

Land-use practices ranging from mining, forestry, and agriculture to intensive urban development may impact wetlands. Mining activities, for example, frequently impact wildlife habitats, and require an evaluation of a wide range of habitat types to understand the relationships between fish and wildlife health and environmental stressors common to mining activities (e.g., physical alteration of habitat, releases of chemicals such as metals

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and other inorganic constituents as part of the mining operation). Amphibians frequent these transition zones between terrestrial and aquatic habitats and consequently face dramatically changing exposure conditions throughout their life histories. Anthropogenic impacts on wetlands and the biota dependent upon these habitats such as amphibians undoubtedly act as one of the stressors contributing to the retraction and decline of amphibian populations (Baringa 1990, Corn 1999). Given their dependence on wetlands, some amphibian species may be adversely affected directly by habitat alteration or destruction, and by nonpoint source run-off and the accumulation of sediments and environmental chemicals that are associated with the displaced matrix. Physical and chemical alterations of habitat may also be associated directly or indirectly with pathogens (see Cary and Bryant 1995, Cranshaw 1999) and the increasingly common world-wide observations of disease and malformations in amphibians (e.g., Cummins 1987, Ouellet et al. 1997, Cranshaw 1999). Moreover, amphibians are key biological indicators of a wetland's status and may be critical for evaluating wetland communities presumably impacted by chemical stressors.

Toxicity evaluations for representative amphibians may be advantageous to an ecological assessment of a wetland, since these species could be exposed to the complex chemical mixtures potentially released from the site and will provide an indicator of exposure. Integrated field and laboratory studies help reduce "lab-to-field" extrapolation error, which may confound evaluations of risks and the development of adapative resource management plans (Walters 1986, Lemly et al. 1999). Field and laboratory work completed as part of a preliminary evaluation for a wetland in western Montana illustrates the integrated approach to risk assessment and causal analysis relative to the adverse effects potentially associated with exposures to mining-related stressors, particularly metals in water and sediments.

Case Study Background

Milltown Reservoir is located on the Clark Fork River in western Montana, six miles east of Missoula, Montana (Fig. 1). The reservoir was formed in 1907 following the construction of a hydroelectric facility located on the Clark Fork River immediately downstream from its confluence with the Blackfoot River (Fig. 2). Since the dam was completed over 90 years ago, upstream mining activities on the Clark Fork River have contributed a large volume of heavy metal-laden sediment to the reservoir where wetland habitats have been formed. Milltown Reservoir wetland (MRW) was initially identified under CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act, or "Superfund") in 1981 after community well-water samples were found to have arsenic levels that ranged from 0.22 to 0.51 mg/L. Although recently revised, in 1981 arsenic criterion for potable water supplies suggested that concentrations not exceed 0.05 mg/L. In addition to concerns focused on ecological effects associated with arsenic in groundwater and surface water, the impact of contaminated sediments on the wetlands was unclear. Hence, these field and laboratory investigations were designed to evaluate the extent of contamination and its impact on the indigenous wildlife and vegetation characteristic of MRW. As part of the wetland evaluation, amphibians were considered biological indicators and were the target of preliminary field and laboratory screening

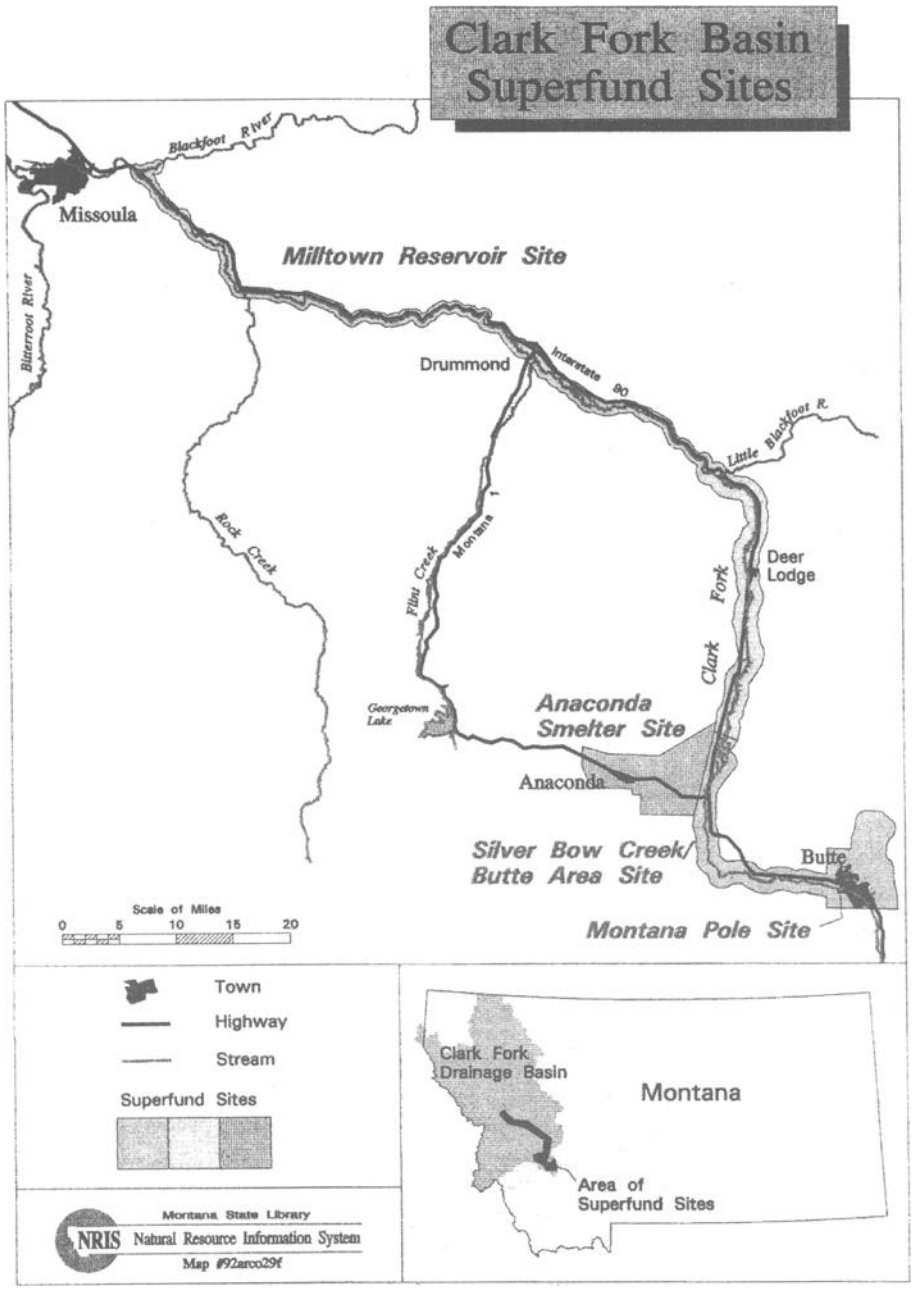


Fig. 1--*Clark Fork River watershed with Milltown Reservoir Wetlands at the western terminus of the Clark Fork River valley, approximately 6 miles east of Missoula, Montana.*

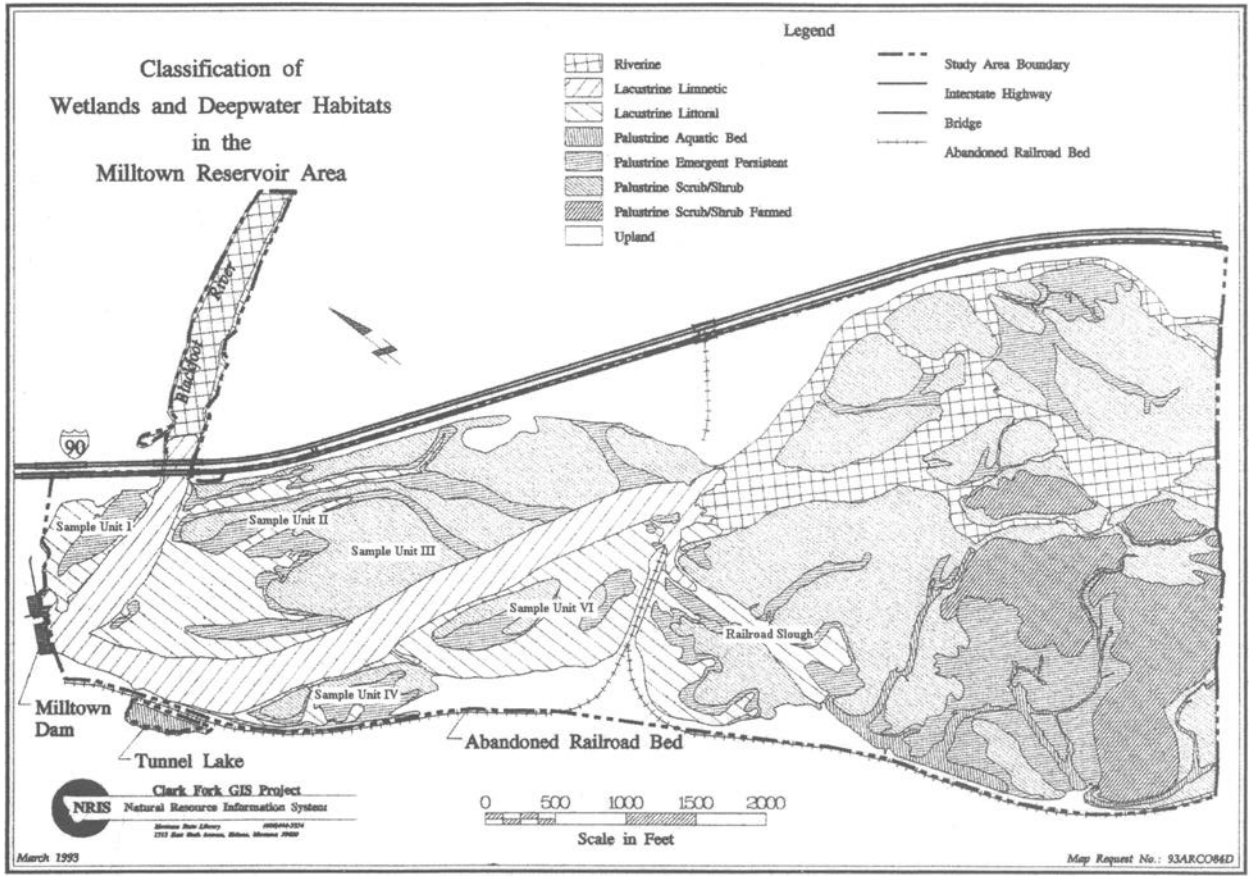


Fig. 2--*Milltown Reservoir Wetlands (MRW) were evaluated through integrated field and laboratory studies using amphibians which are summarized in this paper.*

tests reported in this case study. Arsenic and heavy metals including cadmium, copper, lead, and zinc, were considered the chemical stressors of potential concern in the baseline ecological assessment for the wetlands at Milltown Reservoir (see Linder et al. 1994, Pascoe et al. 1994).

Methods and Materials

Laboratory Toxicity Evaluations

For laboratory testing, a modified Frog Embryo Teratogenicity Assay: *Xenopus* (FETAX), as detailed in the ASTM standard guide (E1439), was used to evaluate surface water grab samples collected at various emergent zone habitats in the Milltown wetlands, including in situ test locations. Additionally, in parallel laboratory toxicity tests, selected metals were also evaluated in single-compound and defined metal-mixture exposures (Linder et al. 1991). In the laboratory, short-term, 96-hour tests were completed through static-renewal exposures in 60 x 15 mm Petri dishes which contained 15 ml control (using well water as the control or diluent) or toxicant solution. Renewals occurred every 24 hours during the 96-hour test. Initially, control and toxicant exposure solutions were dispensed into each Petri dish, then covered and transferred to an environmental chamber (22 ± 2°C) for the duration of the 96-hour exposure. For each renewal, fresh control and exposure solutions were prepared. Toxicant dilution series were designed to yield exposure concentrations that would generate data sufficient for calculations of EC_{50} and LC_{50} endpoints (median effective and median lethal concentrations, respectively). All tests were set up in triplicate with five concentrations plus controls in each of the replicates. Survival data were gathered at the end of 96 hours and chronic effects data were collected coincident with toxicity data. Chronic effects data reflected gross terata (e.g., scoliosis, lordosis, kyphosis and growth reduction) expressed upon termination of the laboratory tests (see Linder et al. 1991).

Field Investigations

During the preliminary field season, screening tests were completed in randomly selected emergent zones at Milltown Reservoir (see Linder et al. 1994 for study design details). In situ evaluations using amphibians were carried out with field-collected eggs and early embryos of *Rana catesbeiana*, which were collected from Cabell Marsh at Finley National Wildlife Refuge located south of Corvallis, Oregon, then transported on ice overnight to MRW where testing was initiated. Time between field collection and test initiation was less than 24 hours. No adverse effect on test performance was noted following observations of embryos held under relatively stable environmental conditions (room temperature incubation) following transit. Preliminary water quality measurements at MRW suggested that no water quality condition should have directly affected in situ test results (Linder et al. 1991, 1994, Pascoe et al. 1994).

In situ toxicity testing with amphibians complemented laboratory tests to reduce the potential lab-to-field extrapolation error. Unlike laboratory methods, in situ tests follow less rigid guidelines that allow for various site-specific contingencies implicit to field testing. While each wetland evaluation is independently designed, the in situ methods

outlined below reflect a testing framework amenable to field conditions at any wetland site. These general considerations were followed during the preliminary field season at MRW.

Conducting Field Tests with Amphibian Eggs and Early Embryos

Site history--Prior to beginning any in situ amphibian testing, site history, toxicity and chemical information presently available should be analyzed. Additionally, any comparative toxicity information relevant to the site-specific toxicity assessment should be collected. Information that identifies spatial or temporal variables should also be incorporated into the initial site work plans.

Life-stage considerations--In these studies, in situ exposures for amphibians were designed with critical early life stages in mind. While later stages in metamorphosis are clearly critical and could easily be tested, the in situ amphibian toxicity test primarily evaluates the first 4 to 10 days post-fertilization. Recognizing the temperature dependency of normal development (Moore 1939, Douglas 1948, McLaren 1965, Licht 1971, McLaren and Cooley 1972, Bradford 1990), the measurement endpoint used to define test termination is the developmental stage rather than a strictly defined exposure period. The significance of a reference or site-equivalent exposure cannot be ignored since stage-specific endpoints in reference locations determine the comparative basis for evaluating site-specific effects. Also, reference locations are critical for site evaluations, since habitat interactions (particularly for in situ methods) and interspecies variability are unavoidable and must be considered on a site-by-site basis.

Habitat considerations--Initially, preliminary water quality characterization for the sites to be tested should be completed in the field. At a minimum, these measures should include water temperature, water hardness, alkalinity, dissolved oxygen, ammonia, conductivity, and salinity. Samples may also be collected for a more complete laboratory analysis, if desired. The extent to which laboratory characterizations of water quality or contaminant concentrations should be completed will be determined in part by preliminary activities supporting these integrated field and laboratory studies (e.g., site history and contaminants presumed present). If possible, sediment samples should be collected, particularly if the in situ exposure chambers provide exposures to sediments directly as well as via the water column. Routine water chemistries should be done prior to initiation of the actual in situ testing, since confounding water quality measures may obscure contaminant effects. If water quality conditions do not preclude a successful start of a field test, then in situ exposures should be initiated.

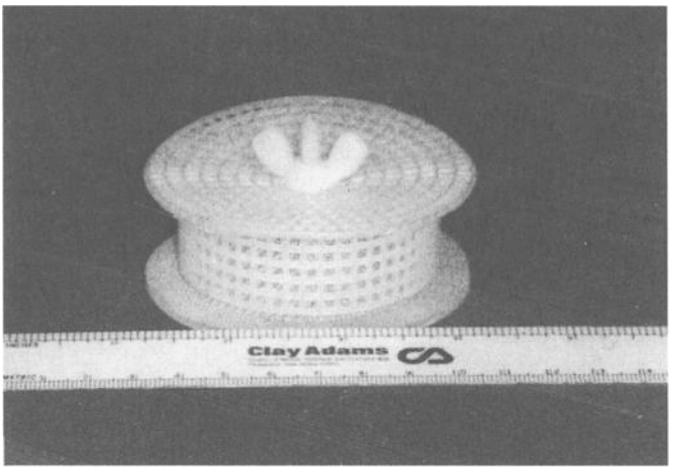
Test organisms--To initiate in situ exposures, fertilized eggs or early embryos from either commercial sources or pristine habitats must be available. If possible, local species may be collected, but the problems of quantity and quality of test organisms must be considered prior to their use in testing. Regardless of the source, to assure the quality of test organisms, reference toxicants should be evaluated using the test species of choice. Ideally, these determinations should be completed in laboratory settings (mobile or fixed), and temperature and water quality measures should be controlled according to

routine laboratory testing guidelines. The fertilized eggs or early embryos should be visually inspected, then sorted to assure high viability, then placed into exposure cages following on-site temperature acclimation. For in situ testing, exposure cages should be constructed of relatively inert materials as specified in ASTM standard guide for conducting field bioassays (E 2122). In these studies exposure cages were made of polypropylene and teflon mesh (Linder et al. 1991). On-site temperature acclimation may be accomplished by placing the shipping container used to transport the eggs and early embryos (e.g., plastic bag) directly in the water to be tested until ambient and shipping container temperatures are within 1 - 2°C. Ideally, water quality conditions in the shipping container should approximate those ambient conditions measured earlier in preliminary field activities.

Exposure--Once temperature equilibration is achieved, test organisms should be transferred from their shipping container to the exposure cages by pipeting. Transfer should be as gentle as possible, and may be expedited by using a plastic tissue culture pipet. When adequate numbers of test organisms have been placed into the exposure cage (preferably 25, but no less than 10), the exposure cage should be secured to prohibit loss of eggs and early embryos (Fig. 3). The entire exposure cage should be placed into the test matrix (e.g., sediment and water column) at on-site locations, then secured with stainless steel stakes or other restraints. Exposure cages should then be allowed to track environmental conditions without interference. To assure adequate sample sizes for comparisons between reference and contaminated sites, a minimum of 4 to 6 exposure cages should be placed on-site in each sample unit being evaluated. The number of exposure cages depends upon the spatial characteristics of the site, the heterogeneity presented by the site, and the time and resources available for the effort. Preliminary field screening may be completed with fewer exposure cages being placed in the field, but statistical interpretations become restricted owing to the limited replicates.

Routine measurements--Temperature should be monitored with recording or maximum/minimum thermometers and periodic water quality measures should be taken. Water quality measures may be taken in the field and are not considered laboratory-dependent unless site work plans so specify. Twice daily inspections of the exposure cages are recommended, preferably early morning and late evening, unless water levels change dramatically as in reservoir settings. When possible ancillary field work (e.g., wetland field surveys; Lemly et al. 1999) should be completed to complement the toxicity assessment.

Endpoints--At termination, all test organisms should be saved for future reference, and if possible, laboratory work should be completed for full measures of teratogenic endpoints not readily accessible in the field. At a minimum, the field data should yield mortality data. Embryos can be saved in histological solutions (e.g., formalin, Bouin's) for future reference or laboratory study. In the field, preparations of MS-222 (tricaine methane sulfonate) may be used as chemical restraint, if nearby facilities are used for reading test endpoints. Endpoints readily measured in mobile facilities or in laboratories



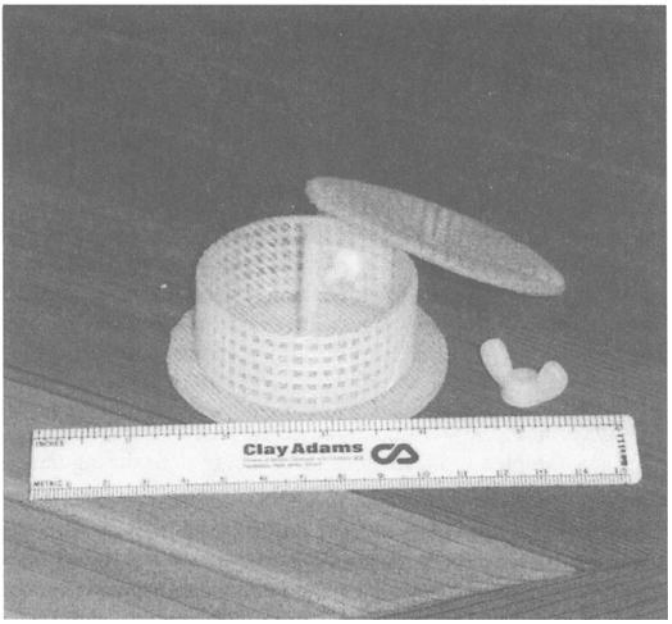


Fig. 3--*Exposure cages. In situ exposures were completed using double-walled cages constructed from polypropylene and Teflon^{R} mesh (cage diameter ca 64 mm).*

include length and gross teratogenic endpoints (e.g., skeletal malformations); field endpoints could also include behavioral observations such as mobility.

Results and Discussion

In the in situ exposures completed at Milltown, no adverse biological effects related to survival were associated with the emergent zones evaluated during the preliminary field season (see Linder et al. 1991). Little to no acute toxicity was noted in laboratory amphibian tests completed with surface water samples (Table 1). Supporting chemical analysis also suggested that metal concentrations in surface water grab samples were not sufficient to mediate acute effects (see Linder et al. 1991, 1994).

However, chronic effects measured on Milltown surface water samples were generally expressed by altered growth and in some of the laboratory tests of MRW surface water samples collected in conjunction with in situ exposures. These adverse biological effects (e.g., decreased survival and developmental anomalies) could be associated with ecological effects in the wetland, e.g., decreased amphibian populations. Chronic effects varied among water samples collected at different locations within the wetland, suggesting contaminant effects, if and when expressed, would not be uniformly distributed across the wetland (Linder et al. 1991, 1994).

Sediment samples were analyzed for total metals (Table 2) and indicated that heavy metal concentrations in sediments are spatially variable across the site, but appear to show consistently elevated concentrations in deposition zones. The sediment deposition pattern suggested that biological effects may be associated, directly or indirectly, with diminished sediment quality. Further characterization of exposure in the field was gained through a limited sampling of resident frog species (*Rana pretiosa*). While sample size and spatial restrictions require guarded interpretations, the metal residue data collected during the study period suggested that tissue concentrations were not elevated relative to comparative tissue data (e.g., Schmitt and Brumbaugh 1990). The presence of adult spotted frogs (*Rana pretiosa*) in these qualitative field surveys suggest that biological effects would not be acute in their expression as indicated by the preliminary laboratory studies.

Results from concurrent laboratory toxicity tests were consistent with in situ evaluations for MRW. Selected metals or metalloids in single-compound exposures were clearly acutely toxic at water concentrations less than 2 to 3 parts per million (ppm), with copper and cadmium being the most toxic metal species (LC_{50}s, 0.11 and 0.8 ppm, respectively; Linder et al., 1991). Similar trends were noted for chronic effects as indicated by the LOEC and NOEC estimates based on growth (Linder et al. 1991). No ambient surface water samples approached these concentrations during these studies, and during the field season (June through September) no exceedance of ambient water quality criteria was observed (Pascoe et al., 1994).

