DEVELOPMENTAL STABILITY IN AMPHIBIANS AS A BIOLOGICAL INDICATOR OF CHEMICAL CONTAMINATION AND OTHER ENVIRONMENTAL STRESSORS

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Estimated Total Cost	Year 1	Year 2	Grand Total
Federal:	**$94,708**	**$73,641**	**$168,349**
State:	**$49,250**	**$38,984**	**$88,234**
Local:	**$49,771**	**$38,588**	**$88,359**
Total:	**$193,729**	**$151,213**	**$344,942**

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PROJECT SUMMARY

The global decline of amphibians is considered a disturbing indicator of environmental degradation because it may forebode of cascading ecological effects, as well as raising health concerns about human populations (Wyman 1990, Wake 1998). Amphibians are ideal biological indicators, because their semi-permeable epidermis and complex life cycle expose them to multiple stressors in both aquatic and terrestrial environments (Wyman 1990). Because of this, amphibians should be among the first vertebrates affected by anthropogenic stressors in either of these environments (Stebbins and Cohen 1995). Furthermore, some of the same stressors affecting amphibians are known to have negative effects on other species, including humans (e.g., PCBs, UV light, etc.; Wake 1998, Carey 2000).

Previous research in our laboratories has shown that developmental stability (as measured by fluctuating asymmetry) is correlated to human-induced stressors in two amphibian species (Whiteman unpubl. data). We propose to continue our studies of developmental stability as a biological indicator of stress in amphibian populations, in an effort to improve the ability of biologists to evaluate the health of these populations, the surrounding ecosystem, and the possible environmental risks to humans. Although we propose to evaluate a wide range of possible stressors, **a major thrust of this project is to correlate amphibian developmental stability with contaminant levels accumulated in their tissues**. Surprisingly, although many studies have used developmental stability as an indicator of such contamination (Moller and Swaddle 1997), none of these studies have concentrated on amphibians.

We hypothesize that amphibian developmental stability will decrease with increased levels of anthropogenic (contaminants, land use practices) and natural (population size and density) stressors. To test this hypothesis, we will sample several species of amphibians, including both aquatic and terrestrial life stages, and taxa from various trophic levels which vary in their potential exposure to stressors. In this way we will be able to discover how well developmental stability operates as a bio-indicator across a wide range of conditions. We will measure developmental stability and various natural and anthropogenic stressors, and use the results to assess the utility of developmental stability as a biological indicator in amphibians.

Development of these methods will allow scientists to determine the population health of a critical group of organisms, to utilize amphibian development as an inexpensive yet accurate proxy for measuring the effects of stressors such as industrial and agricultural pollutants, and will provide methods to separate the effects of anthropogenic stressors from natural ones. This

research will thus be valuable to the estimation and management of ecosystem and human health.

PROJECT RATIONALE

Rationale for Project Selection

This Kentucky EPA/EPSCoR proposal presents a plan of results-oriented, multidisciplinary research aimed at deciphering one of the most timely and urgent environmental problems, the use of biological indicators to understand actual and potential risks to population, ecosystem and human health. Using state-of-the-art, EPA approved methods and protocols, the PIs, graduate students, and undergraduate assistants will obtain baseline data on how anthropogenic and natural stressors affect amphibian development across a range of life stages, species, and habitat types. This research will specifically test the ability of developmental stability to act as a biological indicator of such stressors, and to separate those of anthropogenic versus natural origin.

Institutional Goals

Murray State University is dedicated to developing the future careers of science and engineering students and faculty. EPA/EPSCoR funds will complement recent awards made by the National Science Foundation (Collaborative Research at Undergraduate Institutions program) and the Howard Hughes Medical Institute which are aimed at developing student and faculty research and teaching with an emphasis on promoting undergraduates that are currently underrepresented in science and technology. To this end, the PIs will advertise broadly for graduate and undergraduate students needed to complete this project, and actively recruit women, minorities, and persons with disabilities. Currently, the PIs have eight undergraduates and four graduate students in their laboratories (eleven women, one minority), and have been actively involved in programs designed to encourage minority development within the sciences (e.g., NSF REU and C-RUI, ESA SEEDS).