There are a number of exposure pathways beyond those involving surface waters and sediments. For amphibians, the integration of terrestrial and aquatic habitats as

Table 1. *Results of in situ and laboratory frog tests for surface water samples.*

Sample Unit/Station	Survival		Malformation	Total length (mm)[b,c]
	In situ[a]	Laboratory[a]	Laboratory[b,c]	Mean (std. dev.)
Blackfoot River (upstream reference)	NT	15/20	5/15	7.8 (0.7) +
Clark Fork River at Deer Creek Bridge (downstream reference)	NT	18/20	0/18	9.7 (0.3)
MRW Sample Unit I				
near Station 2	23/25	19/19	1/19	8.5 (0.3) +
near Station 4	23/25	19/19	6/19	8.1 (0.4) +
MRW Tunnel Lake				
near Tunnel Lake, Station 4	22/25	20/20	0/20	9.4 (0.5)
near Tunnel Lake, Station 6	23/25	20/20	0/20	9.5 (0.3)
MRW Sample Unit IV/V channel				
near Station 1	22/25	19/20	5/19	9.2 (0.3)
near Station 1	21/25	18/20	5/18	9.8 (0.3)
near Station 1	22/25	20/20	0/20	10.0 (0.2)
near Station 1	21/25	20/20	14/20	9.8 (0.3)
near Station 1	21/25	20/20	4/20	9.1 (0.3)
near Station 19	21/25	18/19	5/19	9.4 (0.7)
near Station 19	NT[d]	20/20	0/20	9.8 (0.3)
near Station 19	22/25	19/20	0/19	9.9 (0.3)
near Station 19	23/25	18/20	0/18	10.0 (0.2)
near Station 28	NT	20/20	1/20	9.1 (0.4)
MRW Sample Unit IV/VI				
near channel between sample units	NT	9/10	3/9	9.7 (0.6)
near channel between sample units	NT	19/20	6/20	9.7 (0.4)
MRW, Railroad Slough on the Clark Fork Arm				
downstream return to Clark Fork River	NT	20/20	6/20	9.4 (0.6)
oxbow	NT	19/19	4/19	8.9 (0.5)
oxbow	NT	20/20	0/20	9.1 (0.2)
oxbow	NT	19/20	7/19	9.7 (0.4)
oxbow	NT	18/20	1/18	9.7 (0.3)
upstream cut off from Clark Fork River	NT	19/20	0/19	9.9 (0.3)

[a]All survival scores recorded as "number responsive/number exposed"

[b]Malformations scored on survivors; scores for malformations other than growth reduction

[c]Growth results from modified FETAX completed in 96-hr laboratory exposures using *Xenopus laevis*

[d]NT = not tested in preliminary field season

"+" indicates significantly different from other treatments, P = 0.05 (Student-Newman-Keuls; see Linder, et al. 1991 for detail)

Table 2. *Total arsenic and metals in sediment samples collected at Milltown Reservoir Wetland during preliminary field season.*

Sample unit/station	Total analyte concentration[a]				
	As	Cu	Cd	Ni	Zn
Deer Creek Bridge (downstream reference)	4.4	41.0	1.1	4.0	134.0
MRW Sample Unit I					
near Station 1	13.5	26.0	2.5	9.5	45.0
near Station 7	10.3	35.0	2.7	9.0	57.0
near confluence of Blackfoot River with Clark Fork River	5.1	16.0	1.3	5.6	31.0
MRW Sample Unit IV					
near Station 1	50.1	349.0	6.6	7.4	2195.0
near Station 1	59.1	504.0	9.0	11.9	3149.0
near Station 1	47.1	331.0	7.0	9.7	1635.0
near Station 7A	67.6	357.0	6.4	10.0	1405.0
near Station 19A	34.7	299.0	4.3	8.3	635.0
near Station 19A	28.3	303.0	4.5	10.4	657.0
MRW Sample Unit IV/VI channel					
downstream	28.7	230.0	3.7	6.6	455.0
	25.0	198.0	3.3	7.4	463.0
	45.0	272.0	4.4	9.2	656.0
	27.2	221.0	3.4	7.2	441.0
upstream	25.6	242.0	3.5	6.6	443.0
MRW, Railroad Slough on the Clark Fork Arm					
downstream return to Clark Fork River	24.7	267.0	5.5	5.7	740.0
oxbow	64.1	400.0	8.6	7.5	1641.0
oxbow	22.1	229.0	5.2	6.1	1134.0
oxbow	83.8	1219.0	6.2	9.5	1226.0
upstream cut off from Clark Fork River	30.6	174.0	3.7	4.5	564.0

a = concentration, mg analyte/kg dry weight sediment

"continuous fields of exposure" clearly suggests that life history attributes may contribute significantly to the wide range of exposures that potentially link metals with amphibians throughout their life history. For example, most species have early life stages highly dependent on aquatic habitats where exposure may be dominated by direct uptake of metals from water, but subsequent life stages as adults may have exposure dominated by dietary sources, e.g., invertebrate prey items, and may contribute significantly to exposure.

Results from these preliminary field and laboratory investigations at MRW suggest acute exposures to surface waters were not of primary concern under the field conditions present during these studies, although the relatively limited time spent in sampling and testing surface water (i.e., a few weeks during summer) should remind the reader that our results reflect a single "snapshot in time" at MRW. That snapshot, however, suggests that metals in wetland soils, sediments, and surface waters could be associated with chronic effects in longer term exposures to metals, especially through other pathways. While other routes, e.g., dermal or cutaneous uptake, may contribute to exposed dose, direct uptake from water and ingestion pathways (consuming food, coincidentally ingesting soil/sediment, or drinking water) probably dominate exposure to metals at MRW, especially for those metals that bioconcentrate or bioaccumulate and are likely candidates for trophic transfer.

Bioaccumulation of Metals from Aquatic Habitats

Although water column exposures to arsenic and metals were not acutely toxic, for metals that are highly water soluble within the pH range of MRW's surface waters, chronic toxicity and bioconcentration associated with water-column exposures may be the predominant process that mediates transfer of metals from water to aquatic life stages of amphibians. Additionally, dietary exposures to larval stages not evaluated in these studies must be considered (Rowe et al. 1996, 1998a, 1998b). Regardless of the life history-chemical interaction that influences the predominate route of exposure, for relatively water-insoluble metals or metal complexes, bioaccumulation through dietary exposure may be important in aquatic food chains where amphibians occur as either prey or "predator" (e.g., broadly interpreted as herbivorous or insectivorous larval stages).

Bioaccumulation of Metals from Terrestrial Habitats

For amphibians, especially adults, exposed to metals in primarily terrestrial or wetland habitats at MRW, bioaccumulation is probably dominated by dietary exposures unless metals are readily absorbed across dermal epithelia or volatilize from a solid or liquid phase into air (see for example, Noble 1931, Duellman and Trueb 1986, Boutilier et al. 1992, Shoemaker et al. 1992, and Stebbins and Cohen 1995 regarding cutaneous repiratory surfaces and potential routes of metal uptake). Many variables affect the magnitude of bioaccumulation in terrestrial exposures, and the transfer of chemicals within food chains may conveniently be described by transfer coefficients or functions that characterize the relationships between trophic levels in food chains or in food webs (Pastorok et al. 1996, Pascoe et al. 1996, Linder et al. 1998, 1999). These factors may be abiotic, e.g., physicochemical characteristics of chemical or exposure matrix (sediment or soil) or biological in character, e.g., life-history dependent attributes related gastrointestinal or nutritional physiology, foraging or feed preference (see Young 1981, Larsen 1992, Hamelink et al. 1994, Langston et al. 1995, Linder et al. 2002). For example, such factors may characterize the transfer of chemicals from soil to water or from soil and sediment to an organism dwelling within these matrices up the length of the food chain. For the most part, these variables are, at best, empirical descriptors that characterize the transfer of chemicals from an environmental matrix (soil, sediment, or

water) to microorganisms, plants, or animals. In turn, additional transfer coefficients or functions describe the transfer of metals from these biological matrices to the consumers positioned at higher trophic levels. In soils and sediments, the initial transfer functions are influenced by physicochemical characteristics of the matrix such as particle size distribution or texture, cation exchange capacity, degree of carbon enrichment, and porosity. The potential for overestimating or underestimating these transfer functions increases as the complexity of the environmental mixture increases (Linder et al. 1998, 1999).

For example, Linder et al. (1998) focused on the transfer of metals through a wetland food chain; that is, transfer of metals along a "metal-contaminated soil-to-invertebrate prey-to-amphibian predator" pathway. In these designed studies, amphibians (*Xenopus laevis*) were exposed to different metal-contaminated diets for 28 days. While their focus was on cadmium, relationships between anuran predator, invertebrate prey, and wetland soils contaminated with a mixture of metals were fully characterized. Metals bioaccumulated by invertebrates were available to amphibians in a range of concentrations when fed these soil-exposed prey items. Under the given soil conditions (e.g., high TOC, neutral to slightly alkaline pH), metal transfers from soil-to-invertebrate-to-amphibian followed a consistent trend, with highest bioaccumulation factors (BAFs as simple ratio estimators) generally being achieved for metals at their lowest environmental concentrations. Transfer of metals from prey item to amphibian (*X. laevis*) followed consistent patterns as far as metals accumulation in amphibian tissues (Cd >> Cu = Zn > Pb > As), with uptake of zinc, lead and arsenic by soil invertebrates being relatively low in the initial transfer of metals from wetland soils. Maternal transfer of cadmium to developing eggs was clearly noted, however, and subsequent investigations by other workers confirmed that maternal transfer of cadmium (and other metals) to developing eggs was a significant route of exposure and was associated with reproductive effects, e.g., atretic eggs, in laboratory experiments (see Grillitsch and Linder, 2000 for literature summary).

Field sampling efforts parallel to these laboratory feeding trials indicated that cadmium concentration in surface waters of ephemeral wetlands was below ambient water quality criteria, yet exposure via the dietary route was the most significant to amphibians. Cadmium accumulation in invertebrate prey was consistent with early literature suggesting cadmium's hazards to wildlife, including amphibians (e.g., Francis et al. 1984, Eisler 1985, Vogiatzis 1997, Grillitsch and Linder 2000), and its presence in the diet as either tissue residues or as residual soil in the gut dominated the cadmium dose in frogs consuming earthworms exposed to cadmium-contaminated soil. Increases in cadmium concentrations in frog liver and eggs were observed, although relatively little cadmium was present in thigh muscle (Linder et al. 1998). Residues in frog tissues were relatively low for other metals measured in this study, although zinc and copper were found at higher concentrations in the liver than other metals, because of their nutritional role in the frog. Cadmium was the only metal significantly elevated in liver at the termination of the 28-day feeding trial. While the authors stressed the preliminary nature of their findings (Linder et al. 1998), cadmium appeared to be significantly elevated in eggs in higher concentration exposures, although sample size and the intended focus of the study did not allow adequate characterization of cadmium residues in egg samples. Linder et al. (1998) and many other studies (Grillitsch and Chovanec 1995, Rowe et al.

1996, 1998a, 1998b, Raimondo et al. 1998) suggest that metal exposures to amphibian life stages vary with respect to the dominate route of exposure accountable to realized dose and are not exclusively a water-only exposure.

Causal Analysis: Background

Integrated field and laboratory studies are designed to address multiple stressor exposures in the field, and the integrated studies completed in the present work were intended to identify the role that metals might play in mediating adverse effects in individuals that might be expressed as impacts by populations or communities. These integrated field and laboratory efforts implement the iterative process of "asking questions, designing experimental or observational studies, then interpreting data" to support causal analysis, help develop adaptive management practices or refine questions and re-implement the process (Pratt 1964, Hill 1965, Susser 1973, 1977, Heise 1975, Susser 1986a, 1986b, Walters 1986, Fox 1991, Cox 1992, Gustason 1994, Shipley 2000, Pearl 2000). Depending on the number of iterations implemented in the process, one of four outcomes is possible at the conclusion of any one iterate. Given the weight and strength of evidence developed during the integrated field and laboratory process, we can identify the following:

- no linkages between stressors and adverse biological effects appear to occur, or
- there are statistically significant associations between stressors and adverse biological effects which may support identifying a speculative cause, or
- there are sufficient statistically significant results from field and laboratory studies to support an argument of presumptive cause, or
- there are sufficient statistically significant results from field and laboratory studies, including observations of adverse effects in experimental exposures that are similar to those observed in the field, to support an argument of probable cause.

Assignment to any one of these outcomes and the justification for re-iteration of the integrated field and laboratory process largely depends upon the "level of comfort" that a resource manager has when faced with uncertainty in our technical capacity to address the issues the manager believes are "management critical." Often times, adaptive management plans need not require (what may be a costly) identification of probably cause. Rather, a finding of significant associations between stressors and adverse effects may be sufficient to support an adaptive management effort.

In the Milltown example, work moved from exploratory "question-and-answer" studies to those focused on characterizing linkages between specific stressors in environmental mixtures to biological effects. Causal analysis, as demonstrated here, is similar to the process employed in the study of infectious diseases developed by medical microbiologists over 100 years ago during the "Golden Age of Medical Microbiology," a process which has come to be referred to as one that follows Koch's postulates (Rothman 1976, Evans 1978, Rothman 1986, Martin et al. 1987, Thrusfield et al. 1995, Rothman and Greenland 1998). As does the presence of multiple pathogens or other pathological

processes, multiple stressors complicate the relatively simple diagnostic process outlined by Koch's postulates. Here, through integrated field and laboratory studies, causal analysis for multiple stressor exposures was driven by data needs to address these primary questions:

- Do field and laboratory tests of stressor mixtures yield effects similar to those observed in the field?
- Do tests of individual stressors or defined mixtures of stressors yield effects similar to those caused by the mixture of stressors?
- Does experimental manipulation of the exposure matrix (e.g., change in surface water pH, change soil TOC) alter stressor effects?

Causal Analysis: Metal Exposures in Amphibians at Milltown Reservoir Wetland

The biological and chemical information gathered during the preliminary field season at Milltown Reservoir wetlands suggests that no apparent linkage between metals in surface water or sediment occurred during the study period, although other ecological receptors (e.g., sediment benthic invertebrates and fishes) were clearly identified as being linked to ambient metal exposure (Pascoe et al. 1994, Linder et al. 2002). While no significant associations were identified, biological effects may be expressed locally, although not widespread in their appearance. Specifically, while heavy metal-laden sediments occur at Milltown wetlands, particularly in deposition zones (Woessner et al. 1984, Pascoe et al. 1994, Linder et al. 1994), biological indications of widespread expressions of toxicity to amphibians were generally absent, though the preliminary field and laboratory studies suggest that coincident spatial patterns between metals in sediments and biological effects may occur. Teratogenic endpoints expressed by frog embryos in laboratory exposures were subtle, when expressed (e.g., mild abdominal edema, hyperpigmentation). Gross malformations, if expressed, were most frequently characterized by ocular and abdominal edema, and skeletal and ocular malformations. As suggested by the supporting laboratory work, no metal-specific malformations were noted in these exposures.

No single metal in surface waters was linked to adverse effects observed in laboratory studies, and little ambient toxicity was indicated following field tests completed in parallel with preliminary amphibian surveys. No unique "chemical contaminant" signature was evident for field exposures to amphibians, and surface water exposures were unlikely sources of stressors linked to potential adverse effects associated with metal exposures during these studies. Dietary routes, however, were identified as most likely sources of metals and would be most problematic from a resource manager's perspective.

For mining impact studies, chemical stressors may not always dominate the exposure scene, especially when the spatial heterogeneity of habitat yields a gradient of exposure conditions. Physical habitat alteration such as sedimentation associated with soil erosion in areas of disturbance, and interactions between those physical stressors and environmental chemicals have frequently been identified as "major players" in multiple stressor exposures (e.g., Linder et al. 2002) . Also, temporal components have frequently been identified as major contributors to the exposure "equation." For example, season

changes in wetlands (e.g., spring run-off, autumn dry-down) may be management-critical factors in reducing or mitigating risk associated with environmental chemicals. In short, by adopting an adaptive management process that implements integrated field and laboratory studies to support the process, resource managers should become more effective in achieving their goals. This iterative process practiced in integrated field and laboratory studies is not new, but builds from early efforts to address questions regarding environmental hazards (see Dickson et al. 1979), and subsequently risk associated with multiple stressors (Suter et al. 1999, Foran and Ferenc 1999, Ferenc and Foran 2000). Integrated field and laboratory efforts are based on the specific application of technical tools to problem-solving.

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Causal Analysis

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The Role of Multiple Stressor Causes in Declining Amphibian Populations: A Wingspread Workshop Summary

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Numerous studies have documented the decline of amphibian populations over the past decade and no single factor has been the linked to these widespread declines. Determining the causes of declining amphibian populations worldwide has proven difficult because of the variety of anthropogenic and natural suspect agents. A Wingspread workshop, convened by The Society of Environmental Toxicology and Chemistry (SETAC), brought together individuals with expertise in the areas of amphibian biology, ecotoxicology, natural resource management, and environmental policy. This workshop had three objectives: 1) create a network for future discussions on multiple stressor causes of declines; 2) characterize and prioritize technical issues critical to the analysis of the decline problem; and 3) identify and develop resource management approaches to promote sustainable and healthy amphibian populations. The workshop proceedings will be summarized in a book entitled, *"Multiple Stressors and Declining Amphibian Populations: Evaluating Cause and Effect."* This paper summarizes the results of the workshop.

Keywords: amphibian, populations, decline, multiple stressors

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Introduction

Amphibian populations are declining (Wake 1991; Alford and Richards 1999; Houlahan et al. 2000) and this decline has been documented in North America (Fellers and Drost 1993; Drost and Fellers 1996), Europe (Beebe et al. 1990; Beebe 2001), Australia (Laurance et al. 2001), and Central and South America (Crump et al. 1992; Lips 1998; Young et al. 2000). To date, the U.S. Fish and Wildlife Service has listed 19 species of amphibians as endangered and 9 species of amphibians as threatened worldwide (USFWS 2002). Most declining amphibian populations in North America have been recorded in the western United States, but have also been observed in several species in the Midwest and Southeast (Corn 2000). A third of the amphibian populations in the United States are thought to be in peril (Bury et al. 1995)

Amphibians have been referred to as "canaries in the coal mine" - an early warning system signaling environmental danger (Halliday and Heyer 1997). This class of animals is an important indicator of environmental health because, unlike most other organisms, they spend their lives in the water and on land and are exposed to elements through a variety of routes (Blaustein and Wake 1995). Amphibians have been shown to be sensitive to both natural and anthropogenic sources of contamination, and in fact may be more sensitive to ultraviolet radiation (UVB), pesticides, polyaromatic hydrocarbons (PAHs), and acidification than fish (Birge et al. 2000).

The Wingspread Conference

The Workshop

The Workshop on the Decline of Amphibian Populations, was held August 18-23, 2001, at the Johnson Foundation in Racine, Wisconsin. Thirty-four scientists with a variety of expertise in the areas of amphibian biology and ecotoxicology, natural resource management, and environmental policy working in government, academic, and private institutions and companies in the United States and Canada convened. Six groups were formed to discuss the most current information involving the different classes of stressors. These groups were Physiological Ecology/Population Genetics, Synthesis and Integration, Biological Stressors, Physical Stressors, Chemical Stressors, and Habitat Interactions. Each group discussed the latest findings in their area of expertise, developed recommendations, and then exchanged these ideas with other workgroups. Following this interaction, each workgroup summarized their discussions and compiled the proceedings. The following paragraphs summarize the highlights of these proceedings. Discussed are the many stressors involved and their interactions, and the problems faced when determining causality, forming a management decision, and ultimately, taking corrective action.

Highlights of the Proceedings

Amphibians have existed on earth for 350 million years. During this time, they have interacted with other organisms (predators and competitors) and stressors (pathogens and

changing environmental conditions), adapted to these challenges, and continued to thrive. This situation changed as the human population grew and expanded around the globe. Today, amphibians are exposed to countless numbers of new stressors which, in some cases, exceed their ability to adapt to. As a consequence, amphibian populations are declining globally.

Amphibians have a complex life cycle that includes both aquatic and terrestrial life stages. Development requires that the organism transform from an egg, to a larvae, to tadpole, then metamorphose into a froglet, and gradually move out of the water and onto land as an adult. The phases of development from larvae through metamorphosis are controlled by hormones (Duellman and Trueb 1986). Significant energy is required during development which creates additional stress on the organism. Metamorphosis is a particularly stressful phase which involves a series of structural, physiological, biochemical, and behavioral changes. During metamorphosis, the amphibian does not eat because the oral structures are changing. All of the energy needed is gained through the absorption of the tail. Amphibians are challenged by their complex life cycle which alone causes stress on the organism, and if additional stressors are encountered during this period, some individuals may not survive.

The majority of amphibian species experiencing declining numbers in the United States have been documented in the western States. Many of these threatened and endangered species reside in areas of California and Arizona that are experiencing rapid human population growth and development. To meet their increasing needs, humans have altered the environment at an unparalleled rate. Habitat alteration, including the loss of wetlands, degradation of wetland habitat, or fragmentation of wetland complexes, is undoubtedly, the primary cause of declining amphibian populations. Tens of thousands of acres of wetlands are lost each year in the United States and some estimates suggest that as much as 50% of the earth's wetlands have been drained during the past 200 years (Dahl 1990). Human population growth increases the demand for water and food which leads to a depletion of the existing water supply for drinking and irrigation. To provide the necessary homes and commercial development, the need for building materials, including forestry products, has grown. Silviculture management involves a number of activities that can have negative impacts on amphibians, including clear-cutting, selective logging, road building, fire and fire control, and the application of pesticides. The act of removing trees alone causes a reduction in cover, leading to a rise in temperature and reduction in moisture on the forest floor.

Increasing human population has lead to an increased number of roads which divide habitat which can kill migrating amphibians (Ashley and Robinson 1995). Dividing a population may lead to genetic isolation, and if habitat conditions deteriorate, may lead to extirpation of the population (Sjorgren 1991b; Blaustein et al. 1994). Genetic diversity is one of the most important and understudied aspects of amphibian population biology. Environmental stressors can impact the genetic makeup of amphibian populations through chemically induced changes in the DNA as well as reduced genetic diversity within the gene pool. Stressors can lead to population declines by interfering with the dynamics between populations, or metapopulations. However, there is a lack of long-term demographic data for amphibian populations, making it difficult to determine if a population is in decline or experiencing a natural fluctuation in numbers. Although

additional information is needed on metapopulations, there is still a need for focused, site-specific studies.