PROJECT DESCRIPTION

This Kentucky EPA/EPSCoR proposal presents a plan of results-oriented, multidisciplinary research aimed at deciphering one of the most timely and urgent environmental problems, the use of biological indicators to understand actual and potential risks to population, ecosystem and human health. Using state-of-the-art, EPA approved methods and protocols, the PIs, graduate students, and undergraduate assistants will obtain baseline data on how anthropogenic and natural stressors affect amphibian development across a range of life stages, species, and habitat types. This research will specifically test the ability of developmental stability to act as a biological indicator of such stressors, and to separate those of anthropogenic versus natural origin.

Objectives

One of the most important, yet most difficult, tasks associated with understanding and managing the health of ecosystems is the identification of populations subject to stress before such stress has a detrimental effect (Clarke 1995). This is particularly true of amphibians; the global decline of amphibian populations is considered a disturbing indicator of environmental degradation because it may forebode of cascading ecological effects, as well as raising health concerns about human populations (Wake 1998). Amphibians are ideal biological indicators, because their semi-permeable epidermis and complex life cycle expose them to multiple stressors in both aquatic and terrestrial environments (Wyman 1990). Because of this, amphibians should be among the first vertebrates affected by anthropogenic stressors in either environment (Stebbins and Cohen 1995). Furthermore, some of the same stressors affecting amphibians are known to have negative effects on other species, including humans (e.g., PCBs, UV light, etc.; Wake 1998, Carey 2000).

Recently, an increase in incidence of malformed frogs has been observed throughout North America. These observations have typically involved gross changes in morphology such as extra or missing limbs and eyes. Deformed frogs are currently hypothesized to be indicators of developmental problems associated with anthropogenic stress, and evidence testing this idea is currently being evaluated (e.g., Diana and Beasley 1998, Helgen et al. 1998, Rowe et al. 1998ab, Johnson et al. 1999, Sessions et al. 1999). Although deformed frogs serve as a warning for the management of nearby amphibian populations as well as human health concerns, they may appear too late to save local populations, and possibly too late to reduce anthropogenic stress to the surrounding ecosystem as well.

Biologists thus need an early-warning biological indicator that could identify environmentally-stressed animals before the stressor causes population, ecosystem, and/or regional harm. Such an indicator should be able to measure stress-induced effects before drastic changes in morphology or immunocompetence take place which would subsequently decrease the organism's survival and reproductive abilities. One such indicator is developmental stability (Clarke 1995).

Developmental stability is one component of an organism’s ability to withstand environmental and genetic disturbances to produce a genetically predetermined phenotype (Waddington 1942, Lerner 1954; for a review, see Moller and Swaddle 1997). Specifically, developmental stability reduces phenotypic variation resulting from developmental accidents. Under normal conditions, development follows a genetically determined pathway, and minor perturbations are controlled by developmental stability mechanisms. Under stressful conditions

(e.g., increased pollutants), the performance of the stability mechanism may be reduced such that development cannot be restored to the original pathway, resulting in the production of abnormal phenotypes (Waddington 1942, Clarke 1995). Developmental stability can thus provide an indirect measure of an organism's fitness, and numerous studies have found significant correlations between measures of developmental stability and fitness (e.g., Quattro and Vrijenhoek 1989, Moller 1992a, b, McKenzie and O'Farrell 1993). Further, stress-induced changes in developmental stability are typically observed before any detectable change occurs in fitness (Clarke et al. 1986, Clarke and McKenzie 1992), providing an early-warning mechanism for population monitoring.

One of the most widely used measures of developmental stability is fluctuating asymmetry (FA). FA is nondirectional differences between the left and right sides of paired bilateral characters within a population (Thoday 1955, 1958, Van Valen 1962). The underlying assumption of this measure is that development of both sides of a bilaterally symmetric organism is influenced by identical genes, and thus nondirectional differences between sides must be environmental in origin (Waddington 1942, Moller and Swaddle 1997). Because developmental stability acts to reduce such changes, FA will measure the efficiency of developmental stability and the magnitude of the environmental perturbation (Clarke 1995). Because measures of developmental stability, such as FA, can be used to identify stressed populations before more significant deleterious effects are observed, and because such measures can also be used to estimate changes in fitness before they occur, **developmental stability has the potential to be a critical biological indicator**.