Much attention has been focused on the impacts of climate change which affects all landscapes, even pristine environments. Habitat loss and alteration, alone and in combination with other variables, can be caused or enhanced by global changes, including increased ultraviolet radiation (UVB); climate change; drought; El Nino Southern Oscillations (ENSO); and phenology, which involves the effects of climate changes on breeding and migration. These can manifest themselves locally by altering critical terrestrial and aquatic environments through changes in vegetation, soil moisture and compaction, temperature, and an increase in the incidence of climatic events (Donnelly and Crump 1998). A small increase in temperature can have a dramatic effect on a microhabitat, and can lead to either a positive or a negative impact on a population. Increased temperatures can extend the breeding and development period leading to an increased number of temperate amphibian species. However, an increase in temperature coupled with a decrease in precipitation and increased evaporation can have disastrous effects, especially for aquatic life stages. UVB levels have also increased over the past several decades and substantial scientific attention has been focused on the effects of UVB on amphibians. No study has conclusively demonstrated that excess mortality in natural populations has been caused solely by UVB. However, UVB can have substantial impacts on other environmental stressors, such as chemicals causing them to form more toxic or persistent products.

There are many different classes of chemicals, including pesticides, metals and metalloids, organic chemicals, and drugs and pharmaceuticals, and much attention has been focused on the effects of single chemicals on organisms in the laboratory. Chemicals can also directly or indirectly influence an organism's sensitivity to other stressors, ultimately affecting the individual's health and survival. There has been less attention focused on the breakdown products of these chemicals which can be more toxic and persistent in the environment than the parent compound. Field data documenting the effects to amphibians exposed to single or multiple chemical mixtures is limited, and even less is known about potential interactions between chemicals in mixtures and chemicals and non-chemical stressors.

Multiple stressors may act in synergy, creating a new effect or one that looks very similar to an effect caused by a different stressor. The amphibian malformation issue is a good example. Parasites (Sessions and Ruth 1990; Johnson et al. 1999); chemicals (La Claire et al. 1998; Fort et al. 1999; Sparling 2000); ultraviolet radiation (Ankley et al. 1998); and water quality factors, such as high ammonia, lack of essential ions (Tietge et al. 2000), and low pH (Dunson and Connell 1982) have been shown to cause malformations. In some locations, parasites appear to play a major role in the production of malformations. Certain species of trematodes are carried by a bird, mammal, fish, or reptile hosts and use amphibians and snails as intermediate hosts during their complex life cycle. The trematode, *Riberoria ondatrae*, has been shown to cause hindlimb abnormalities, including multiple limbs, in both the field and in the laboratory (Sessions and Ruth 1990; Johnson et al. 1999). Deformed amphibians are more likely to be preyed upon, but the overall effect of high abnormality rates on the long-term survival of the population is not known. Why amphibians are being impacted by these naturally

occurring trematodes remains unclear, but habitat degradation and fragmentation, introduced species, and poor water quality may play a role.

Habitat alteration, physical stressors, and chemical stressors have a common trait - they lower an organism's immune functions which increases its chance of acquiring a disease while decreasing its ability to fight it. Increased human development has brought new pathogens into contact with amphibians. These new pathogens have affected large numbers of species and caused widespread, rapid declines in populations.

Biological stressors, such as diseases and introduced species, are responsible for die-offs (Berger 1998) and displacing native species (Gill and Matthews 1998; Schwalbe and Rosen 1998). Two pathogens have been shown to cause mass die-offs - the chytrid fungus (*Batrachochytrium dendrobatidis*) and a group of iridoviruses (genus *Ranavirus).*

Chytridiomycosis has been described as an emerging infectious disease and has been documented in portions of the United States, Canada, Central America, Australia, New Zealand, Europe, and Africa. Individuals infected with chytrid fungus may demonstrate a range of disease manifestations, from no observable signs to sloughing or a discoloration of the skin. Behavioral signs include sitting unprotected in the daylight, abnormal resting procedures, and avoidance of the water. This fungus is responsible for the rapid decline of several amphibian species, including two toad species in the western United States, the boreal toad (*Bufo boreas*) and the Wyoming toad (*Bufo baxteri*). Both species' numbers declined rapidly during the 1970s, however, they live in different geographic locations and habitats, making it unlikely that there is one common stressor or group of stressors that lead to the declines. Chytrid fungus does not appear to require a co-factor, such as UVB or a chemical, to kill (Daszak et al. 1999); however, lethal chytridiomycosis occurs frequently at cold temperatures (Carey 2000).

Iridoviruses were first recognized in the 1960s (Granoff et al. 1965; Wolf et al. 1968) and include many virulent amphibian ranaviruses that infect a wide range of frog and salamander hosts. Most recent research focuses on a group of closely related ranaviruses that cause tiger salamander die-offs. The virus appears to live solely in tiger salamanders, persisting in metamorphosed salamanders before reinfecting others during the breeding season. These die-offs can occur on a regular basis over a period of years and can potentially threaten the existence of tiger salamander populations.

Diseases have spread rapidly within the past several decades infecting populations over a wider geographic area. Stopping the spread of disease is of paramount importance to scientists and land managers alike. Diseases are often spread through the introduction of infected organisms into uninfected populations. For example, tadpoles acquired through biological supply companies frequently are infected with chytrid fungus which can infect a native population if these tadpoles are released into the wild. The ranavirus responsible for the tiger salamander die-offs has been introduced to healthy populations of tiger salamanders when diseased salamanders are collected and used as fishing bait.

Introducing a species into a new environment poses potential consequences. Besides the potential threat of spreading a disease, the introduced species may become a nuisance and invasive species. Prime examples of invasives are the brook and brown trout species and the bullfrog, two species which were introduced into the lakes and ponds of the western U.S. These invasives have played a major role in decimating the populations of at least two species of amphibians, the mountain yellow-legged frog and

the Chiricahua leopard frog by predating upon or out-competing these native species for other resources.

Having many stressors present in a situation complicates the determination of the potential cause(s) and requires that an assessment process be employed. The assessment process should be accompanied by an adaptive management approach which utilizes risk management techniques. A monitoring program and evaluation period should follow to determine if additional assessment and management actions are needed. Figure 1 demonstrates how to evaluate causation and Figure 2 illustrates the conceptual model by diagraming the effects associated with road construction and use and sport fisheries management. Such models are helpful in illustrating the interactions among drivers, stressors, effects, and endpoints and demonstrate why a population may be declining. An accurate evaluation of the situation will lead to effective management actions necessary to correct the problem.

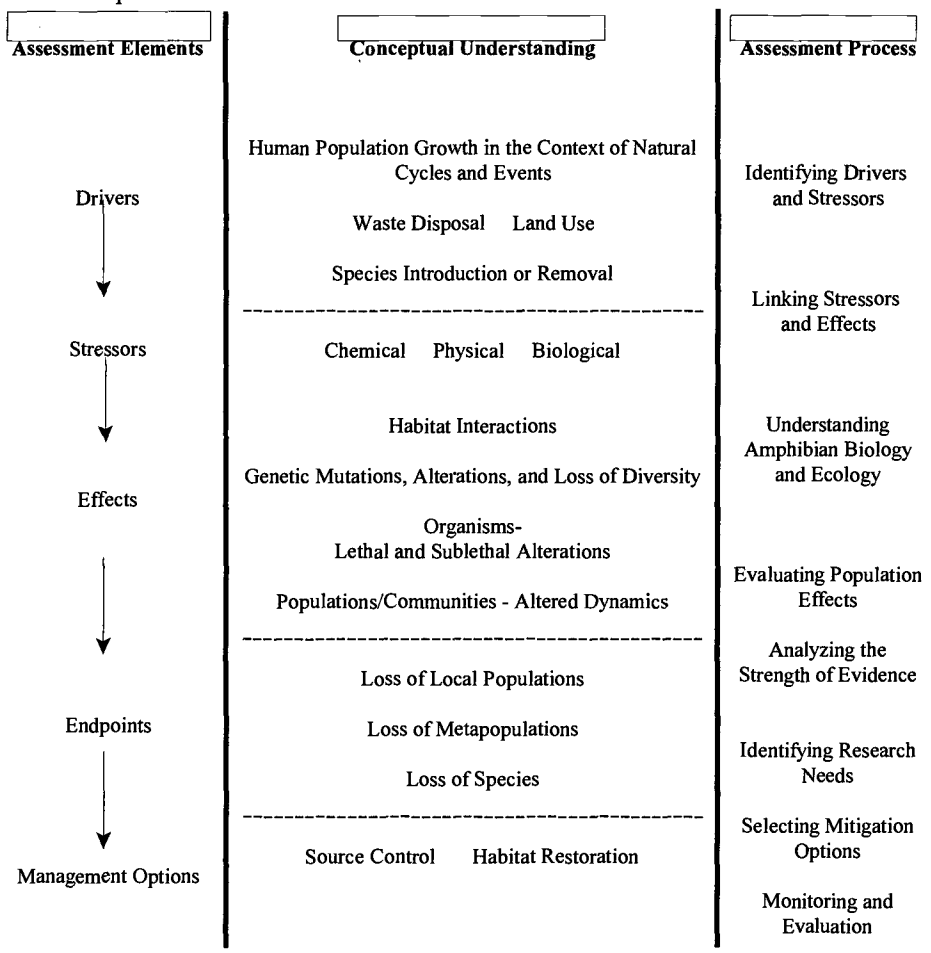

Figure 1 - Evaluating Causes of Amphibian Decline

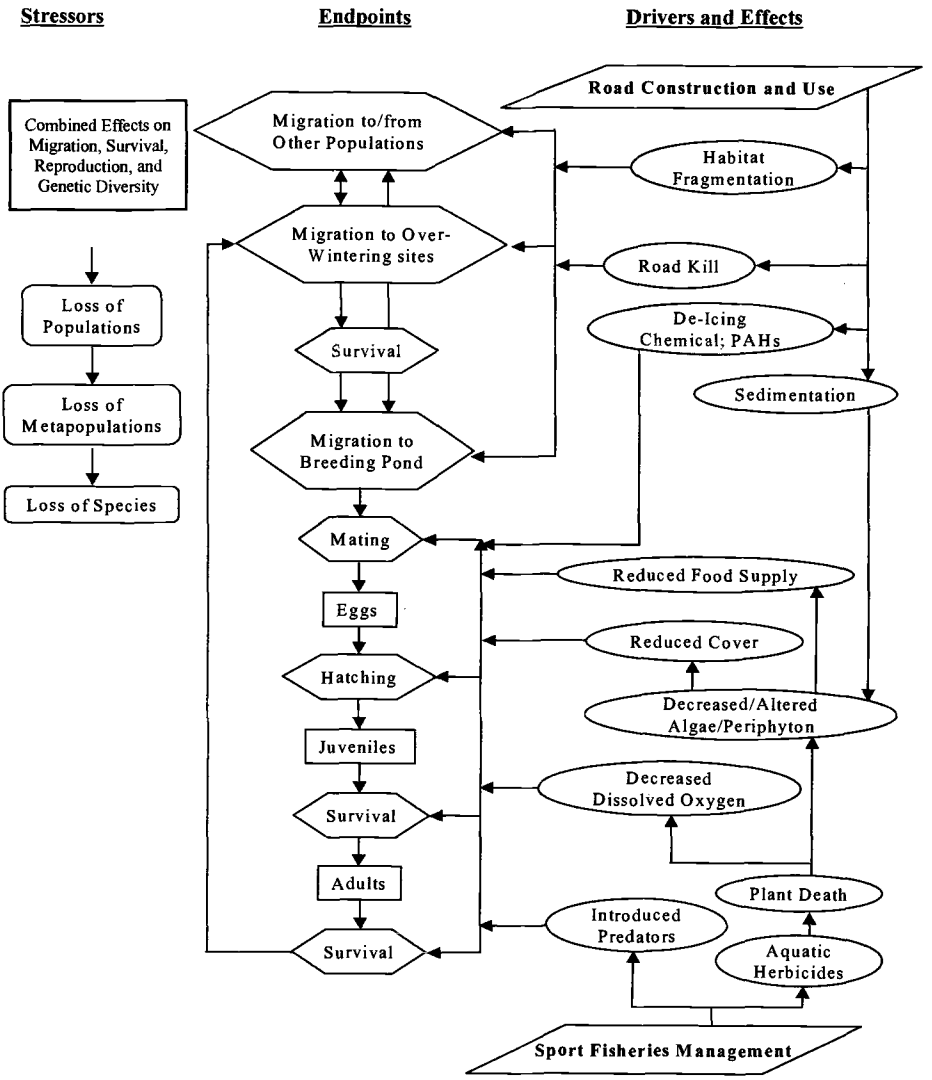

Figure 2 - Conceptual Model for Amphibian Decline

A conceptual model applicable to causal evaluations of amphibian decline illustrating effects associated with road construction and use and sport fisheries management practices. In this diagram, drivers are parallelograms, stressors are ovals, rectangles are life history stages of amphibian receptors, and hexagons are activities of the receptors. Endpoints are shown as rectangles with rounded corners.

If practitioners are to institute accurate management recommendations to correct land-based problems, they must have thorough and practical information on which to base their decisions. Therefore, scientists need to move away from single stressor, lab-only research and begin to consider the multiple stressor scenarios that occur in the field. They should examine non-traditional endpoints (behavior, biomarkers, etc.), and ultimately investigate the potential effects on population. Scientists must communicate with experts in other fields, including policy-makers and land managers. The purpose of this Wingspread conference was to bring together professionals with a variety of expertise and begin the communication process.

Conclusions and Recommendations

The goal of these proceedings is to inform practitioners, such as land managers, researchers, and policy makers, about the amphibian decline issue and its complexity, and provide the necessary tools to put the recommendations into action. Traditionally, managers have focused their attention and efforts on a single wetland or tract of land, game species, or a particular issue of concern. Due to the interactive nature of ecosystems and their inhabitants, managers, scientists, and policy-makers must begin to look at the broader picture and consider landscape issues, natural and anthropogenic substances and mixtures, and less charismatic species, including amphibians. Documenting the absence or presence of a species, rather than a specific number of individuals at a particular site will provide more reliable information when observing population status over time. The potential impact on metapopulations should be the level practitioners consider when selecting an action, such as where to build a road, how much buffer area to include, or method of controlling an invasive species. Land managers must be cognizant of the hydroperiod when controlling the water level of a pond or lake. Biologists need to take special precautions to not unintentionally introduce a pathogen into a study area by washing boots and equipment with detergent and bleach, and should collect voucher specimens of the non-listed species being studied, especially when disease is suspected. Contaminants should be considered when declining numbers are observed, even in situations where the source is not obvious. When identified, chemically impacted media can be remediated and the appropriate environmental quality department and specialists should be contacted. Unfortunately, controlling or counteracting global physical stressors, such as UVB light and climate change, are beyond the direct control of the individual. However, practitioners and biologists should remain aware and knowledgeable about the potential impacts these stressors can have on amphibians. Best management practices, such as the addition of buffer areas, retaining snags, constructing amphibian tunnels, reducing speed limits, maintaining roads, providing habitat corridors, and avoiding chemical applications near breeding area are in existence and can be employed immediately by land managers. Recommended mitigation and restoration practices suggest that programs to introduce species (e.g. stocking hatchery-reared game fish) be terminated and the native amphibian population re-established, once the threat of the invasive species being introduced again has been eliminated.

This workshop provided the participants the opportunity to share knowledge and

expertise and to formulate a broader network for future interaction on the issue as it evolves. It is the group's hope that a similar workshop will convene in the future, that will incorporate experts from other corners of the world to create a global network dedicated to promoting sustainable amphibian populations worldwide.

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Establishing Cause-Effect Relationships for Chemical Stressors in Amphibians: Providing Adequate Data for the ERA

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Abstract: Most studies with amphibians in the past have been designed to address either the effect or the potential cause. Thus, data collected may provide suitable documentation of field effects on local populations with little information on causality, or the data may provide suitable information on possible causes with little concrete explanation of effects on the local populations. In order to establish such a relationship at this level, a study must be designed to answer two primary questions. First, what is the effect on the local population? Second, what factors are causing this effect? To establish an effect on a local population of amphibians, population characteristics must be surveyed. This survey should include information on habitat suitability, life stage distributions, and reproductive and developmental success. Establishing the cause of the noted effects requires the collection of adequate field-based ecotoxicological data combined with well-designed and highly controlled laboratory studies, ultimately providing adequate lab-to-field extrapolation potential. Field-based ecotoxicological data can be collected using a combination *in situ* exposure and evaluation at various life stages, and laboratory examination of specimen collected from the field at appropriate life stages. More controlled laboratory-based studies involving the culture of indigenous species under simulated field exposure conditions should also be considered. Cross-over studies in which organisms collected from uncontaminated areas are cultured under contaminated conditions, and those from contaminated locations are cultured under uncontaminated conditions to help establish exposure and impact scenarios. Appropriate chemical analysis of environmental samples including water, sediment, soil, and tissues from field collected and laboratory-reared specimen should be performed to determine exposure concentrations relative to the effects observed in individuals and accumulation potential. Highly controlled spiking studies can then be performed to help confirm potential causes of the effects observed. Overall, in many cases a combination of parallel field and laboratory studies can be used to provide adequate data to establish causality

Keywords: amphibians, ecotoxicology, risk assessment, lab-to-field extrapolation, *in situ*, study design

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Many hypotheses have been provided to explain the global declines of amphibian populations; however, few studies have provided the clear cause-effect results required to demonstrate effects at the local population level. This deficiency does not diminish the efforts of the investigators providing these hypotheses, but is rather a tribute to the complexity involved in establishing cause-effect relationships at the local population level. Considering the current level of interest in amphibian ecotoxicology and the resultant research data that is now available, it is somewhat surprising that amphibians are not being more widely incorporated into formal regulatory ecological risk assessments (ERAs). Several factors could potentially limit the use of amphibians in regulatory ERAs including, general data limitations, suitability as a receptor, ecological significance, trophic equivalency with other potentially similar species, and capacity to establish causality. Limitations in any one of these areas could potentially lead to data gaps in establishing the multiple lines of evidence needed for adequate ERAs. In this report, these factors and methods for effectively establishing causality are discussed using a specific case study to illustrate experimental design considerations.

The Current State of the Science – Data Limitations

In order to put the historical use of amphibian species in ERAs into perspective, we searched the Biological Abstracts and Medline/PubMed literature bases from 1980 to 2000. The search strategy was based on keywords such as chemical stressors, amphibians, and ERAs using the Boolean Operators "and" and "or." The search was further defined using keywords such as field studies, lab studies, effects, causality, and cause and effect. This exercise was conducted as an extension of a search conducted by Sparling et al. (2000a) who conducted a more generalized search of the Wildlife Review and Sports Fisheries CD-ROM abstracts from 1972 through 1998 using the keyword union of pollution, toxicology, or contaminants with each vertebrate class. Of the 1271 citations found by Sparling et al. (2000a), only 312 (2.7%) were for amphibians.

Similar database findings were found in our search in which 878 studies were identified as involving the study of a chemical stressor or stressors to an amphibian species. Of these studies, 402 and 444 of the studies involved field components or lab components only, respectively. Only 32 studies actually involved both field and laboratory components. Of the 878 studies generally identified as being involved in a chemical stressor study with amphibians, 196 studies were used in some form of risk assessment and 192 of these studies specifically documented toxicological effects. Of the 192 citations identifying toxicological effects, 12 papers claimed to establish causality and 3 studies provided a detailed link between cause and effect. However, none of the studies queried were used in a formal regulatory ERA. Overall, these literature searches confirmed the presence of general data gaps in ecotoxicological data for amphibians and the limited use of this data in ERAs.

Amphibians as Receptors

Does the lack of amphibian data considered in regulatory ERAs imply that amphibians are not suitable ecological receptors? Most likely this is not the case (Meyers-Schöne 2000). Based on the life history traits of amphibians, likelihood of

exposure to various chemical stressors is high, making it an ideal model for evaluating exposure. In fact, amphibians may be particularly vulnerable to waterborne environmental contaminants due to their largely aquatic life histories and their highly permeable skin. A recent special report on environmental endocrine disruption by the U.S. Environmental Protection Agency (EPA) cited no reports of such effects in amphibians, although it concluded, "this class of vertebrate represents a unique sentinel animal model for laboratory and field exposure studies" (U.S. EPA 1997).

In spite of increasing alarm over global population declines in amphibian species over the last 20 years, only recently has evidence of adverse effects due to exposure to a variety of different physical and chemical stressors been provided (Sparling et al. 2000b). It is likely that several of these factors are working together as multiple stressors. In some cases, amphibians also provide a critical link to identifying potential hazards for other animals including humans (Fort et al. 1999a, 1999b, in press). Thus, amphibians currently carry a special status and are integral within their trophic level making them suitable receptors for ERAs.

Broad Limitations in Using Amphibians in ERAs – Basis or Bias?

Because amphibians are suitable receptors for ERAs in many cases, broad limitations in the use of this class of animals must exist that has resulted in their exclusion from formal regulatory ERAs. Selection of species to incorporate into ERAs is often based upon the species with the largest toxicological database and species that are considered to be "the most sensitive." Large databases and species sensitivity represent an obvious advantage except in the cases where relevance to the site and phylogenetic relevance are not considered. For example, fish may represent an aquatic species with a seemingly similar exposure scenario to amphibians; however, differences in the life history traits between fish and amphibians do not make fish a suitable surrogate for amphibian species.

On the other hand, data gaps in the amphibian toxicology data often make it difficult to establish the multiple lines of evidence required for ERAs. Data gaps represent a legitimate limitation in the use of amphibians in ERAs. To establish multiple lines of evidence, ecological significance, causality, and ecological recovery need to be established. Understanding ecological significance requires establishing a larger scale effect of a stressor or stressors on a local population. Establishing causality is more specifically addressed in the following section. Generally, causality is established by demonstrating exposure, toxicity, and a mechanistic connection between exposure and effect. Finally, ecological recovery refers to some positive change in the ecosystem rendered by natural or induced remedial actions. Establishing ecosystem restoration following the removal of key stressors is important, but often difficult to accomplish since most biological systems do not return to their original state once they are disturbed.

Establishing Causality

Establishing causality represents one of the greatest obstacles in providing multiple lines of evidence for the ERA. Establishing causality requires identification of the

effect or effects, proof of exposure, demonstration of the effect, and identification of a mechanism of action (Meyers-Schöne 2000). To demonstrate the effect, ecologically significant studies must be constructed that provide adequate laboratory-to-field extrapolation. This establishes the connectivity between the effects observed in more controlled laboratory studies and field studies. Finally, identification of mechanisms of action of COPECs provides the link between the presence of the COPEC and the effect induced by the COPEC at varying biological levels ranging from molecular to organismal. Because establishing causality is one of the most difficult processes in establishing multiple lines of evidence, the remainder of this manuscript is dedicated to experimental approaches to specifically establish causality, including a case study illustrating one type of approach.

Experimental Design Considerations

Local Population Effects

Demographic indicators must be integrated both spatially (i.e. how widespread is population decline) and temporally (short term versus decade) in relation to normal population cycles. To understand the significance of the effects on the larger scale, the effect of stressors at the local population needs to be evaluated. Local population characteristics including habitat suitability, life stage distributions, and reproductive and developmental metrics should be considered. In addition, a thorough evaluation of physical and chemical stressors should be performed to identify confounding factors that may be attributed to effects at the local population level.

Causality

The two essential keys for establishing causality are generating adequate laboratory-to-field extrapolation data and well-designed and highly controlled laboratory data. The field component of this data may include examination of field-collected specimen and/or *in situ* exposure studies. Field study design requires highly complex experimental design considerations. The laboratory component may include the culture of indigenous species of interest collected from the field at designated. Culture of surrogate amphibian species in the laboratory under field-simulated conditions can also be considered in the experimental design.

Design considerations for the laboratory-derived data is also important since studying in the laboratory provides a greater level of control over the experiments performed. Studies performed in the laboratory also afford flexibility in experimental design. The case study discussed in the following section describes the use of four different experimental approaches to evaluate effects and help establish causality. The first design consideration is the use of different life stages to evaluate specific sensitivities. These studies might include partial and full lifecycle assessments using, for example, early developmental, metamorphosis, or adult reproductive endpoints. Cross-over exposure designs in which indigenous specimens from reference areas are exposed to contaminated water and sediment, and specimens from the contaminated sites are exposed to reference site waters and sediments should also be considered. In

the case of developmental studies, cross-over experiments help determine whether the effects induced in indigenous species are the result of environmental exposure to contaminants, transgenerational transport of toxicants from the parents to the progeny, or both factors. Spiking studies in which reference site water or sediment are laced with COPECs should also be considered to demonstrate that the COPECs are capable of inducing reasonably similar effects to that observed in specimens cultured from the contaminated sites. Tissue residues, including whole body and target organs, should also be measured particularly in longer-term studies to document exposure and accumulation of COPECs in relation to the effects induced. Tissue residues in short term studies, particularly early developmental studies, are generally less-effective in establishing exposure/accumulation-effect relationships. In these cases toxicants which induce early developmental effects do not necessarily accumulate as the effect is elicited as the result of exposure during a relatively short, but developmentally critical window of time. Finally, a biomarker study provides links between a particular biochemical process that is affected by the COPECs and an organismal effect observed, thus providing mechanistic information. These experimental design approaches represent only several of many different experimental tactics that can be used to establish causality.

Case Study – South Central U.S.

Background

The present study encompassed a 12.5 K stream reach with adjacent floodplain, backwater areas, and established intermittent pools in a prominent river in the south central U.S. This study area comprises a natural habitat for several ranid species including *Rana sphenocephala* (Southern Leopard frog), *R. blairi* (Plains Leopard frog), *R. clamitans* (green frog), and *R. catesbeiana* (bullfrog). The identity of the river and test sites was excluded from this report to protect the identity of the stakeholders. Diesel-range organic, and to a lesser extent gasoline range organic, hydrocarbon contamination (TPH-GRO/DRO [total petroleum hydrocarbons–gasoline/diesel range organics]) in the sediment of this river has been dated back to the mid 1970s. Eight target intermittent pools within the adjacent floodplain containing varying levels of TPH-DRO contamination and three upstream reference pools were used in the study. TPH-GRO/DRO concentrations in sediment and premetamorphic larval tissue from each site are provided in Table 1.

Local Population Effects

Local Population Comparisons - Local species abundance surveys were conducted for frog species in accordance with the methods of Burton and Likens (1975), Berven (1990), Blaustein et al. (1994), and Bishop et al. (1999). Because the focus of the study was primarily on ranids species, the following species were evaluated in the present study: *R. sphenocephala*, *R. blairi*, *R. clamitans*, and *R. catesbeiana*. In comparison to the upstream reference sites, a reduction in local populations estimates was noted in each species with the exception of *R. catesbeiana* (*R. sphenocephala*>*R. blairi*>*R.*

clamitans>>R. catesbeiana). As previously indicated, specific data form the field studies is privileged and therefore, cannot be provided in this report.

Table 1 – *Total Petroleum Hydrocarbon (Gasoline Range Organics/Diesel Range Organics) [TPH-GRO/DRO] Levels in Sediments and Tissues*

Sample Location	Sample Type[1]	Total GRO/DRO[2] (mg/Kg)/(mg/Kg)	Sample Location	Sample Type[1]	Total GRO/DRO[2] (mg/Kg)/(mg/Kg)
1	Sediment	0.8/12.8	6	Sediment	0.2/1.1
	Tissue	0.1/2.8		Tissue	0.1/1.6
2	Sediment	2.3/78.3	7	Sediment	5.3/52.5
	Tissue	0.7/12.9		Tissue	1.1/15.6
3	Sediment	0.5/9.6	8	Sediment	0.4/2.8
	Tissue	0.2/3.2		Tissue	0.2/1.1
4	Sediment	0.2/7.6	Reference	Sediment	<0.01/<0.01
	Tissue	0.1/2.1		Tissue	0.01/0.02
5	Sediment	0.3/1.7			
	Tissue	0.2/2.5			

[1]Sediment samples consisted of a composite of four core samples for each location. Tissue samples were comprised of a composite of 50 whole body metamorphic *Rana sphenocephala* tadpoles collected fromeach site.

[2]Samples were extracted in accordance with EPA method 8021 and analyzed by gas chromotography with mass selective detection (GC-MS) in accordance with EPA method 8015 (U.S. EPA 1986).

Field deformities - Recently, metamorphed specimen were collected from the reference sites and target locations and evaluated externally for malformations. Of the *R. sphenocephala*, *R. blairi*, *R. clamitans*, and *R. catesbeiana* collected within the target locations, 22.9% (n=83), 17.4% (n=138), 11.1% (n=171), 1.2% (n=321) were malformed. Of the *R. sphenocephala*, *R. blairi*, *R. clamitans*, and *R. catesbeiana* collected within the reference sites, the rate of abnormality in any species did not exceed 0.7% (n=194, 256, 192, 258, respectively).

The primary malformation syndromes observed in the field for *R. sphenocephala* and *R. blairi* were facial, mouth, eye, spine, and hind limb defects; and facial, mouth, eye, and forelimb anomalies. Eye and hind limb mal-development was found in *R. clamitans* specimens. Of the defects noted, the incidence of limb malformations did not exceed 3.0% of the specimens surveyed. On the contrary, the incidence of other malformations associated with the deformity syndrome was appreciably greater.

Other Information - The tissue residue data presented in Table 1 indicated that petroleum hydrocarbons were accumulating in developing *R. sphenocephala* larvae. Separate from the metamorphic tadpoles analyzed and reported in Table 1, several adult

R. sphenocephala from both the contaminated sampling sites and the reference sites (n=5 for both) were also composited and analyzed for whole body TPH-GRO/DRO. Whole body TPH-GRP/DRO for adult specimen from the contaminated sites contained 4.3/16.2 mg/Kg. Whole body TPH-GRP/DRO for specimen from the reference sites contained <0.5/<0.5 mg/Kg. Other miscellaneous COPECs, including PCBs, dioxins, furans, and organophosphorus pesticides were note detected. Trace levels of 2,4-D and lindane were detected (ca. 0.5 mg/Kg). Parasitology reports on a cleared sub-sample of the adult specimen collected with limb deformities were negative, including the trematode, *Ribeiroia ondatrae* (*Rib*). This was perhaps the most substantial finding from the field studies and indicated that the limb deformities were not the result of parasite infections and were the result of other stressors including contaminants.

Controlled Exposure Studies

In situ studies - In situ exposure studies were performed with *R. sphenocephala* based on the availability of egg masses at the site and their relevance to the study area. Developing embryos/larvae were maintained in 3 m x 3 m enclosures at each site through metamorphosis. Three enclosures with 40 embryos/larvae were maintained at each sampling location (n=120). Teflon screening surrounded the enclosures to reduce predation and to allow light to penetrate. Approximately 20 premetamorphic tadpoles per site were collected randomly from the enclosures for tissue residue analysis (Table 1). Since the primary emphasis of the study was to evaluate the normalcy of development through metamorphosis, specimens were evaluated for external deformities at three critical periods during development. These periods included embryo-larval stage (premetamorphic), limb bud stage (prometamorphic), and newly metamorphosed stage. The incidence of malformation induced in premetamorphic *R. sphenocephala* collected from each of the sampling sites is provided in Figure 1. The greatest incidence of malformation at the embryo-larval stage was recorded in specimens collected from sites 1, 2, and 7. However, malformations were most prevalent in prometamorphic specimens collected from sites 4 and 8 (Figure 2). Similar to the embryo-larval specimens, sites 1, 7, and most notably 2, demonstrated the greatest incidence of malformation in metamorphs collected from the *in situ* cultures (Figure 3). Although the accumulative malformation rates in each of the sites corresponded reasonably well with the level of TPH contamination (r^2=0.77), the variation in malformation frequency within the study based on the evaluated stage of development was quite dramatic. Several of the lesser contaminated sampling locations showed greater sensitivity during later larval development, whereas the more contaminated sites demonstrated greater sensitivity during the early embryo-larval stage and metamorphic stage. These results suggested that differences in stage sensitivity may be observed when evaluating development, therefore evaluation of different stages of development is important in ecotoxicological evaluation of amphibian development. Furthermore, since the COPECs evaluated in most studies of this type are complex mixtures, it is extremely difficult to predict which stages of development will be the most sensitive, requiring the use of multiple stages of development in the assessment.

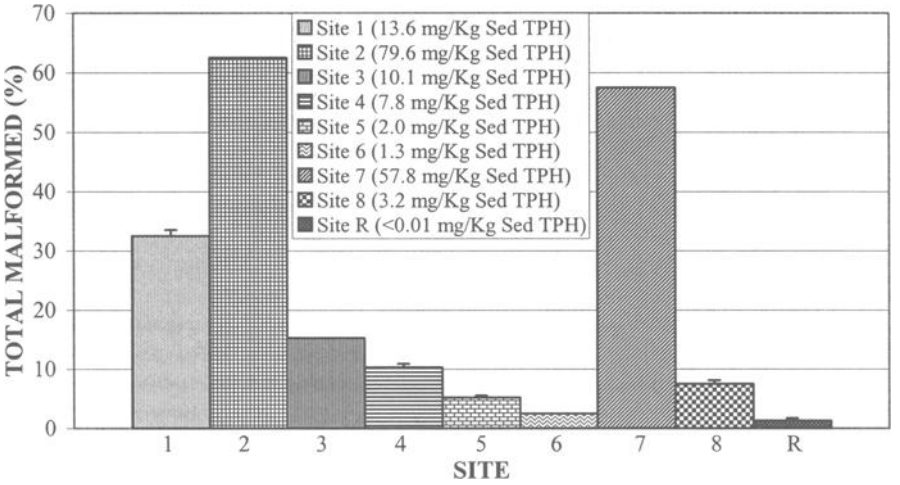

Figure 1 – *Effect of Sediment Petroleum Hydrocarbons on Embryo-Larval Malformation in Rana sphenocephala Cultured in situ*

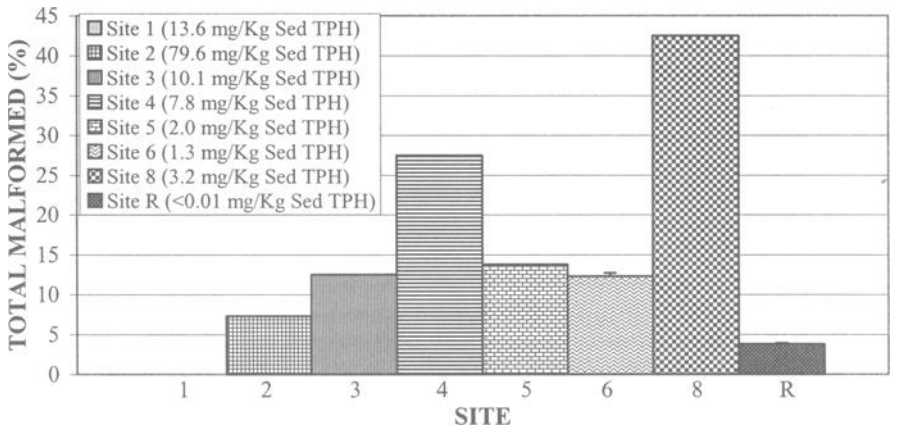

Figure 2 – *Effect of Sediment Petroleum Hydrocarbons on Prometamorphic-Larval Malformation in Rana sphenocephala in situ*

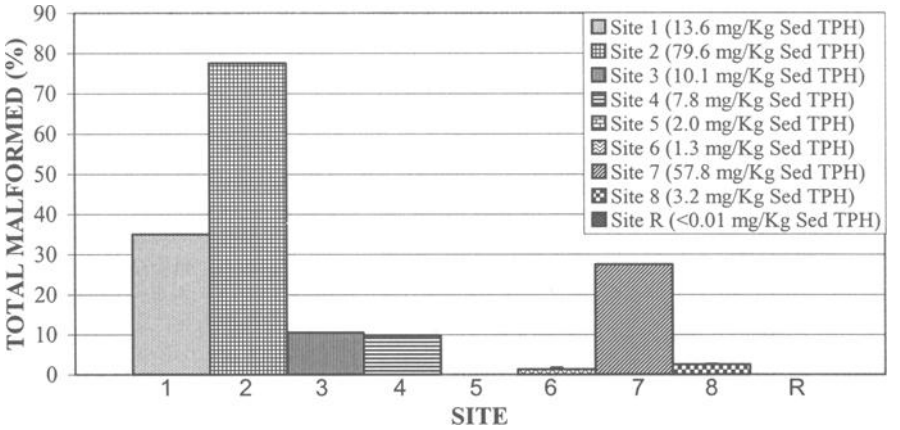

Figure 3 – *Effect of Sediment Petroleum Hydrocarbons on Metamorph Malformation in Rana sphenocephala in situ*

The malformation syndromes induced by the target sites locations were reasonably similar. Characterizing malformation syndromes in *in situ* and laboratory studies when evaluating laboratory-to-field extrapolation is often useful. For example, the occurrence of specific malformations during the *in situ* culture period from site 2 is presented in Figure 4. The malformation syndromes observed in *R. sphenocephala* cultured *in situ* were similar to those observed in field specimen (Figure 5).

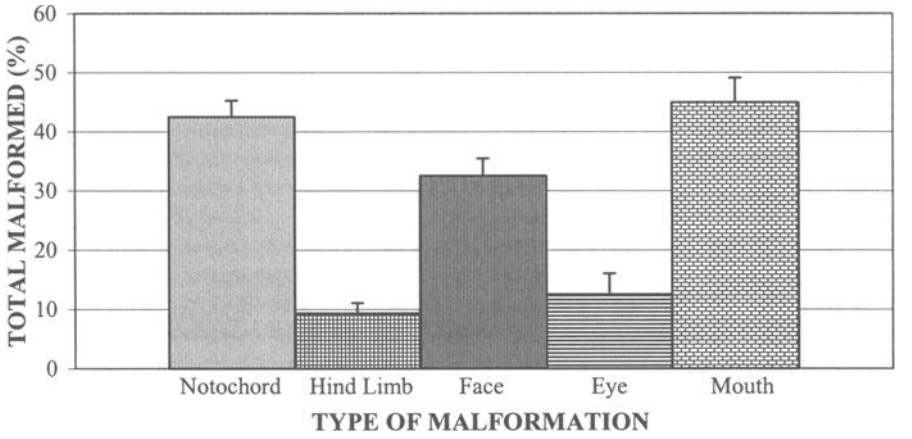

Figure 4 – *Types of Malformations Induced in Rana sphenocephala Cultured in situ at Site 2 – Representative Syndrome*

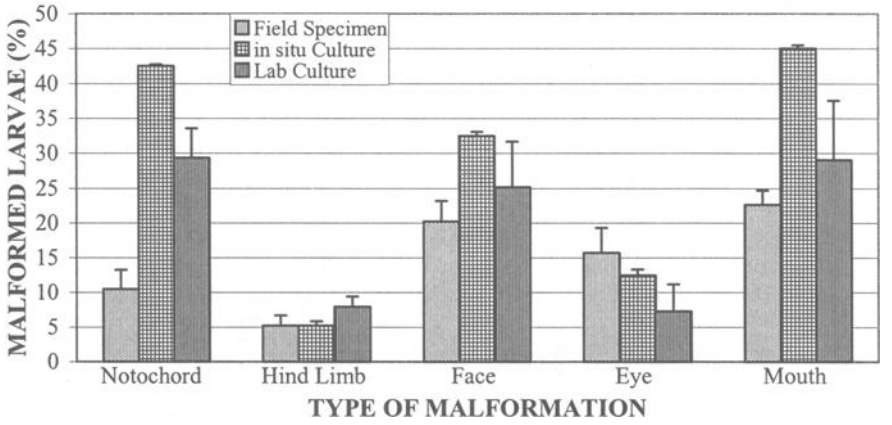

Figure 5 – *Comparison of the Types of Malformations Induced in Rana sphenocephala Cultured in situ and in the Laboratory from Site 2 in Relation to Malformations Observed in Field Specimen*

Laboratory-Based Studies - As observed in the *in situ* exposure studies, *R. sphenocephala* cultured under laboratory field conditions induced malformations at each of the more highly contaminated sampling locations. The mean malformation rates observed throughout the exposure period, which extended from egg mass to metamorphosis, were greatest in specimen cultured from sites 2, 3, 4, and 7 (Figure 6). No adverse developmental effects were observed prior to hatching, and hatching success was not appreciably altered at any of the test sites studied. The malformation syndromes observed in laboratory cultured *R. sphenocephala* (Figure 7) were similar to those observed in both field specimen and specimen cultured *in situ* (Figures 5).

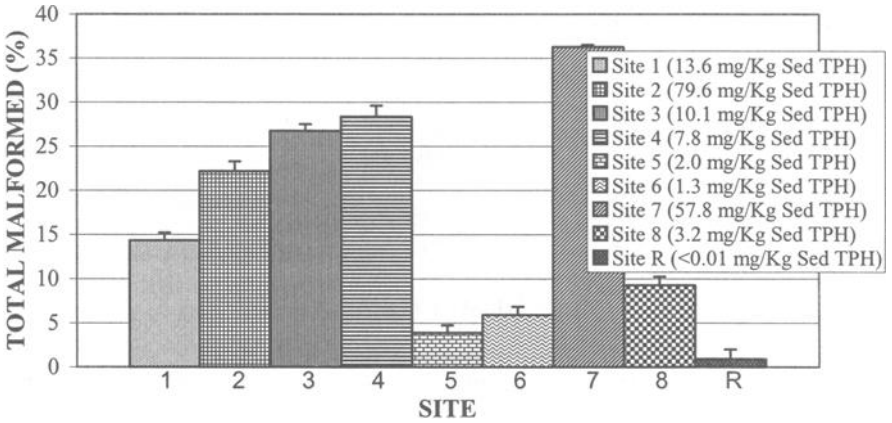

Figure 6 – *Effect of Sediment Petroleum Hydrocarbons on Prometamorphic Larval Malformation in Rana sphenocephala Cultured in the Laboratory*

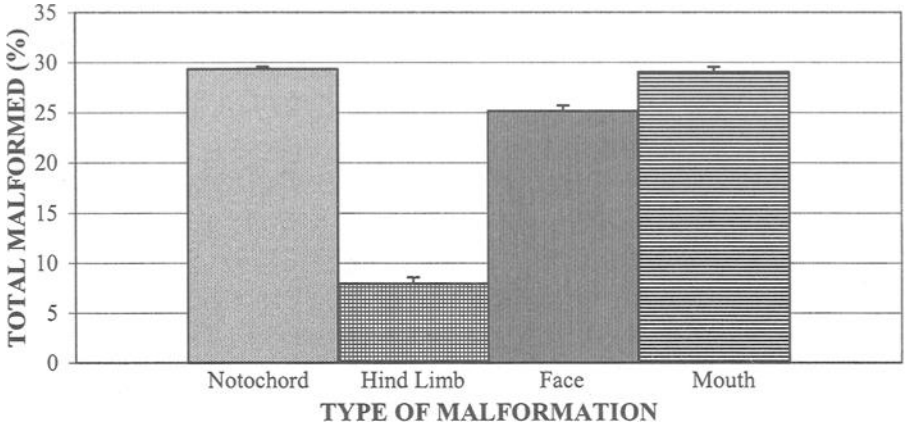

Figure 7 – *Types of Malformations Induced in Rana sphenocephala Cultured in the Laboratory from Site 2 – Representative Syndrome*

In effort to evaluate whether the developmental effects observed in specimen were the result of transgenerational transfer of COPECs from the parents to the progeny or the result of exposure of the COPECs to the developing organisms, cross-over exposure studies were performed in the laboratory. Results from these studies indicated that both routes of exposure contributed to the developmental toxicity observed (Figure 8).

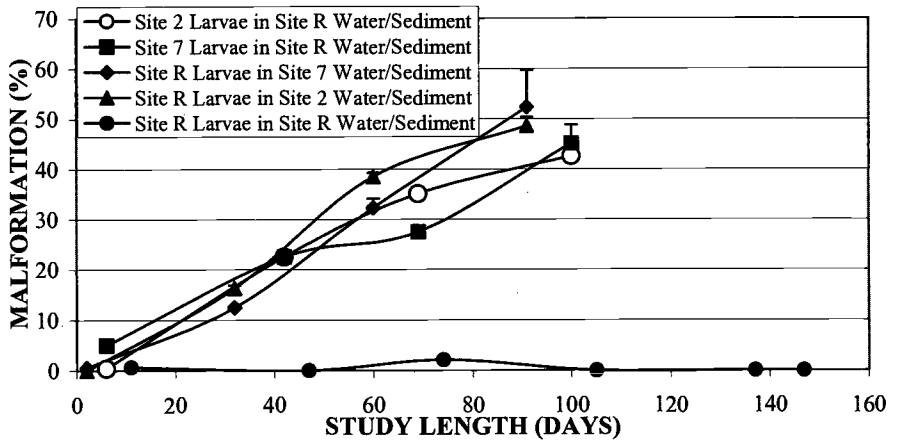

Figure 8 – *Results of Cross-Over Exposure Study in which Specimens from Contaminated Sites 2 and 7 were Cultured in Reference Site Water and Sediment and Specimens from the Reference Site were Cultured in Contaminated Water and Sediment (Sites 2 and 7)*

To determine whether TPH-DRO was capable of inducing malformations, a reference sediment was spiked with 25 mg/Kg of diesel fuel and evaluated for long-term developmental toxicity. Result from these studies suggested that the spiked sediment was capable of inducing larval malformation (Figure 9). In addition, the malformation syndrome including mal-development of the face, mouth, notochord (osteolathyrogenesis), and hind limbs (phocomelia) was similar to that observed in both the laboratory exposure studies, the *in situ* exposure studies, and the field specimens collected. The final component of the study was to evaluate a mechanistic link between exposure to the COPECs and the effects observed in the specimen. In this case, we focused on abnormal hind limb development in specimen exposed to the diesel contaminated reference sediment. A subset of specimen collected from this exposure were fixed and stained (hemotoxylin-eosin) at Gosner stages 25-27 (Gosner 1960) to evaluate the development of the early limb bud. The ectodermal ridge in specimen exposed to the diesel spiked reference sediment was a highly disorganized cell mass disjointed from the underlying inducing mesoderm. It appeared from these observations that the diesel fuel induced abnormal early development of the limb bud by disrupting cellular migration and communication processes, ultimately resulting in phocomelia. Eye malformations induced by exposure to diesel spiked sediment resulted in incomplete fusion of the lens and blistering of the pigmented retinal cone. Similar to induction of hind limb development, the induction of lens development may also be affected by diesel range petroleum hydrocarbons.