Many studies have used developmental stability as an indicator of stressors, including lead, benzene, mercury, PCBs, DDT, various pesticides, and UV radiation in a diverse array of organisms from algae to grey seals (Moller and Swaddle 1997). Surprisingly, although development has been studied more extensively in amphibians than perhaps any other group (Duellman and Trueb 1986), and many stressors have been shown to have major developmental effects on amphibians (Diana and Beasley 1998, Rouse et al. 1999; Appendix 1) **developmental stability is only now being applied to amphibians.**

Previous studies by the PIs and their students have shown that developmental stability can be used as a biological indicator of anthropogenic stressors. Studies of bullfrog (*Rana catesbeiana*) tadpoles and tiger salamander (*Ambystoma tigrinum*) adults revealed that FA increased with increasing levels of agricultural disturbance and water quality degradation (Fig. 1). We have also documented tissue contamination associated with developmental deformities in bullfrog tadpoles. Normal tadpoles exhibited total PCB congener and total pesticide concentrations ranging from detection limit (0.35 ng g^{-1}) to 10.36 ng g^{-1} wet weight and detection limit (0.35 ng g^{-1}) to < 6 ng g^{-1} wet weight respectively. Grossly deformed tadpoles (tumors, axial deformations), in contrast, exhibited elevated total PCB concentration (>20 ng g^{-1} wet weight), but did not differ from normal tadpoles in total pesticide concentration (Seaford et al. 2000). Unfortunately, lack of funds has limited our ability to test whether FA correlates with these tissue contamination levels, and to analyze amphibian tissues for other contaminants such as butyltins and PAHs.

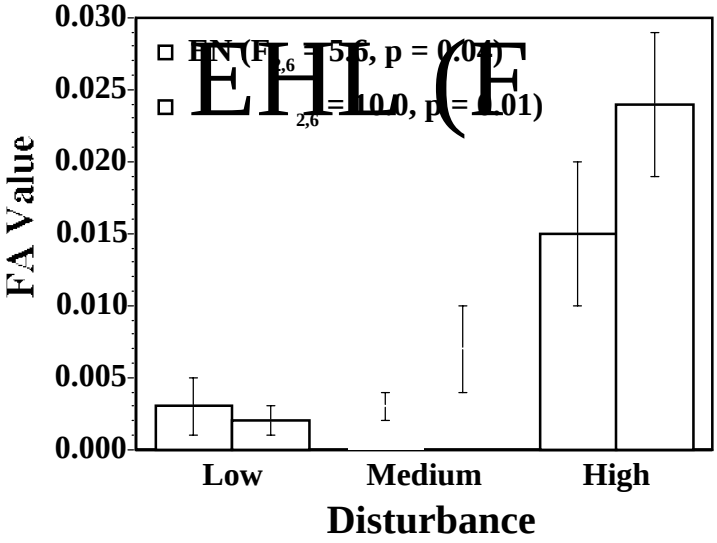

Fig. 1: FA increases with anthropogenic disturbance level (low, medium, high) in bullfrog(*Rana catesbeiana*) tadpoles from western Kentucky. Low disturbance areas are ponds surrounded completely by forest, medium are farm ponds with associated trees and shrubs, and high areas are farm ponds without any trees or other non-agricultural vegetation. Two different FA measures are shown: EN (eye to nares) and EHL (eye to hind limb).

We propose to continue our research into this biological indicator by correlating FA with a number of natural and anthropogenic stressors that might influence variation in developmental stability. These variables include: 1. amphibian tissue concentrations of organic, organometallic, and trace elements commonly associated with industrial and agricultural pollution (Appendix 2), which have been implicated in endocrine disruption and developmental problems, including amphibian abnormalities (Appendix 1); 2. important water chemistry measures, which have been associated with amphibian mortality and deformities (Appendix 1) and have been correlated with significant FA in previous studies (see above); 3. land use practices, i.e., undisturbed forested sites, moderately disturbed agricultural sites and highly disturbed industrial sites; 4. population density, which at high levels can induce stress and FA (Clark and McKenzie 1992); 5. adult population size, which may increase FA via inbreeding depression in small populations (Quattro and Vrijenhoek 1989). Population estimates will allow separation of natural stress levels from those that may be human induced, which a recent NSF workshop identified as a critical goal of amphibian conservation (Wake 1998). We generally predict that FA will increase with increased tissue contamination, decreased water quality, increased land use degradation, and in populations with high densities (particularly in larvae) and/or very small adult population sizes.