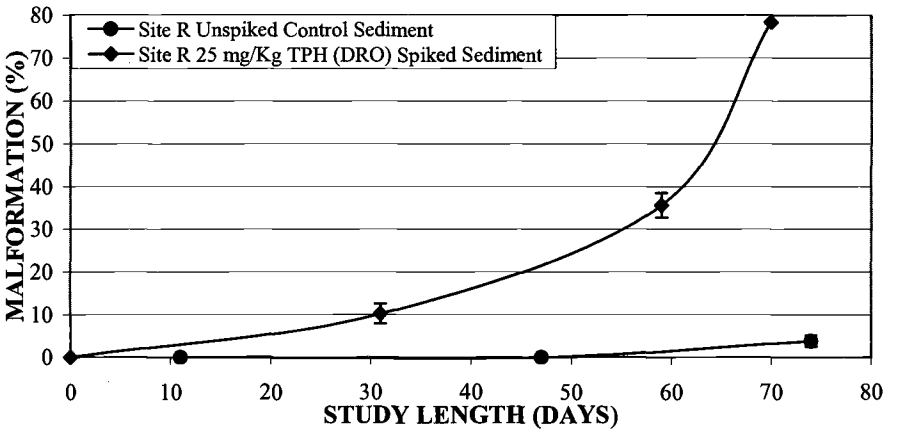

Figure 9 – *Effect of TPH Spiked Reference Sediment (25mg/Kg TPH[PRO]) on Rana sphenocephala Development in Laboratory Culture*

Conclusions

Establishing causality requires identification of the effects, proof of exposure, demonstration of the response, and identification of a mechanism of action (Meyers-Schöne 2000). Identification of the effect requires specific definitions of the endpoints

to be measured and levels (i.e., meta-population, community, and individual species) at which they will be measured. Proof of exposure requires measurement of the presence of COPECs in physical samples, as well as measurement in tissue residues. To demonstrate the effect, ecologically significant studies must be constructed that provide suitable laboratory-to-field extrapolation. This establishes the connectivity between the effects observed in more controlled laboratory studies and field studies. Finally, identification of mechanisms of action of COPECs provides the link between the presence of the COPEC and the effect induced by the COPEC at varying biological levels ranging from molecular to organismal. Because of the need for adequate causality data for ERAs, experimental designs for hazard assessment should be addressed in each of the four basic components described to establish multiple lines of evidence.

Acknowledgments

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Multiple Causes for the Malformed Frog Phenomenon

Reference: Lannoo, J. J., Sutherland, D. R., Jones, P., Rosenberry, D., Klaver, R. W., Hoppe, D. M., Johnson, P. T. J., Lunde, K. B., Facemire, C., and Kapfer, J. M., **"Multiple Causes for the Malformed Frog Phenomenon,"** *ASTM STP 1443, Multiple Stressor Effects in Relation to Declining Amphibian Populations*, G. Linder, S. Krest, D. Sparling, and E. Little, Eds., ASTM International, West Conshohocken, PA, 2003.

Abstract: Progress has been made in understanding the malformed frog problem, yet we still cannot identify with assurance specific causes of malformations at particular locations. To address this problem we assembled a team of specialists and present here results on geographic distribution, water quality, parasite infection, and morphological patterns from Minnesota malformed frog sites and reference sites. Malformed frog hotspots (> 5% malformed animals) tend to occur in a broad line from northwest to southeast across Minnesota associated with the North Central Hardwoods and Driftless Area ecoregions, and are less associated with Lake Agassiz Plain, Northern Glaciated Plain, and Western Corn Belt Plain ecoregions. Few hotspots occur in the southwestern grassland and northeastern boreal forested portions of the state. There is a tendency for

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hotspots to occur at ecoregion junctions. No single water quality feature correlates with hotspots. Heavy *Ribeiroia* infections always indicate hotspots, but lesser *Ribeiroia* infections may or may not. Conversely, certain hotspots show no evidence of the presence of *Ribeiroia*. Among reference sites, two have no evidence of *Ribeiroia*. The most common hindlimb malformation type was ectromelia, followed by micromelia and the presence of spongiform bone. Limb hyperextension, amelia, and polymelia were the least common malformation types. Malformed frog hotspots are typically associated with altered wetlands and any solution to the malformed frog problem must include restoring these sites.

Keywords: amphibian declines, malformations, habitat alteration, parasites, chemical contamination, habitat restoration

Despite no longer making headline news, the malformed frog problem has not been solved. It is true that progress has been made: malformation types have been described and general causes of amphibian malformations have been identified. Yet we still cannot answer the question foremost in the public's mind: Can we identify with assurance specific causes of malformations at particular locations? It is generally suspected that various causes are probably working at different sites, either individually or in combination. This explanation does little to assuage the concerns of affected landowners, especially those with young children. (If this sounds like hyperbole, realize that at one point during the malformed frog investigation, the state of Minnesota issued bottled water to some of these families [Souder 2000]).

There are several reasons why we have failed to achieve firm answers to questions about amphibian malformations (Souder 2000). One of the most important is that modern scientific inquiry favors specialists, and it is the tendency of specialists to see what they know and ignore what they do not (Steinbeck and Ricketts 1941). Given this nature, and given the multiple causes known to cause amphibian malformations, it is unlikely that individual researchers or individual research laboratories will generate results that fully explain the broader phenomenon. One solution to this problem is to assemble a team of specialists in relevant disciplines, and we have done this. Our team was funded through the U.S. Geological Survey's Amphibian Research and Monitoring Initiative (ARMI) and the Minnesota Pollution Control Agency (MPCA) and was divided into field biology (Hoppe, Lannoo, Sutherland, Kapfer), hydrology (Rosenberry, Jones), parasitology (Sutherland, Kapfer, Johnson, Lunde), landscape ecology (Klaver), and morphology (Lannoo). We present here our results for a subset of malformed frog sites in Minnesota, reference sites in Minnesota and in northwest Iowa, as well as sites from across the country (data from Johnson, Lunde, Facemire) that have relevance to our regional data.

Methods

We identified 17 sites for study (Fig. 1; site data summarized in Table 1) using techniques detailed in Helgen et al. (1998) and U.S. Geological Survey (2001). The majority of our sites (11; BUR, CBA, CTG, CWB, DOR, HIB, HYD, NEY, ROI, SUN, TRD) are considered "hotspots" by the Minnesota Pollution Control Agency (≥ 5% of animals with malformations in any single sample). Four Minnesota sites (BLO, GEL, IWPA and MHL) represent reference sites located within the same region. Two other sites (OKB1, OKB2) are located 150 km SW from the nearest known hotspot and represent reference sites distant from the region where hotspots are prevalent.

We sampled these sites for malformed frogs and characterized the sites in terms of origin (natural, human created [hereafter referred to as created], and

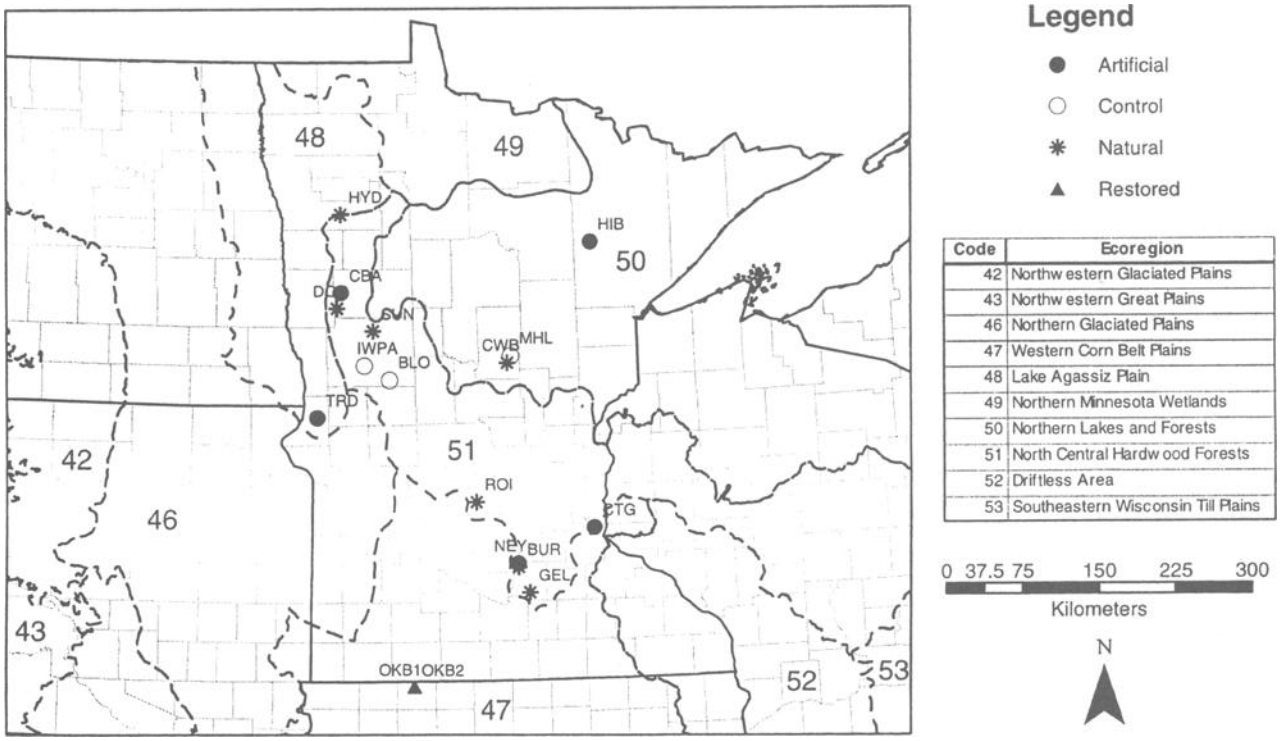


Figure 1 - *Malformed Frog Study Sites and Reference (Control) Sites Sorted by Origin (Artificial Hotspot, Control, Natural Hotspot, Restored).*

Table 1 – *Description and Classification of Each of 17 Sites Sampled for this Study.*

BLO. Semipermanent to permanent wetlands located immediately east of Block Lake, Ottertail County, Minnesota. One wetland was forested and located across a road, near lake level. A second wetland is located in an old field grassland about 200 m upland, separated from the first wetland by forested hillside. During our frog sampling visit both wetlands were nearly dry, making an assessment of macrophytes difficult. Block Lake leopard frogs have been sampled for many years by David Hoppe and Robert McKinnell because of the high frequency of burnsi morphs, which are leopard frogs exhibiting an autosomal dominant gene generally found in lower frequencies through the eastern portion of the Upper Great Plains (Merrell, 1965). Forty-four leopard frog and 11 wood frog adults were sampled. Classification: Natural, Reference site.

BUR. Small, permanent wetland in Le Sueur County, Minnesota originally chosen as a reference site for nearby NEY hotspot (see below), but malformations have appeared here. The wetland is located about 15 m from an agricultural field, which is separated from the wetland by a gravel road. The wetland is surrounded by willows (*Salix* sp.); emergent vegetation includes rushes (*Scirpus* sp.) and sedges (*Carex* sp.). The center of the wetland was mostly open water with some duckweed (*Lemna minor*). Macrophytes were sparse. Brook sticklebacks (*Culaea inconstans*) were present, as were a variety of aquatic invertebrates, including a planorbid snail (*Helosoma trivolvis*). Eighty-six leopard frog adults were sampled. Classification: Created, Hotspot.

CBA. A constructed, permanent wetland in Ottertail County, Minnesota. Wetland was made by diking a hillside punctuated with springs and fens. Water was cold (8 °C below air temperature at 1000 h), consistent with being spring fed. Dead trees were scattered along the periphery. In the water there were thick macrophytes, mostly coontail (*Ceratophyllum demersum*). Sticklebacks and mudminnows (*Umbra lima*) were present. A large number of invertebrates were also present including planorbid snails. One-hundred twenty-nine leopard frog adults and seven leopard frog tadpoles were sampled. Classification: Created, Hotspot.

Table 1 (continued)

CTG. Permanent wetland constructed to control stormwater runoff in the city of Cottage Grove, a southeastern suburb of St. Paul located in Washington County, Minnesota. The site is located in Pinetop Park, surrounded by housing developments. The wetland shoreline is partially earthen, partially paved with asphalt. Garbage, including broken lawn chairs, a television, beverage cans and (mostly broken) bottles, litters the shoreline and the wetland bottom. Rainwater enters this basin from the west. This area has fluctuating water levels and contains stands of cattails. The water is colored pea soup green with phytoplankton. Planorbid snails and clam shrimp (concostracans) were present. Twenty-six American toads (*Bufo americanus*) were sampled. Classification: Created, Hotspot.

CWB. Natural lake located near Mille Lacs Lake, in Crow Wing County, Minnesota. Wetland is surrounded by mostly deciduous forest, which in turn is interspersed with agricultural land and roadways. One lakefront house is present. Cattle from an adjacent dairy farm use a portion of the wetland and have greatly eroded the shoreline bank adjoining our collection site. Cattails (*Typha* sp.) ring the shoreline, with stands of water lilies (*Nuphar* sp.) and arrowheads (*Sagittaria* sp.) beyond the cattails, and abundant three-way sedge (*Dulichium arundinaceum*) in the areas disturbed by fallen trees and a dock. Sunfish (*Lepomis* sp.), sticklebacks, and minnows (*Pimephales* sp.) are present, as are a variety of invertebrates, including planorbid snails. The wetland is the only site visited where carcasses of frogs and fishes were observed. Eighty-eight mink frogs and one green frog (*Rana clamitans*) were sampled. Classification: Natural, Hotspot.

DOR. Large, natural, permanent wetland located in Becker County, Minnesota. Water level is generally controlled by beaver dams, but a local lake association controls against extreme fluctuations. Cattails (*Typha* sp.) ring the wetland and also occur in floating mats in deeper water. Dead trees are scattered throughout the wetland, indicating historic times when the water levels were lower. Fifty-three leopard frog adults were sampled. Classification: Natural, Hotspot.

GEL. A large permanent wetland connected to Lake Jefferson in Le Sueur County, Minnesota. Bordered by both public and private land, the wetland smelled of cattle manure. The public land is mowed to keep noxious plants especially Canada thistle

Table 1 (continued)

(*Cirsium arvense*) in check. Cattails ring the shoreline. In the water, dense and extensive mats of duckweed were present. Macrophytes include coontail and at least 2 species of pondweed (*Potamogeton* sp.), including sago pondweed (*P. pectinatus*). Bowfin (*Amia calva*) and green sunfish (*Lepomis cyanellus*) were seined from the water. Fifty leopard frogs and 19 American toads were sampled. Classification: Natural, Reference site.

HIB. A small, constructed permanent wetland in St. Louis County, Minnesota, built in the shape of a ring (to facilitate ice skating) in 1996. Lawn grades to the pond edge, some rushes, sedges, and cattails are present. In the water, sago pondweed predominates. Snails were observed but were not sampled. One American toad, seven wood frogs, and 11 leopard frogs were sampled. Classification: Created, Hotspot.

HYD. Permanent wetland in Polk County, Minnesota, ringed with cattails, willows, swamp milkweed (*Asclepias incarnata*) and Canada thistle. Agricultural fields are more distant. Site is unusual in that the bottom is covered with 50–70 cm of loose muck, likely representing erosion from adjacent fields. Frogs were not found in association with the wetland but instead were found feeding in a mowed field across a gravel driveway. Sixty-five leopard frogs were sampled here. Classification: Natural, Hotspot.

IWPA. Small, semi-permanent wetland on state property in Ottertail County, Minnesota, ringed and dotted with cattails and sedges, duckweed and star duckweed (*Lemna trisulca*) in open water. The basin is surrounded by restored prairie with little bluestem (*Andropogon scoparius*) predominant; woods were more distant. Eight wood frogs and 11 leopard frogs were sampled. Classification: Natural, Reference site.

MHL. Lake located in Crow Wing County, Minnesota that served as a reference site for CWB (see above). Includes a sphagnum bog on the southeast side. The lakeshore is wooded and partially developed with cabins. Sunfish (*Lepomis* sp.) were observed in the water. Four mink frogs and nine green frogs were sampled. Classification: Natural, Reference site.

Table 1 (continued)

NEY. Large, constructed wetland located in Le Sueur County, Minnesota and surrounded by old fields and agricultural fields now associated with a nature center. Sago pondweed predominates macrophytes and forms thick beds that restrict water currents and tend to produce pockets of warm water. Snails predominate. One-hundred fifteen leopard frogs were sampled. Classification: Created, Hotspot.

OKB1. Large, semipermanent wetland restored about 5 years ago under the U.S. Fish and Wildlife Service's Waterfowl Production Area program located 0.8 km west of Welch Lake in Dickinson County, Iowa. Ringed by cattails with a variety of macrophytes and invertebrates. Planorbid snails were present. Nine American toads and 20 chorus frogs (*Pseudacris triseriata*) were sampled. Classification: Restored, Reference site.

OKB2. Small, semipermanent wetland restored about 5 years ago under the U.S. Fish and Wildlife Service's Waterfowl Production Area program located 0.8 km west and 0.8 km south of Welch Lake in Dickinson County, Iowa. This site is ringed by cattails with a large cattail stand along the west side. Several species of macrophytes and invertebrates, including planorbid snails, were present. Eleven leopard frogs were sampled. Classification: Restored, Reference site.

ROI. Shallow, semipermanent natural wetland in Meeker County, Minnesota. The basin is surrounded by forest on the north and west sides and by mobile homes and lawns on the south and the east sides. More trash was found here than at any site except CTG. Duckweed covered over 80% of the water's surface. Emergent cattails and grasses were present, indicating a history of drying. A large number of invertebrate species were observed. One American toad, 11 leopard frogs, and four wood frogs were sampled. We also captured a gray treefrog (*Hyla versicolor*) tadpole and one salamander in the *Ambystoma laterale* complex. Classification: Natural, Hotspot.

SUN. Deep fringing wetlands associated with Paul Lake in Ottertail County, Minnesota. The wetlands are situated partially in woods, partially in the open and are gradually being filled and used for expensive lakeshore housing. The remaining habitat appeared healthy with a large number of invertebrates. Eleven leopard frog and nine

Table 1 (continued)
wood frog adults were sampled. Classification: Natural, Hotspot.

TRD. Small but deep constructed wetland on public school grounds located in Traverse County, Minnesota. The basin is composed of hardpan clay with steep sides, ringed by cattails. Well water is pumped in periodically to prevent pond drying. In August, during our sampling visit, few macrophytes were established in the open water, although by September coontail was dense (Hoppe, personal observations). Few invertebrates were observed and in three years of sampling, no snails of any species have been collected (Hoppe, personal observations). Various species of fish, including bullheads (*Amieurus* sp.) and fathead minnows (*Pimephales promelas*) have been introduced, although no fish were observed or captured in 2001 (Hoppe, personal observations). Two American toads and 14 leopard frogs were sampled. Classification: Created, Hotspot.

restored), and malformed frog history (hotspot or reference site; below, see also Table 1). A subsample of frogs from each site was collected (Minnesota Department of Natural Resources Special Use Permit No. 10510), dissected for parasites (Johnson et al. 2002), then radiographed (Table 2; Lannoo 2000). Our team of hydrologists also sampled these sites using a battery of water quality tests, including major ion composition (Brinton et al. 1996, Mitko and Bebek 2000), nutrients (Antweiler et al. 1993), and dissolved organic carbon (Wershaw et al. 1983, http://water.usgs.gov/owq; and below). These various datasets were then summarized and analyzed with reference to each other.

Results

Sample Sites

From the 17 sites visited (Table 1) we sampled a total of 837 amphibians, 274 of which were subsampled for parasite and radiographic analyses (Table 2).

Geographic Distribution —During the course of our sampling, it became apparent that hotspots are brought to the attention of authorities when there is a congruence of amphibian malformations and humans interested in the outdoors. This occurs with school group and scouting group trips, and in areas where people enjoy wildlife (see also Souder 2000). We found one previously unreported hotspot (9.1% malformation frequency) simply by being curious about a roadside wetland. Therefore, we must view this map as the product of a non-systematic sampling effort. If we believe the map to be generally representative (and there is independent evidence of this, see USGS 2001 and NARCAM 2001 for similar patterns) malformed frog hotspots tend to occur in a broad line from northwest to southeast across Minnesota—that is, few reported hotspots occur in the southwestern grassland and northeastern boreal forested portions of the state (Fig. 2). One way to characterize this pattern is through the use of Level 3 Ecoregions (originally defined by Omernik 1987; but since revised [see U.S. Environmental Protection Agency 1999]). In this analysis, hotspots are more associated with the North Central Hardwoods and Driftless Area ecoregions, less associated with Lake Agassiz Plain, Northern Glaciated Plain, and Western Corn Belt Plain ecoregions (Fig. 2). There may be some tendency for hotspots to occur at the junctions of recognized ecoregions (Fig. 2).

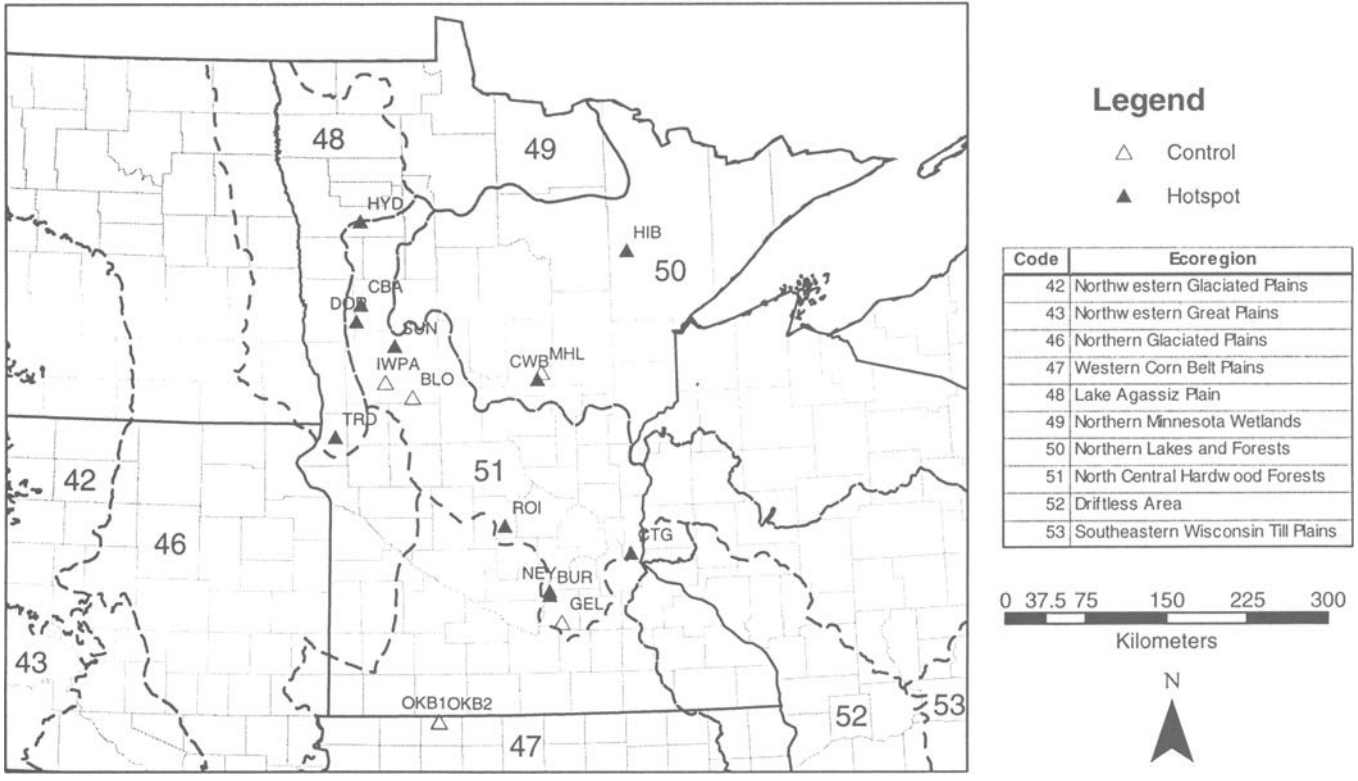
Water Quality Data —Few distinguishable patterns emerged with respect to a ranking analysis of the twenty-two water quality features that were considered (Table 3). No single water quality feature was related to hotspots. A few conclusions can be drawn, however. For example, water conductivity was highest in two northwest constructed wetlands (CBA, TRD) and lowest in two northeast natural wetlands (CWB, MHL). Similarly, alkalinity was highest in one northwest constructed wetland (CBA) and one northwest natural wetland (IWPA), lowest in two northeast natural wetlands (CWB, MHL). CWB and MHL also had the lowest calcium, magnesium, and sodium values.

Hotspots versus reference sites, and natural versus artificial sites could not be distinguished based on measures of pH, nitrogen, phosphorus, dissolved organic carbon, hardness, potassium, chloride, sulfate, fluoride, silica, iron, manganese, or dissolved solids.

As a second attempt to determine patterns in the water quality data, we identified the two highest and two lowest values for each feature measured and asked which wetlands were notable for these outlying values. IWPA (natural, reference site) had the highest number of outliers (10 out of 22 possible) followed by CBA (9; created, hotspot)

Table 2 – *Numbers of Anurans Examined, Sorted by Species, from Each Wetland Site. Numbers in Parentheses are the Animals Kept, Dissected for Parasites, and Radiographed. See Table 1 for Full Site Names and Descriptions.*

Site	*Bufo americanus*	*Pseudacris triseriata*	*Rana clamitans*	*Rana pipiens*	*Rana septentrionalis*	*Rana sylvatica*	Total
BLO				44 (10)		11 (10)	55 (20)
BUR				86 (12)			86 (12)
CBA				136 (13)			136 (13)
CTG	26 (26)						26 (26)
CWB			1 (1)		88 (9)		89 (10)
DOR				53 (9)			53 (9)
GEL	19 (10)			50 (10)			69 (20)
HIB	1 (1)			11 (11)		7 (7)	19 (19)
HYD				65 (10)			65 (10)
IWPA				11 (11)		8 (8)	19 (19)
MHL			9 (9)		4 (4)		13 (13)
NEY				115 (11)			115 (11)
OKB1	9 (9)	20 (20)					29 (29)
OKB2				11 (11)			11 (11)
ROI	1 (1)			11 (11)		4 (4)	16 (16)
SUN				11 (11)		9 (9)	20 (20)
TRD	2 (2)			14 (14)			16 (16)
Total	67 (49)	20 (20)	10 (10)	618 (144)	92 (13)	30 (38)	837 (274)



Code	Ecoregion
42	Northwestern Glaciated Plains
43	Northwestern Great Plains
46	Northern Glaciated Plains
47	Western Corn Belt Plains
48	Lake Agassiz Plain
49	Northern Minnesota Wetlands
50	Northern Lakes and Forests
51	North Central Hardwood Forests
52	Driftless Area
53	Southeastern Wisconsin Till Plains

Figure 2 - *Location of Hotspot versus Reference (Control) Study Sites.*

Table 3 – *Basic Water Quality Data for the 17 Sites Sampled in the Minnesota Malformed Frog Survey. For Details on How These Data are Collected and Analyzed, see http://water.usgs.gov/owq. Bold Numbers Indicate the Two Highest and the Two Lowest Values Measured for Each Test.*

Station Name	Sp. Cond. (mS/cm)	DO (mg/L)	pH (units)	Alk (mg/L as $CaCO_3$)	Nitro Amn & Org Dis (mg/L as N)	Nitrogen Amm + Org Tot (mg/L as N)
BLO	237	**8.32**	8.36	108	0.864	**1.26**
BUR	291	7.49	7.71	132		
CBA	**738**	2.18	7.25	**311**	**0.47**	**0.71**
CTG	143	**7.95**	**9.37**	32	**0.672**	2.656
CWB	**45**	7.59	**9.00**	**21**		
DOR	425	**0.18**	**6.67**	204	**2.087**	**2.728**
GEL	316	0.72	7.05	142	1.712	**2.963**
HIB	255	5.06	8.92	69	0.941	1.272
HYD	458	6.79	**6.81**	188	1.602	2.074
IWPA	531	0.20	7.01	**266**	1.267	1.641
MHL	**30**	6.79	8.44	**12**		
NEY	336	6.95	7.42	141	1.757	1.958
OKB1	433	1.33	8.99	214	2.541E	2.46
OKB2	465	6.33	7.85	239	2.017	2.179
ROI	306	0.41	**6.81**	145	**2.588**	2.686
SUN	535	**0.15**	7.52	211	1.294	1.685
TRD	**627**	4.01	7.71	236		

Table 3 (continued)

Station Name	Phosphorus Total (mg/L as P)	Phosphorus D (mg/L as P)	Carbon Organic Dis. (DOC)	Ion Balance (% Difference)	Hardness (Total) mg/L as CaO3	Sodium D mg/L as Na	Potassium D mg/L as K
BLO	**0.0704**	0.0173	9.66	-5.3	110	2.19	6.49
BUR			11.50	2.93	160	1.2	1.11
CBA	0.168	0.1029	**5.47**	-1.67	**380**	6.43	2.64
CTG	0.239	0.025	**8.80**	**-11.11**	**24**	17.81	2.17
CWB		**< 0.01**	12.10			**0.8**	**0.81**
DOR	0.343	0.276	17.61	1.14	210	3.42	3.12
GEL	0.6	0.1069	17.99	5.38	160	4.98	2.38
HIB	**0.0769**	0.0292	14.96	-3.27	**79**	**19.58**	3.11
HYD	0.1718	0.0631	19.67	-2.29	210	2.73	4.35
IWPA	0.218	**0.1306**	18.14	**0.24**	**260**	1.48	5.28
MHL		**< 0.01**	9.40			**0.48**	**0.57**
NEY	0.208	0.1199	11.08	**8.87**	170	3.13	3.13
OKB1	**0.428**	0.363E	**29.23**	4.11	220	2.46	**10.85**
OKB2	0.241	0.1231	21.15	3.49	250	4.71	6.29
ROI	**1.533**	**1.078**	**33.66**	6.21	140	5.88	**11.15**
SUN	0.1233	0.0748	15.13	**0.04**	200	**30.34**	1.59
TRD		**< 0.01**	10.50			82	7

Table 3 (continued)

Station Name	Chloride D mg/L as Cl	Sulfate D mg/L as SO4	Calcium D mg/L as Ca	Magnesium D mg/L as Mg	Flouride D mg/L as F	Silica D mg/L as SIO2	Iron D (ug/L as Fe)
BLO	5.32	2.19	23.15	12.17	0.085E	5.41	42.35
BUR	3.76	12.73	37.72	15.05	0.25	1.01	17.29
CBA	4.59	**87.16**	**84.84**	40.2	0.192	23.28	62.74
CTG	19.5	3.5	6.39	1.86	<0.2	4.31	**15.59**
CWB			**5.3**	**1.4**		0.58	
DOR	5.79	4.07	42.31	25.16	**0.17**	19.49	229.3
GEL	10.72	1.5	37.61	16.38	**0.167**	15.25	295.76
HIB	**36.57**	1.92	18.68	7.81	0.095E	**0.28**	95.45
HYD	10.22	**25.87**	49.16	20.7	0.267	13.63	**12.93**
IWPA	**1.24**	0.48	**58.57**	**26.83**	0.110E	**67.13**	178.34
MHL			**4.3**	**0.89**		**0.45**	
NEY	8.58	0.92	33.12	21.37	0.207	13.65	**564.82**
OKB1	**3.03**	1.08	50.65	22.57	**0.528**	1.14	71.3
OKB2	5.96	1.1	49.43	**30.22**	**0.349**	3.3	259.18
ROI	4.5	**0.36**	39.77	10.51	0.087E	2.15	**495.97**
SUN	**41.29**	**0.47**	43.36	22.53	0.084E	**26.04**	125.49
TRD			25	14		19	

Table 3 (continued)

Station Name	Manganese D (ug/L as Mn)	Dissolved Solids (mg/L)
BLO	38.33	**132**
BUR	30.79	152
CBA	215.14	**436**
CTG	**<3**	**86**
CWB		
DOR	296.05	226
GEL	626.58	175
HIB	**4.42**	136
HYD	99.65	240
IWPA	**1251.45**	**322**
MHL		
NEY	**889.14**	170
OKB1	2.21E	220
OKB2	175.44	245
ROI	428.73	162
SUN	261.54	292
TRD		

and CTG (8; created, hotspot), CWB (8; natural, hotspot), MHL (8; natural, reference site) and ROI (8; natural, hotspot). Again, no patterns emerged.

Parasite Data

Several trends emerged from the parasite data (Table 4). Encysted echinostome metacercariae were found in the kidneys of animals from every site (Table 4). Other metacercariae, such as *Fibricola cratera* (Fib) and ochetosomatids (Mano), were found in frogs from a majority of wetlands. For the remaining parasite species there was strong site specificity; they could be present, often in high numbers, in amphibians from one site and completely absent in another. This was true of *Ribeiroia ondatrae*, which is important here because of the role it plays in causing amphibian malformations (Johnson et al. 1999, 2002).

Heavy *Ribeiroia* infections were indicative of hotspots (e.g., CTG, CWB, HIB) but lesser *Ribeiroia* infections might (e.g., BUR, GEL, NEY, ROI) or might not be associated with malformations (e.g., MHL; Table 4). Conversely, hotspots such as CBA, DOR, HYD, SUN and TRD showed no evidence of the presence of *Ribeiroia*. Among the four reference sites, the two Iowa wetlands (OKB1, OKB2) and IWPA also showed no evidence of the presence of *Ribeiroia*.

In 1999, four severely malformed mink frogs necropsied from CWB harbored a mean intensity of 110 *Ribeiroia* metacercariae (range 96-125). In 2000, 12 northern leopard frogs (10 malformed) from HIB were infected with *Ribeiroia* (mean intensity 155.5, range 51-266). Interestingly, the only two apparently normal frogs in the 2000 HIB sample had the two smallest *Ribeiroia* infections (51 and 52 metacercariae). Malformations at HIB in 2000 included cutaneous fusions, truncations, bony protuberances and soft tissue protuberances.

Where *Ribeiroia* occured, metacercariae were not found in every species of amphibian inhabiting the wetland. For example, in MHL (natural, reference site) *Ribeiroia* metacercariae were found in mink frogs (n = 4) but not in green frogs (n = 9); in ROI (natural, hotspot) *Ribeiroia* were found in leopard frogs (n = 11) but not in wood frogs (n = 4) or American toads (n = 1). This pattern may be due to sampling artifact. In general, species with longer larval stages have higher rates of *Ribeiroia* infection, and higher rates of infection among these species often correspond to higher numbers of amphibian species being infected.

At wetlands where *Ribeiroia* was present in every species of amphibian, infection rates varied across species. For example, at CWB (natural, hotspot) mink frogs (n = 9) averaged 35.4 *Ribeiroia* metacercariae per animal while the single green frog sampled had 12, a lower number than any mink frog. Similarly, at HIB (created, hotspot), wood frogs (n = 7) averaged 17.4 *Ribeiroia* metacercariae, leopard frogs (n = 11) averaged 7.9, and the one American toad had 3. An alternate explanation is to tie low American toad infection rates to their small body size (and therefore small target area for roaming cercariae). This may in part be true, but note the high *Ribeiroia* values for CTG (created, hotspot) American toads (17.1; n = 26), and HIB wood frogs (17.4; n = 7), both small-bodied anurans.

Perhaps the most surprising finding is the distribution of amphibians infected with *Ribeiroia* metacercariae (Fig. 3). Prior to this study, none of us had realized the strong tendency for *Ribeiroia* to occur predominantly in eastern Minnesota wetlands. This tendency extends to other sites in southeastern Minnesota and western Wisconsin (D. Sutherland, unpublished data). The majority of these sites are within the North Central Hardwood ecoregion although three occur in the Northern Lakes and Forest ecoregion. In our sample, no *Ribeiroia* sites occurred in grassland ecoregions.

Table 4 – *Parasites Dissected from Frogs Sampled During this Study. See Table 1 for Site Names. The First Number in each Column Represents Percent Occurrence. The Number in Parentheses is Mean Prevalence (Number of Parasites/Number of Animals Sampled).*

Site Code	Frog Species	Rib[1]	Fib[2]	Alaria	Globbie[3]	Mano[4]	Clino[5]	Thickie[6]	Kid Echin[7]	Gill Echin[8]	Masses	Choled
BLO	Rasy		20 (0.4)	80 (45)		40 (4.1)			90 (43.7)		20 (1)	
	Rapi		60 (2.8)		30 (0.3)	100 (62.8)			80 (16.1)	30 4.7	10 (0.6)	
BUR	Rapi	58 (3.08)	58 (5.5)		67 (4.7)	92 (3.2)			100 (55.7)			
CBA	Rapi		77 (58.4)	31 (9.3)	46 (2.2)	100 (118.9)			85 (13.0)	23 (4.2)	15 (1.0)	
CTG	Buam	100 (17.1)					4 (0.04)		42 (1.3)	4 (0.04)		
CWB	Rase	100 (35.4)		78 (62.8)	67 (1.9)	22 (0.2)	11 (0.1)		100 (241.4)		89 (36.1)	
	Racl	100 (12)			100 (1)				100 (275)			
DOR	Rapi		33 (1.9)			100 (37)			100 (25.7)	11 (22.2)		
GEL	Rapi	20 (3.4)	30 (9.4)	50 (60.9)	40 (1.2)	20 (0.6)	22 (2.0)		89 (40.1)		11 (1.5)	
	Buam		10 (1.2)	10 (0.4)								

Table 4 (continued)

Site Code	Frog Species	Rib[1]	Fib[2]	Alaria	Globbie[3]	Mano[4]	Clino[5]	Thickie[6]	Kid Echin[7]	Gill Echin[8]	Masses	Choled
IWPA	Rapi		9		55	73			82	9	18	
			(0.2)		(6.8)	(29.0)			(39)	(2.7)	(0.7)	
	Rasy		50	63	12	75			75		12	
			(7.2)	(6.9)	(0.4)	(9.2)			(86)		(0.7)	
MHL	Rase	25			100		50		50		100	
		(5)			(9)		(2)		(18.2)		(16.7)	
	Racl		11		44	11			44		100	
			(2.1)		(2.4)	(0.1)			(16)		(19.1)	
NEY	Rapi	36	27		91	18	27		91			
		(1)	(3.6)		(30.5)	(2.1)	(0.9)		(13.2)			
OKB1	Buam		44						11			
			(3.5)						(1.3)			
	Pstr		15						25			35
			(11.6)						(1.2)			(2.6)
OKB2	Rapi		91	9	45	55	9		82		45	
			(112.4)	(10.6)	(43.8)	(146.9)	(0.1)		(114.4)		(8.1)	
ROI	Buam								100			
									(10)			
	Rasy			50					25			
				(9.2)					(4)			
	Rapi	36	18	18	64	64			91		36	
		(2.1)	(21)	(18.4)	(24.8)	(7.6)			(96.9)		(2.9)	

Table 4 (continued)

Site Code	Frog Species	Rib[1]	Fib[2]	Alaria	Globbie[3]	Mano[4]	Clino[5]	Thickie[6]	Kid Echin[7]	Gill Echin[8]	Masses	Choled
SUN	Rasy		89 (22.7)						33 (1.6)			
	Rapi		55 (62.2)	18 (6.4)	45 (5.4)	82 (6.5)	9 (0.3)	18 (1)	45 (2.7)	55 (10.5)		
TRD	Rapi		93 (67.2)			93 (27)			79 (6.8)			
	Buam		50 (3)									

[1] Rib = *Ribeiroia ondatrae*

[2] Fib = *Fibricola cratera*

[3] Globbies = a cohort of metacercariae that all possess globular excretory bladders; this group includes *Glypthelmins quieta*, *Auridistomum chelydrae* and numerous undetermined metacercariae

[4] Mano = ochetosomatid metacercariae; this group includes a number of trematodes that inhabit the lungs, pharynx and oral cavity of snakes (short for *Manodistomum* which is the worm Sessions and Ruth (1991) originally thought was the cause of malformations in *Hyla regilla* in California)

[5] Clino = *Clinostomum* is the yellow grub common in inshore fishes (but also common in frogs); adults are found in pharynx of herons, egrets and perhaps black-crowned night herons

[6] Thickie = *Apharyngiostrigea pipientis* possesses an extremely thick walled cyst

[7] Kid Echin = encysted echinostomes in kidney; Rebecca Cole (personal communication) believes there are several species involved

[8] Gill Echin = encysted echinostomes along cranial nerves exiting brain near gill resorption site

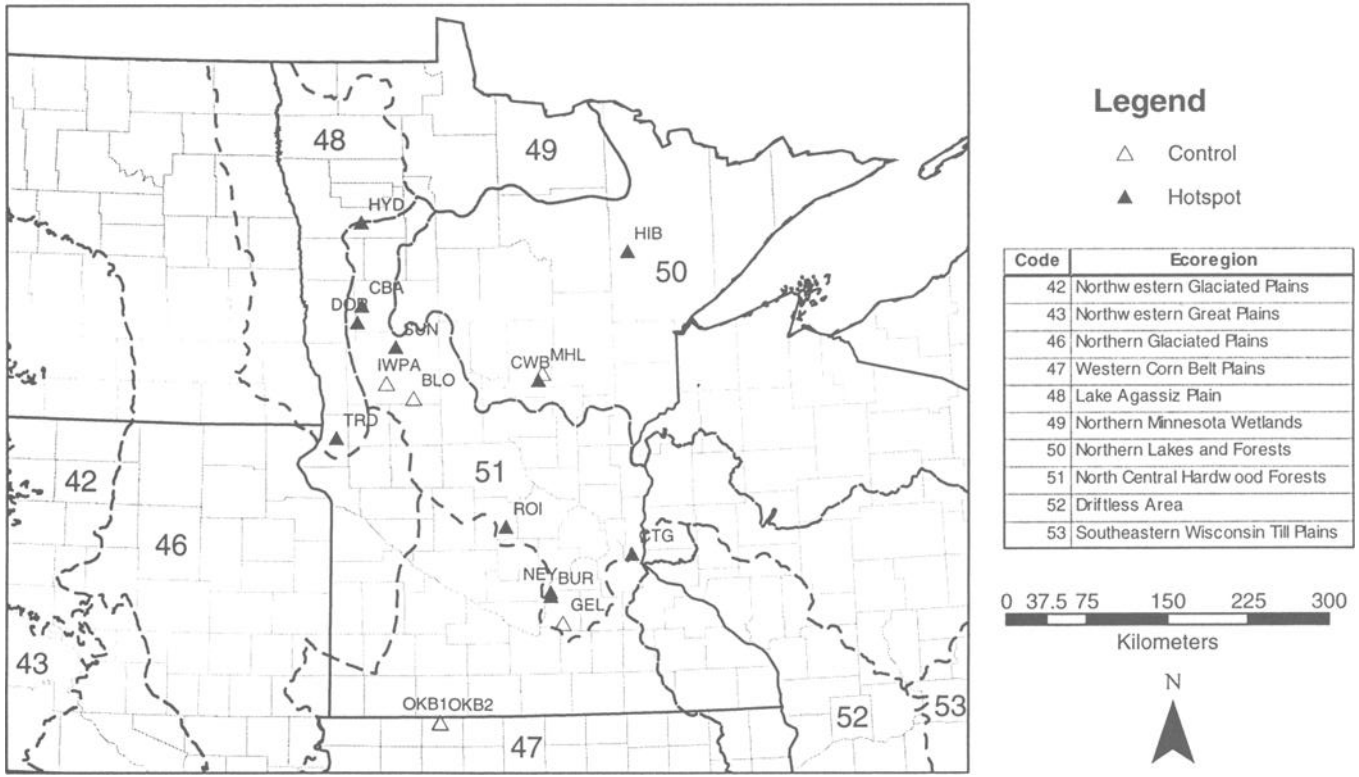


Figure 3 - *Location of Sites with the Trematode Parasite* Ribeiroia *Present Compared with Sites where the Parasite was Absent.*

Morphological Analysis

The 274 animals examined morphologically represented 6 species from 17 sites in Minnesota and Iowa. They form a subset of the 977 animals representing 16 species one of us (Lannoo) has examined from 73 sites in 13 states. For this analysis, we follow Meteyer et al. (2000, see also Johnson et al. 2001b, Ouellet 2000, Ouellet et al., 1997) and focus our attention on hindlimbs. Our malformation categories are summarized in Table 5 and include: amelia (completely missing limb, often times associated with missing pelvic elements); polymelia (duplications of limbs or limb elements); hemimelia (missing leg elements); micromelia (limb elements present but small); taumelia (or bony triangles, right angle, or nearly right angle bends in bones, see Gardiner and Hoppe 1999); limb hyperextension (rigid leg with immobility at hip, knee, and ankle joints; and spongiform bone (expansion of the cancellous bone at the distal tip of ectromeliac limbs or associated with taumelias; expansions are typically terminal, irregularly shaped, and only present on the affected limb).

We present our data by presence of malformation type by wetland (Table 5). Note that we do not present absence, nor do we present percentages, because neither our samples, nor samples from any other field studies conducted to date, have been done with sufficient rigor (i.e., drift fence studies) to determine that all malformed animals were sampled (and therefore that we can be truly sure malformation types were absent). Therefore, the only facts we can state are that animals with particular malformation types were found at particular wetlands (see Discussion for a more complete rationale).

The most common hindlimb malformation type (Ouellet et al. 1997, Meteyer et al. 2000) was ectromelia, followed by micromelia and the presence of spongiform bone. Limb hyperextension, amelia, and polymelia were the least common malformation types.

ROI, NEY, and CWB had the largest number of hindlimb malformation types, and aside from reference sites (GEL, IWPA, OKB1, OKB2), DOR, SUN, and TRD had the fewest (although HIB has produced large numbers of malformed frogs in past years, we collected no malformed animals in 2001 from HIB).

Discussion

Sites

Location — Malformed frog hotspots sampled in this study were not evenly, randomly, or even haphazardly distributed across Minnesota (Fig. 2); instead, most hotspots occurred in ecoregions with a mixed forest component. Hotspots do not tend to occur in ecoregions with a predominantly grassland or boreal forest component. Hotspots also tend to occur along the borders of ecoregions, not in their centers. Interestingly, humans also tend to live along the borders of ecoregions. Knowing this, several questions arise, including: does this pattern of hotspots reflect the true pattern of malformation locations? If it does, does this then reflect the direct influence of humans on the environment? If it does not, does it simply reflect the bias that human outdoor activities bring to the discovery of events in nature?

Origin — Hotspots can occur in natural or created wetlands; CWB is a natural site, HIB and NEY are created. It is important to realize that none of the 17 sites sampled were natural in any true sense. From among the reference sites, IWPA is surrounded by restored prairie, OKB1 and OKB2 are restored (although rich) wetlands, MHL has cabins along its north and east sides. Each of the other sites was associated with agriculture or housing developments. Some of the natural sites, such as ROI, HYD and

Table 5 – *Malformation Types Found at Each of Our Study Sites. See Table 1 for a Description of Sites, and the Text for a Description of the Malformation Types.*

Site name	Amelia	Polymelia	Ectromelia	Micromelia	Taumelia	Limb Hyper-extension	Spongiform Bone	Total
BLO					X			1
BUR				X			X	2
CBA								0
CTG	X		X		X		X	4
CWB		X	X		X	X	X	5
DOR			X	X			X	3
GEL								0
HIB								0
HYD	X		X	X			X	4
IWPA								0
MHL								0
NEY	X	X	X	X	X	X		6
OKB1								0
OKB2								0
ROI	X	X	X	X	X	X	X	7
SUN			X	X			X	3
TRD		X	X	X		X		4
Total	4	4	8	7	5	4	7	

CWB appear highly to moderately affected by human development and agricultural practices.

Water Quality — The battery of limnological tests we conducted did not reveal patterns of water quality, including acidification, underlying malformations. There can be several reasons for this, including that there are no patterns—that the chemistry of water does not influence the presence of malformations. This notion certainly contradicts some assumptions made during the course of the Minnesota Pollution Control Agency's investigation (Helgen et al. 1998, see Souder 2000); it also contradicts some results conducted from FETAX studies (Fort 1999a, 1999b) as well as other studies (Sparling 2000). A second reason may be that the problem is in the water, that it is chemical, but that it is separate from the more naturally-occurring chemicals sampled during limnological studies. Chemicals that would escape detection include pesticides, retinoids, and hormones, each of which have been implicated as causing amphibian malformations. We sampled for retinoids and certain hormones during the course of our study (analyses were not completed in time to make the manuscript deadline) but pesticides would not be detected in our sampling scheme. This explanation would be consistent with assumptions made by the Minnesota Pollution Control Agency and the results of Fort (1999a, 1999b).

A third reason for the lack of correspondence between water quality features and frog malformations may be that the problem is in the water but is biological rather than chemical. At least two lines of evidence (parasites, predation) suggest that biological causes can be important. The problem here is that biological causes cannot be generalizable to all hotspots. For example, failed predation can produce missing limbs, but the frequency of malformations at some sites in some years (> 60% of animals affected) and the absence of predators, or high densities of predators, at many sites argue against predation as a general cause. Similarly, the trematode parasite *Ribeiroia* has been shown experimentally to cause a wide range of malformation types in anurans (Johnson et al. 1999, 2001a) but these parasites do not occur at all malformation hotspots. Indeed, in one of our most intriguing results, *Ribeiroia* were only present in samples from the eastern half of Minnesota (Fig. 3).

A fourth reason for the lack of correspondence between water quality features and frog malformations could be that the problem is genetic. However, arguments for a genetic cause to malformations are undermined by three observations. First, hotspots are frequently found near sites that are not considered hotspots; close enough that individual frogs could, and probably do, migrate between normal sites and hotspots. Second, within hotspots, all or most species of frogs are affected. If there were a genetic cause underlying malformations, the observation that all species in one wetland are affected while no species in an adjacent wetland are affected would be highly improbable. Third, if there were a genetic component, one would expect that malformation types would sort by wetland types (that one wetland would produce, for example, amelia while another might produce polymelia; or that within wetlands, one species might produce amelia while another species would produce polymelia). So far, these patterns have not been observed. Additionally, Hoppe (in Volpe 2000) has experimental data that argue against genetic causes.

Morphological Signatures

This idea that morphology gives clues to malformation causes is implied by the title of the most recent paper on Minnesota frog malformations (Meteyer et al. 2000): "Hind limb malformations in free-living northern leopard frogs [*Rana pipiens*] from Maine, Minnesota, and Vermont suggest multiple etiologies." If true, this notion can be a

powerful tool. Most malformed frogs are observed following metamorphosis. But hindlimb development occurs days, weeks, or months before this, depending on the species and local environmental conditions. This temporal disparity may be sufficient to allow whatever caused the malformations to leave, be washed out, degraded, or be diluted to the point where it is (they are) undetectable. It is for this reason that the concept of morphological signatures—malformation types that uniquely identify malformation causes—has been attractive, because it allows causes to be inferred from morphology. Less desirable but still useful is the idea that certain malformation types may allow the exclusion of potential causes (Lannoo 2000).

For example, early in the investigation of U.S. amphibian malformations, "bony triangles" (taumelia) were seen as indicative of retinoic acid involvement (Gardiner and Hoppe 1999, see also Souder 2000). However the demonstration by Johnson et al. (1999) that *Ribeiroia odonatrae* metacercariae can also cause this malformation type (perhaps through the secretion of chemicals with retinoid properties) forced us to abandon the idea that bony triangles were unique morphological signatures for water-born exposure to retinoid, or retinoid-like compounds.

In another example, Ankeley et al. (1998) showed that exposure of tadpoles to UV-B radiation produces bilaterally symmetrical ectromelias. However, bilaterally symmetrical ectromelias are rarely found in nature (Souder 2000, Lannoo, unpublished data; see also Meteyer et al. 2000, Johnson et al. 2002). Further, when bilaterally symmetrical ectromelias are found, in our experience these malformations are associated with spongiform bony expansions. None of the animals produced by Ankeley et al.'s (1998) experiments exhibited spongiform bone. What we can say from Ankeley et al.'s data is that there is no evidence that unilateral malformations, or malformations with spongiform bone associations, are caused solely by overexposure to UV-B.

Hindlimb malformations may also be produced by failed predation attempts (see Lannoo 2000 for a full discussion of this possibility). However, 74% of the ectromelias in animals we sampled showed spongiform bone. Our results with experimental amputations (Lannoo, in preparation) show that (as with UV-B exposure) laboratory amputations also fail to produce spongiform bone. However, we cannot exclude the possibility that spongiform bone is produced by conditions in the wetland, perhaps by other animals aggravating the wound; conditions that are not duplicated in more controlled laboratory settings). What we can say is that there is no experimental evidence that ectromelias associated with spongiform bone are caused by failed predation attempts.

Johnson et al. (1999, 2001a, 2002) reported a range of malformations induced experimentally by the trematode parasite *Ribeiroia* and from field-collected animals containing encysted *Ribeiroia* metacercariae throughout the western USA. We radiographed 125 of these frogs. Eight species are represented and there appears to be no morphological signature for *Ribeiroia* infections. Hemimely, ectromely, and polymely were common malformations in 104 *Ribeiroia* infected animals. These malformation types were also common in 21 field-collected animals from sites with no evidence of parasites. Spongiform bone was not exhibited in Johnson et al.'s (1999) experimental animals and tends not be associated with *Ribeiroia* infections. We continue to explore this dissociation but have drawn no conclusions. Therefore, we determine that in contrast to the title (but, interestingly, not the text) of Meteyer et al. (2000) there is little evidence from Minnesota frogs that causes of malformations can be inferred from morphological signatures.

At the national level, however, three sites have produced malformation types that are so bizarre or unique that we suspect their causes to be unique. In Trempealeau County, Wisconsin, Sutherland sampled 27 newly metamorphosed, malformed green frogs (*Rana clamitans*) from a site that included 16 unilateral hindlimb ectromelic animals, 5 bilateral hindlimb ectromelic animals, 4 unilateral hindlimb ectromelic animals, one

hindlimb polydactly, and one animal with a unilateral taumelia. One animal had a unilateral hindlimb hemimely and a contralateral ectromely. This ectromely and one other ectromely were associated with missing pelvic elements, including iliums. Sixteen of the hemimelic animals (all but two cases) were associated with bony expansions. But unlike the spongiform bony expansions described here (above) and seen in most circumstances, expansions also occurred in the contralateral, normal (gross appearance) limbs in two hemimelic animals with bony expansions from this site (Fig. 4a). Also unlike spongiform bone, these expansions could be either terminally or subterminally positioned and were symmetrical. Subterminal expansions were not associated with the site of penetrating arteries. In four cases cancellous bone was clearly expanded in conjunction with compact bone. But in ten cases compact bone, not cancellous bone, was differentially expanded. Therefore, this Wisconsin site was unique in producing regularly-shaped, subterminal or terminal bone expansions characterized by abnormal compact bone growth. Furthermore, these expansions could occur in both ectromelic and the contralateral normal limbs. It is worth noting that in the four years of studying this site, not one metacercaria of *Ribeiroia* has been found.

One of Facemire's Switzerland County, Indiana, sites contained a sample of 13 late tadpole stage or newly metamorphosed bullfrogs (*Rana catesbeiana*) and demonstrated several types of malformations. Four animals had edematous swellings, which we term hygromas (subcutaneous, serous fluid-filled swellings), surrounding their lower limbs or associated with their ventral pelvic regions (Fig. 4b). Two of these animals had bilaterally symmetrical, serially arranged hygromas, with separate edemas occurring in femoral and tibiofibular segments—that is, when these hygromas were associated with a limb they encompassed a limb segment, extending from joint to joint, for example hip to knee, knee to ankle. One of these animals had a pair of duplicated hindlimbs. Other malformations occurring in this sample included a malformed mandible, a kinked tail, and a hindlimb ectromely. Radiographs of the proximal femur of this limb showed no spongiform bony expansion.

One of Johnson and Lunde's Santa Clara County, California sites contains bullfrogs with *Ribeiroia* infections. These animals are characterized by having long bones that bend at the site where nutrient arteries penetrate (Fig. 4c). This is true for all long bones in a hindlimb, true bilaterally, and true for forelimbs bilaterally.

Can We Identify with Assurance Specific Causes of Malformations at Particular Locations?

Whether they are considered hotspots or not, sites with *Ribeiroia* are likely to support malformed animals, and some percentage of these malformations are no doubt caused by the metacercariae. In our sample, these *Ribeiroia* positive sites are all located in the eastern half of Minnesota (Fig. 3) and include BUR, CTG, CWB, GEL, HIB, MHL, NEY, ROI (Table 4).

In hotspots where *Ribeiroia* infections were absent, this parasite cannot be the cause of observed malformations. These sites include DOR, HYD and TRD. In one of these sites (TRD), macrophyte beds that provide habitat for planorbid snail hosts were undeveloped at the time of our sampling, and in three years of sampling this site Hoppe (unpublished data) has failed to find snails of any species. In the absence of host habitat, hosts, and metacercariae it is difficult to argue for parasites as a cause for malformations. It is also unlikely that metacercariae have died and been "cleared" from animals, thus escaping detection. As amphibians metamorphose and proceed from being aquatic organisms to becoming terrestrial, their parasitic fauna changes—they lose some aquatic-associated species and gain terrestrial-associated species such as the lung fluke, *Hematoloechus* sp. that the frog acquires from ingesting infected dragonflies (Sutherland

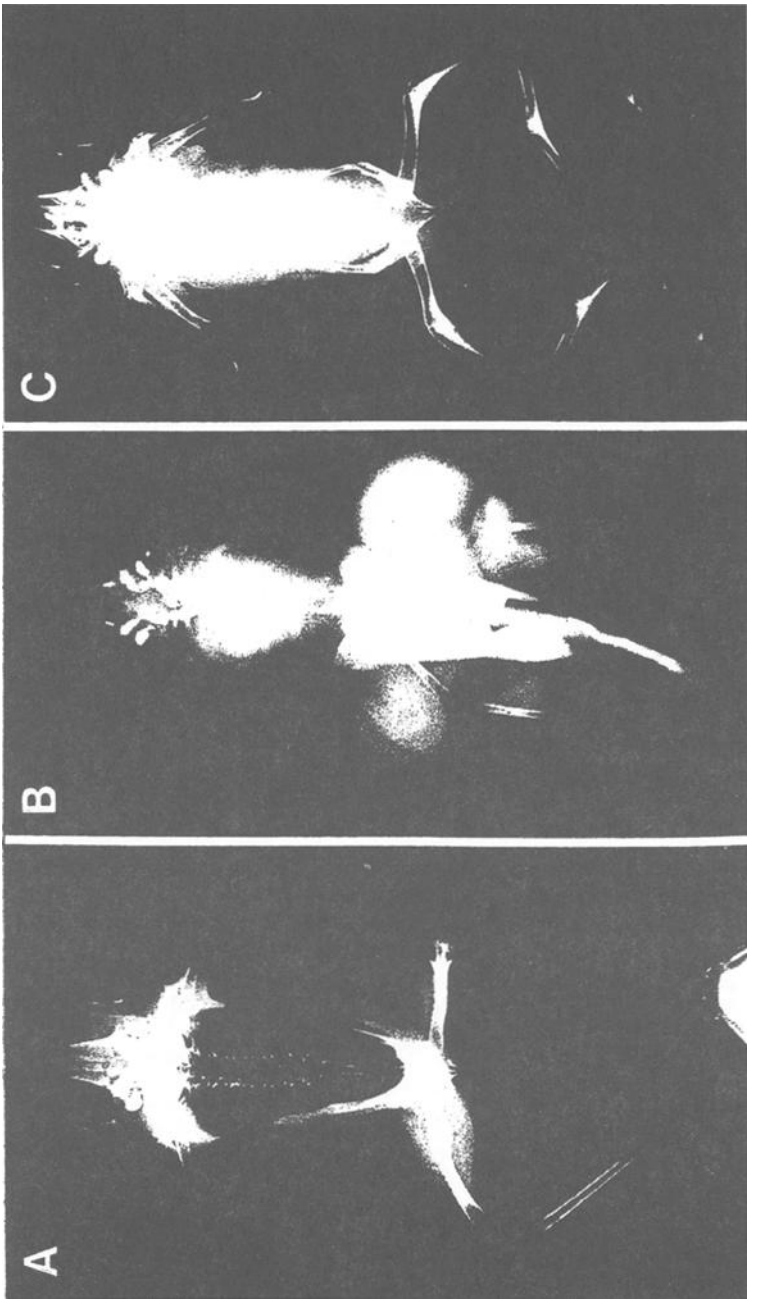


Figure 4 - *Malformation Types Characteristic of Particular Wetlands. A) A Northern Leopard Frog from Trempealeau County, Wisconsin. B) An American Bullfrog from Switzerland County, Indiana. C) An American Bullfrog from Santa Clara County, California. See Text for Details of these Malformations.*

and Kapfer, unpublished data). Therefore, the absence of *Ribeiroia* from an aquatic-associated parasitic fauna indicates a lack of infection, not infection with subsequent clearing (also consider the time from hindlimb development to metamorphosis in *Bufo* species can be as short as one week). On the other hand, if sufficient time has occurred to develop a terrestrial parasitic fauna, we can reasonably expect that evidence of some aquatic infections will wane.

At this point, two questions come to mind (which may have related answers). The first is: In sites with *Ribeiroia*, what environmental factors affect their abundance (intensity) such that, for example, mink frogs in CWB have an average of 35.4 metacercariae/animal while mink frogs in nearby MHL have an average of 5.0—a 7-fold difference? There are many differences between CWB and MHL including size (CWB is much smaller), biology (MHL has a sphagnum bog), and land use (MHL has cabins; CWB hosts dairy cattle that create an erosion problem along a portion of the shoreline). Environmental conditions that produce differences in *Ribeiroia* infection intensity may be natural or not. If not, it is useful to consider that eutrophication associated with cattle use will provide more and richer plant life (phytoplankton and macrophyte beds) when compared to less abused wetlands, and this plant life provides food and habitat for the planorbid snail hosts of *Ribeiroia* (Johnson and Lunde 2002). It is also useful to consider that with modern farming techniques, including feed additives, cattle feces and urine contain many more substances than the natural byproducts of ingestion and metabolism (as we write this, pollution by such additives is being perceived as an unrecognized, but large problem in U.S. waterways - see http://toxics.usgs.gov/regional/emc.html). We also await the analyses of data we collected on the presence of hormone and retinoid compounds.

The second question is: What effect does the presence of *Ribeiroia* have on our consideration of other potential causes of malformations? In part this is a question with a social component. There has been so much contention surrounding the discovery of causes of malformed frogs that finding one cause has tended to be interpreted as excluding other causes (see Souder 2000). This is a false assumption. While CWB contains *Ribeiroia*, because this wetland was littered with frog and fish carcasses this trematode cannot be the only problem at CWB. *Ribeiroia* is not known to cause mass die-offs in normally appearing tadpoles, adult frogs, or fishes. Further, the absence of *Ribeiroia* in the western portion of Minnesota indicates some factor other than parasites must be causing malformations, and it is not clear why this factor should partition itself in an east-west pattern mutually exclusive of the distribution of *Ribeiroia*.

Conclusions, With Recommendations for Land Owners and Managers, and a Larger View

Amphibian malformations have several causes and in the best of all possible worlds each hotspot would be carefully examined, the cause determined, and the source of the cause eliminated. This process is not only costly, but time consuming, and given the current funding crises experienced by governments at all levels, it is also not likely to happen anytime soon. Instead, it might be better to recognize that hotspots tend to be altered wetlands. These alterations grade from what appears to be benign causes (simply being created and perhaps not having the buffering capacity of more mature systems; these sites include TRD and perhaps BUR, CBA, NEY), through what is perhaps simple eutrophication (which would lead to increased plant growth, increased snail populations, and increased *Ribeiroia* levels [Johnson and Lunde 2002]; these sites include HIB, perhaps DOR), through eutrophication with suspected additional chemical inputs (CWB, ROI, perhaps CTG, HYD, SUN). It is this latter category that should be of most concern to humans.

Recognizing that hotspots are altered wetlands, probably the quickest and least expensive way to reduce malformations is to recognize the nature of the alterations and take steps to eliminate them. For example, is part of the eutrophication and suspected chemical input to ROI due to leaky septic systems? This is easily tested and if so, these waste control systems should be upgraded. Is part of the eutrophication and suspected chemical input to CWB due to the utilization of this wetland by cattle? This is an especially instructive question, because while the debate on malformation causes has tended to focus on proximate causes (for example retinoids versus parasites; see Souder 2000) the fact is that both could be present, and both could be caused by a single factor: cattle usage (nutrients produce eutrophication which produces snails which produce trematodes; retinoids enter the water as a non-digested component of feed additives). In either case, parasites or retinoids, the solution at CWB might be to remove the cattle.

Such solutions, however, are rarely simple because they involve value systems. For example, at CWB, does the right of a homeowner to live without health concerns caused by environmental degradation trump a farmer's need to water cattle? At ROI do health concerns trump the cost of upgrading septic systems? Does a state's right to manage public property trump the cost of private individuals practicing agricultural erosion control? Value systems then, in our view, become a central issue in solving the malformed frog problem, and as a society, we have found these decisions difficult to make.

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Establishing Causality in the Decline and Deformity of Amphibians: The Amphibian Research and Monitoring Initiative Model

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Abstract: Research to date has indicated that a range of environmental variables such as disease, parasitism, predation, competition, environmental contamination, solar ultraviolet radiation, climate change, or habitat alteration may be responsible for declining amphibian populations and the appearance of deformed organisms, yet in many cases no definitive environmental variable stands out as a causal factor. Multiple stressors are often present in the habitat, and interactions among these can magnify injury to biota. This raises the possibility that the additive or synergistic impact of these stressors may be the underlying cause of amphibian declines. Effective management for the restoration of amphibian populations requires the identification of causal factors contributing to their declines. A systematic approach to determine causality is especially important because initial impressions may be misleading or ambiguous. In addition, the evaluation of amphibian populations requires consideration of a broader spatial scale than commonly used in regulatory monitoring. We describe a systematic three-tiered approach to determine causality in amphibian declines and deformities. Tier 1 includes an evaluation of historic databases and extant data and would involve a desktop synopsis of the status of various stressors as well as site visits. Tier 2 studies are iterative, hypothesis driven studies beginning with general tests and continuing with analyses of increasing complexity as certain stressors are identified for further investigation. Tier 3 applies information developed in Tier 2 as predictive indicators of habitats and species at risk over broad landscape scales and provides decision support for the adaptive management of amphibian recovery. This comprehensive, tiered program could provide a mechanistic approach to identifying and addressing specific stressors responsible for amphibian declines across various landscapes.

Keywords: chemical, physical, biotic variables, environmental stress, amphibians, risk assessment, causality, deformity and decline

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Introduction

The loss of amphibian populations has been observed worldwide and the decline of populations (Alford and Richards 1999) and the appearance of deformities have been observed throughout North America (Lanoo 2000). Clearly, a range of potential environmental variables is responsible for the decline and deformity of amphibians. However, establishing causality has been difficult because of the complexity of factors extant at any particular site and the paucity of data available on the status of the population. Whether population decline and the appearance of deformities arise from similar etiologies is controversial, though it could be argued that a high frequency of deformities may impair the long-term viability of the affected population, since deformed organisms seldom mature to reproduction. The recovery of species at risk, including those that are endangered, threatened, or suffering localized declines requires an analysis of stressors likely linked to their peril. Once this is done, then effective steps can be taken for their restoration.

Organisms like amphibians pose significant challenges to this evaluation because some species are unfamiliar to resource managers. Knowledge of such organisms may be limited because of their reclusive habits, remote locations of their habitats, or because they are rare in number and seldom encountered. As a result, the organism's habitat requirements, especially terrestrial habitats of amphibians, may not be fully known. Amphibian life history and behavioral habits may also be poorly understood. Amphibians have simply not been studied as adequately as other vertebrate classes. Compounding these difficulties is the common lack of information about the status of an amphibian population. To determine that a population is truly in decline requires life-table data on their population size, reproductive success, age distribution, and mortality rate (Green, 1997). Amphibians often display a large variation in abundance over time (Semlitsch et al. 1996) that can be related to abiotic factors such as pond hydroperiod, and biotic factors such as competition and predation. This variation makes it difficult to distinguish declines from natural variation because there is insufficient long-term data for many populations (Pechmann et al. 1989). The unknowns associated with population dynamics can significantly hamper identification of critical limiting factors in their environment. The situation with amphibians is further complicated because many species have requirements for both aquatic and terrestrial habitats. An additional challenge for species in decline arises from the lack of advocacy that they receive. Unfamiliar, cryptic organisms tend not to be charismatic species, and thus, fail to draw the public attention that more familiar organisms attract in the allocation of resources necessary for evaluation and restoration.

A Case for Multiple Stressors

Plants and animals must adapt to a range of environmental variables in order to survive and reproduce. When the magnitude of an environmental variable exceeds the normal range for which the organism is adapted, stress occurs (Lack, 1954). Such stress places energetic demands on the organism that would otherwise be directed toward physiological maintenance, growth and reproduction as it attempts to maintain

homeostasis (Wedemeyer et al. 1980). Failure to cope with stress constitutes injury to the organism that can result in a loss of vitality, reproductive failure, or death.

Environmental assessments of causal factors relevant to species in decline are often the result of regulatory activities directed toward anthropogenic alterations of the environment that may pose an existing or potential future hazard to the environment such as the release of a hazardous material, commercial development, or mineral extraction activities. In such cases, the focus of the investigation is on the most salient environmental variables likely to result from the activity, such as habitat alteration or contamination. The weakness of a priori assumptions is that environmental variables do not exist in isolation from other variables that may contribute to the cumulative impact to the organism or even directly act upon the presumptive variable to increase (or decrease) its impact. Whereas risk assessment strategies may be employed in identifying potential cause, most often such approaches are based on single stressors, such as contaminants. A priori assumptions as to the causes for the loss of viability within a population can sometimes be misleading and cause unnecessary delays and expense in identifying and addressing the cause of injury. The "obvious" problem may not be the problem or may not be the only problem. The environmental situation may also be ambiguous with many apparent problems, no apparent problems, or transient challenges apparent only in combination with certain variables or lifestages.

Such focus on the presumptive causality of individual variables has been criticized as being "inappropriate, inefficient, and potentially expensive analyses, [that] may delay the identification of cause beyond the time frame in which appropriate remedial action could reasonably have been taken" (Fairbrother et al. 1997). For example, initial investigations of the catastrophic release of the herbicide, Agent Orange, to soils at a storage facility near a playa lake in the Great Basin of the western U.S. revealed a pattern of scattered mortality of creosote bushes (*Larrea mexicana*), as well as the apparent mortality of livestock. Agent Orange appeared to be the apparent cause of this mortality. However, further study revealed that the plume of Agent Orange (the presumptive cause) in ground water was not clearly linked to stressed vegetation. Further, the creosote bushes had a high tolerance for this herbicide under existing soil conditions such as highly alkaline pH (Linder et al. 1995). While other receptors may have been adversely impacted by the chemical's release, the stressed vegetation was not causally linked to herbicide exposures via the rhizosphere. Further assessment determined that the creosote bush is intolerant of low oxygen environments. A shallow water table discovered at the site would have likely resulted in water stress of the creosote bushes in years of high rainfall, and livestock would have been entrapped in the attendant muddy conditions (Dobrowolski et al. 1992). Remedial actions, if pursued, would likely focus on the risk posed by contamination of groundwater to receptors other than creosote bushes.

When multiple environmental variables are considered in regulatory investigations they are often evaluated as competing factors in their impact to the target organism or function rather than stressors interacting simultaneously. For example, in the natural resource damage assessment of mining activities in the Clark Fork River of Montana, the impact of toxic metals in the river to the salmonid population were weighed against impacts resulting from dewatering by irrigation, overharvest by sport fishing, and natural fluctuations in water quality (Audet 2000).

Interactive Effects of Stressors on Amphibians

In combination, stressors can have not only additive or synergistic effects in their cumulative impact to the organism and its population, but minimalizing effects as well. Most often, stressors such as contaminants, competition or predation by non-native species, or environmental changes resulting from climate change or habitat alteration are assessed in the absence of other environmental variables. This can result in an incorrect assignment of the causal linkages between the presumed stressor and the endangerment or decline of a population or a species. For example, acid deposition from power generation can directly impact the biota and can also increase the rate of breakdown of humic acids leading to an increase of harmful ultraviolet (UV) radiation in the water column (Schindler et al. 1996). A series of cascading effects can occur that normally would not be predicted based on a single stressor model. Thus, a number of environmental variables are important to consider in habitat restoration because they are site-specific and likely to impinge simultaneously upon the community.

There is an increasing literature on the interactive effects of environmental stressors to amphibians. Carbaryl is a neurotoxic insecticide with known direct and indirect effects on amphibians (Bridges 2000). However, tadpole density, predator presence and pond hydroperiod can also influence the effects of carbaryl (Boone and Semlitsch 2001, 2002; Boone et al. 2001). For instance, when gray tree frogs (*Hyla versicolor*) were exposed to carbaryl in mesocosms with high tadpole density, they had greater survival to metamorphosis than gray tree frog tadpoles in low density mesocosms (Boone and Semlitsch 2002). Additionally, Boone and Semlitsch (2001) found that exposure to carbaryl induced greater body mass of tree frogs at metamorphosis when predators, (newts), were present, than when predators were absent. There is evidence that the effect of a pesticide stress differs with environmental conditions such as density (Boone and Semlitsch 2000) and pond hydroperiod (Boone and Semlitsch 2001; Boone and James, in review) and biotic interactions such as predation and competition (Mills 2002). Further, additive or synergistic toxicity occurs when sunlight converts certain chemicals to more toxic forms. Photoenhanced toxicity of contaminants such as pesticides, petroleum, and industrial chemicals reduced amphibian survival (Little et al. 2000). The toxicity of the insecticide carbaryl to gray tree frogs significantly increases in the presence of UV (Zaga et al. 1998). Bridges et al. (in review) have shown that exposure of developing northern leopard frogs (*Rana pipiens*) to the complex chemical mixtures in environmental extracts alone and in combination with UV can lead to deformities and reduced growth.

These studies of the interaction of stressors emphasize the importance of manipulating environmental variables with a presumptive stressor such as a pesticide. The studies indicate, for example that the interactions are not always predictable because impacts often occur indirectly through changes in the food web (Mills 2002) that result from the interactions among pesticide exposure, predators and competitors on the survival and development of amphibians.

Approaches to Assessing Multiple Stressors

The evaluation of multiple environmental stressors has been considered in national workshops over the past 10 years (See Foran and Ferenc 1999) and several approaches

have been proposed for assessing multiple stressors relative to regulatory objectives. The assessment of multiple variables at landscape scales has been emphasized in the origins of landscape ecology (Forman and Godran 1986) and the application of these methods in landscape ecotoxicology (Cairns and Niederlehner 1996). The identification of unknowns in risk assessment, for example, have provided a means of weighing impacts to biota caused by contaminants from those caused by other environmental variables. For example, extensive surveys of environmental variables are emphasized in deriving the index of biological integrity for state streams by the Ohio EPA (Yoder and Rankin 1995). Similarly, the Cumulative Effects Assessment used in Canada considers present and future stressor loading capacity relative to proposed developments in the watershed (Munkittrick and McMaster 2000). Often these approaches are directed at systems that are relatively well known, or that have sufficient resources for the proposed assessment, and allow sufficient time to generate such information. For most populations of amphibians neither information, resources, nor time are sufficient for multiple stressor risk assessment.

There are several aspects of these regulatory applications that limit their utility in the broader assessment of causality required for amphibian deformity and decline. Regulatory approaches emphasize extensive data collection or sufficient background information about the site and the species, and also assume sufficient funding and time for analysis (CERCLA 1980; NRDA 1986). The determination of causality issues in amphibian decline (as well as other poorly known classes of organisms) is beset by severe limitations in time and funding, and incomplete knowledge of the species, its status, or the quality of its habitat. In addition the appraisal must consider a more extensive spatial range than envisioned in landscape-scale regulatory efforts that are commonly focused on a specific watershed or site of apparent damage.

Establishing Causal Linkages to Biological Injury

Establishing causality involves development of weight of evidence, including experimental evidence, ecoepidemiological assessments, and bioassessment monitoring to link the presumptive cause with the biological injury. Ecoepidemiology characteristically involves several lines of association (Susser 1986) including the following:

- Probability or statistical significance of relationship observed between disease and circumstance,
- Time order of hazard and occurrence of injury,
- Strength of association or precision with which the occurrence of hazard (to the exclusion of all others) will predict injury,
- Specificity or plausibility of effects induced by the hazard,
- Predictive performance relative to the extent that previously unknown corroborating evidence supports the association between hazard and injury,
- Consistency of effects compared to those observed with other organisms, species and test setting, and
- Coherence with existing theory and information.

Causality is disproven when the presence of the stressor does not procede the appearance of biological injury, by a lack of plausibility that the type of injury could

result in the biological injury, and by a lack of consistency in replicated coincidence of stressor and injury. Causality is affirmed by strength of association, consistency, predictive performance and coherence (Fox 1991).

Determination of cause-effect relationships involves a number of considerations including the identification of pathways (in time and space) between the stressor and the affected organism. In the case of contaminants and disease, this includes air, water, sediment/soil and food-chain, as well as maternal exposures. The relationship should be readily replicated in controlled studies and should induce similar responses in related species. The stressor should also induce plausible responses that are consistent with the mode of action that may affect the organism or its food web. It is important to emphasize that correlations between the presence of a stressing variable and biological effect do not establish the link. Experimental manipulations are necessary to confirm the causal link. It is also important to determine that the effect has measurable consequences at the population level.

The assessment of multiple stressors can include retrospective analyses common to risk assessments as well as prospective analyses conducted to determine impact (Foran and Ferenc 2000). In addition, the analysis can be stressor-driven where the emphasis is on the presence, pathway, and magnitude of causal variables. Stressor-driven efforts begin with an inductive listing of possible stressors or spatial temporal correlations to establish cause and effect relationships, in addition to mechanistic explanations to determine causality (Suter et al. 1999). It also can be effects-driven wherein the "measured response of biological components of the ecosystem are used to identify and estimate the effects of stressors on the ecosystem component with the goal of ultimately managing stressors to the system" (Klump et al. 1999).

Basic steps in this bioassessment include:

- Identifying ecosystem components
- Determining methods of measurement
- Designing the survey
- Conducting the survey to identify biologically significant changes
- Conducting ecoepidemiological and diagnostic approaches to identify causal relationships
- Conducting remediation
- Confirming the effectiveness of remediation
- Conducting risk management

The spatial and temporal extents that may be required in assessing an amphibian metapopulation may exceed the boundaries usually defined in risk or impact assessments. Regulatory approaches for establishing causality tend to focus investigations on a few presumptive factors that affect only a limited spatial area such as a site where hazardous substances have been released, or development has occurred. The decline of major animal groups such as amphibians appear to occur over broad geographic areas such as the Intermountain Western U.S. (Corn 2000). Risk and impact assessments conducted for regulatory purposes tend to be expensive. A systematic approach would likely be required for determining the causes for amphibian decline and deformity in view of limited funding and expanded boundaries that may be involved.

Amphibian Research and Monitoring Initiative (ARMI)

The U.S. Geological Survey's Amphibian Research and Monitoring Initiative (ARMI) was mandated by the U.S. Congress as a part of the Department of Interior's Amphibian Initiative of 2000 to develop a program for monitoring trends and to conduct research into the causes of amphibian declines. The objectives of ARMI:

- Establish a network for monitoring the status and distribution of amphibians on Department of Interior lands;
- Identify and monitor environmental conditions known to affect amphibian populations;
- Conduct research on the causes of amphibian population change and deformities;
- Provide information to resource managers, policy makers, and the public in support of amphibian conservation.

The ARMI monitoring effort involves intensified monitoring and research at one or two sites (high-intensity sites) within each of seven regions across the nation. These sites include areas that have been extensively studied in the past to develop baseline information on population dynamics. A more generalized inventory and monitoring effort encompassing broader areas (low-intensity sites), such as National Parks, is being conducted within each ARMI region. In addition to monitoring for species numbers, abundance, distributions, and health, the monitoring effort includes collection of physical and chemical data on the habitat. Manipulated studies are conducted in support of the monitoring effort to identify factors that are limiting amphibian viability.

The ARMI budget for causal research is limited; thus the investment must be efficient to ensure a meaningful assessment. An examination of factors involved in the deformity of amphibians in Minnesota revealed a number of potential stressors. At one particular site, two stressors, parasitism (Johnson et al.1999) and contaminants (Bridges et al. in review), appear to be independently responsible for deformity. Had only one avenue of investigation been followed, the other source of injury at this particular site would not have been identified and subsequent efforts to restore the habitat based on a single stressor would likely be unsuccessful. Thus, to initiate research on specific variables without understanding what other potential variables are acting upon the population would be imprudent. An assessment for pesticides in a particular region, for example, could exhaust the annual research budget, and may overlook the main factors affecting the populations.

Research to determine cause-effect relationships between potential stressors and amphibian deformity and decline involves an initial screening of potential stressors. Subsequently, an assessment of correlations among stress variables and biological endpoints should be conducted to form hypotheses for subsequent investigations. Subsequent investigations of selected variables would include more specific (and more expensive) assays. This iterative process is intended to provide justification for the research focus and will ensure consideration of a range of potential stressors, focus diagnostic action, and increase assessment efficiency with limited resources.

A program of national scope requires uniformity of appraisal to ensure consistent effort and breadth of assessment, but of sufficient focus to address local or regional landscapes and species that inhabit them. Guidelines will be developed for conducting a systematic, multiple stressor approach for identifying environmental factors responsible for population declines and deformities. This will require a tiered approach of increasing complexity to determine correlation between stressors and decline. The tiered approach

includes: 1) initial synoptic survey to determine the potential stressors; 2) experimentally-based research to determine causality between a stress and decline gradually increasing the number of factors in the studies; and 3) evaluating the relative risk in nature and eliminating risk factors.

Proposed Three-Tiered Research Strategy

The purpose of the synoptic assessment is to evaluate existing information and habitat-specific data related to potential stressors within the watershed/landscape that can be used to identify potential hazards. The basis of the synoptic assessment will be a catalog of information on habitat quality, climate, disease, contaminants, predation/competition, and species characteristics. The synoptic appraisal will include GIS evaluations of expected stressor distributions upon which to base later hypotheses and design studies. The catalog will be used throughout the ARMI program to ensure a consistent, unbiased, uniform appraisal of potential hazards within the area being monitored. The catalog would likely be modified to facilitate assessment of various habitat types (e.g. terrestrial, riverine, wetlands). The catalog entries might also be scored relative to the magnitude of potential hazards.

Tier 1 Assessments- The objective of the Tier 1 synoptic environmental evaluation is to begin the diagnostic process by identifying potential factors that could harm a population and gathering sufficient information to develop hypotheses. The first tier will provide a comprehensive preliminary evaluation of potential stressors that may cause injury to the population (Table 1). This tier is intended to provide uniformity of evaluation at sites across the country. It will build upon information developed by the ARMI monitoring effort and will be conducted in coordination with the Regional Principal Investigators (RPI). The synoptic environmental evaluation manual, possibly similar to the ARC-VIEW format used by the Contaminant Assessment Program in National Parks may facilitate the evaluation.

Tier 1 will include desktop and onsite activities. The desktop effort will evaluate existing information about the site/population. Sources of information will include ARMI RPIs and collaborators, the ARMI database, and existing databases such as National Weather Service, U.S. Department of Agriculture, and USGS National Water Quality Assessment. Tier 1 will also include the measurement of a range of environmental variables, including water quality, examination for disease, survey for non-indigenous organisms, survey for potential contaminant sources during onsite verification visits.

Tier 1 evaluations should be conducted at all sites, particularly for low-intensity sites. An abbreviated Tier 1 assessment may be conducted at high-intensity sites that have been well-studied in the past. However, it is important to ensure that all potential stressors have been examined, even in well-studied sites. The classes of environmental variables to be included in the Tier 1 evaluation include habitat use/alteration, climate including solar UV, water quality, disease/parasitism, non-indigenous species, and contaminants. Tier 1 evaluations will provide the basis for developing hypotheses about the potential environmental factors causing decline and deformity.

Table 1 - Categories of Potential Stressors

Habitat Quality	
Habitat extent	Habitat fragmentation
Sensitivity to alteration	Water supply/hydroperiod
Food web	Aquatic community health
Habitat Alterations	
Urbanization	Agriculture/silvaculture
Water source/quantity	Wildland fires
Construction (roads,dams)	Mining/mineral extraction

Climate

Departures from decade trends for precipitation/ temperature
Fluctuations in current monthly mean temperature/pecipitation
Fluctuations in daily temperature, precipitation, humidity
Dates of first freeze/thaw
Winter degree-days, number of days above freezing
Flooding, drought, extreme, or catastrophic events
UV radiation
Changes in cloud cover (number of sunny days)
Air quality (particulates, nitrogen and sulfur oxides)
Changes in vegetative cover
Turbidity and dissolve organic carbon concentration

Chemical

Historic use of chemicals in watershed
Proximity to contaminant sources (agricultural fields, feedlots, land fills, incinerators)
Non-point source pollution(urban, industrial, agricultural)
Exposure pathways (wind direction, aquatic pathways, food chain)
Water quality characteristics (nutrients, acid neutralizing capacity, ions organic carbon)
Soil/sediment quality (composition, metals, organics, pH, cation exchange capacity)
Air quality (Acid deposition, smoke, particulates, contaminants)

Biotic

Disease

Factors that increase the susceptibility of the host (immune function, physiological state population density, naïve populations)
Factors that increase success of the pathogen (vector/host lifestage and species) timing of infection, pathogen ecology, pathogen adaptation/resistance)
Non-infectious diseases (malformations, neoplasia, lesions, size, development, coloration, asymmetry, abnormal behavior, non-anthropogenic toxicity, physiological markers of stress and exposure)

Ecological

Predation and competition from non-native species.
Disruption of food web, loss of food chain organisms
Disruption of community dynamics (regulatory processes, loss of critical species)

Tier 2 Assessments - The objectives of Tier 2 studies are: 1) to complete the diagnostic process, in order to determine cause-effect relationships between stressor and amphibian decline and deformity; 2) to identify contributing environmental factors that lead to harmful conditions; and 3) to define critical variables for routine, broad scale monitoring. The second stage of assessment should be hypothesis-driven experimental research that would be conducted in a tiered process of increasing complexity (Table 2). This level of assessment would begin with rapid evaluation methods that are easy to use in the field to determine presence/absence or magnitude of stressing conditions. This might include a toxicity test, or use of ELISA assays to detect a pesticide. The determination of the stressing condition from initial studies would be followed by more specific studies of greater complexity. Once causality is implicated for a particular variable, regional monitoring of that stressor would be incorporated in the monitoring effort. GIS evaluations could also be initiated.

Table 2 - Example of a potential iterative sequence to determine contaminants in Tier 2 assessments

• Initial toxicity test with common test species (e.g., *Daphnia magna, Ceriodaphnia dubia, Pimephales promelas*).
• Toxicity test of environmental extracts with early lifestage amphibians. This can be tested in combination with another environmental stressor (e.g., temperature, UV).
• Developmental assay of environmental extracts using early lifestage amphibians.
• Analyze environmental extracts for specific chemical content.
• Conduct tests to identify general class of compound(s).
• Initiate focused investigations to determine contaminant exposure pathways to the organism.

Research would comprise a range of activities including manipulative lab and field tests, comparative monitoring, analyses of ARMI and other national databases. This tier of research is in progress at the high-intensity sites and would probably also be applied in low intensity sites. The second tier emphasizes the evaluation of multiple environmental variables, each of which may cause injury individually or in combination. For example, an evaluation of petroleum products at sublethal concentrations may indicate hazard when solar radiation is included in the evaluation. The iterative process is intended to identify the major environmental variables potentially responsible for decline and deformity; thus, initial steps consider many variables, and through the process of elimination focus on a few.

Once major problems are identified, the researcher may intensively examine those variables. At this stage it is especially important to examine the problem within an ecological context, such as the food web or community. Should parasitism, for example, be identified as a major problem, the research may include an evaluation of conditions that support vector/host organisms and their relationship to the amphibian's habitat. If non-indigenous organisms are determined to be a major problem, then an evaluation of competition or predation might include food web and habitat structure.

Although the main focus of research at this stage may be either a parasite or a contaminant, for example, the research would be conducted in a manner that would include multiple environmental variables, such as rainfall or degree-days, sublethal contamination, or pathogens. Research at this stage will likely have a regional or local focus; however, the outcome of the effort might be of broader, national significance such that the variable would be included in the general monitoring program.

Tier 3 Assessments-The objective of the third tier in the evaluation of causal factors in amphibian decline and deformity is to provide a predictive basis for identifying species or habitats at risk on a regional, ecosystem, or national scale. For example, if the interaction of drought, virus, and contaminant were found to be associated with species decline, then the distribution of these variables could be mapped. From this landscape appraisal, predictions might be made as to hazardous areas, or populations potentially at risk. Information generated by research would be used to develop restoration strategies. In an adaptive management process, research can provide critical information about the effectiveness of various restoration activities. The third stage of assessment would provide an iterative decision support process that includes probabilistic risk assessment models based on the magnitude of stressor effects on a species, margins of error for injury, and focused monitoring activities of the identified causal factors. These models would determine the degree of risk posed by the identified hazard(s). The models could also be applied from a species-specific perspective to consider the risk of injury posed by the environmental conditions. Risk assessment modeling would also be important in the adaptive management of remediation activities.

Conclusions

Systematic appraisals for determining environmental factors leading to the loss of natural populations have been proposed, but the application of these has mainly be limited to studies in support of litigation or regulation with a site-specific or species-specific emphasis. Such approaches are inadequate in the face of the spatial scope and scale of complexity of the biodiversity crisis, of which amphibians are but one example. Landscape-scale appraisals and recent advances in mapping distributions of organisms and environmental variables provide an opportunity for unprecedented appraisals of potential correlations between stressors and populations. Statistical methods are being developed for analyzing spatial data bases and should significantly enhance our ability to detect potential interactions of environmental variables on amphibian populations. In addition, advances in cell biology offer new tools for the analytical appraisal of certain stressors in the environment. Methods and models arising from the nexus of community ecology and ecotoxicology should expand our predictive capabilities to monitor lifetable

trends and resolve interactive processes. Most importantly, the application of this tiered approach will bring economy and uniformity to the assessment of species at risk.

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