Approach and Methods

The goal of this project is to utilize developmental stability as an biological indicator in several amphibian species and life stages that vary in habitat, life history, behavior, and resource utilization (Table 1). All of these factors should influence the exposure and susceptibility of these organisms to toxins through biomagnification. We will explore these general questions across:

A. areas with variable exposure to toxins, including relatively pristine sites, moderately disturbed (agricultural) sites, and heavily disturbed (industrialized) sites.

B. a variety of species and life stages that we predict will have differential exposure to stressors, including: i. larval vs. adult forms; ii. species that vary in their use of aquatic vs terrestrial habitats; and iii. species that vary in trophic level, and thus bioaccumulation and biomagnification levels.

Clearly, studying FA across habitats with different potential stress levels (A) will directly evaluate the use of FA as a surrogate estimator of contamination and ecosystem health. Testing a wide variety of species and life stages (B) will allow us to determine which kinds of species and stages are the best ecological indicators, and thus which should be utilized in future investigations.

Table 1: Life stages, trophic levels, and habitats of proposed study species.

Species	Larvae		Adult[1]	
	Habitat	Trophic Level	Habitat	Breeding Habitat
Bullfrog (*Rana catesbeiana*)	aquatic	herbivore[2]	semi-terr.	aquatic
Leopard frog (*Rana utricularia*)	aquatic	herbivore[2]	semi-terr	aquatic
Spotted Sal. (*Ambystoma maculatum*)	aquatic	carnivore[3]	terrestrial	aquatic
Slimy Sal. (*Plethodon glutinosis*)	terrestrial[4]	carnivore[3]	terrestrial	terrestrial

[1]All adults are carnivorous. [2] primary consumer [3]secondary consumer [4]larvae metamorphose within the egg; thus we will sample juveniles.

Each of these different comparisons set up tests of different potential exposure levels to assess FA as an biological indicator. For example, in most of the proposed study species there are aquatic larval stages and terrestrial adult stages (Table 1). From a temporal standpoint, adults should have accumulated more contaminants and thus have increased FA compared to larvae. However, aquatic larval stages may experience increased exposure due to their aquatic nature when compared to terrestrial (often fossorial) adults. Further, adults may reflect selection against heavily exposed individuals, i.e., larvae may reveal indications of asymmetry better than adults because heavily exposed and potentially asymmetric larvae never survive to adulthood (see also Burger and Snodgrass 2000). Our comparisons of salamander species with aquatic versus terrestrial reproduction should help test this hypothesis.

Similarly, different amphibian species spend various amounts of time in aquatic vs. terrestrial habitats (e.g., adult habitat vs. breeding habitat in Table 1). This differential exposure to the aquatic environment may also influence the degree of exposure and bioaccumulation of contaminants, and thus the predicted level of FA. Bioaccumulation and FA should also differ among species and life stages that vary in their trophic level, with herbivorous tadpoles having the lowest levels, carnivorous salamander larvae intermediate ones, and carnivorous adults the highest.

Targeted Contaminants

Although we will be assessing a wide variety of water and soil chemistry parameters, as well as tissue contaminantion (Appendix 2), we will target a number of variables which have particular relevance to amphibian development and life history (Appendix 1), and which are found in the western Kentucky area (Table 2). These include polychlorinated biphenyl (PCB) congeners, chlorinated hydrocarbon pesticides, polynuclear aromatic hydrocarbons (PAHs), butyltins, trace inorganic elements (including trace metals such as mercury, cadmium, and aluminum), hydrogen ions (i.e., pH) and associated aluminum, and nitrogen (ammonium, nitrate).

Table 2: Selected Industrial and Agricultural Pollutants in Western Kentucky

	Sample Medium	References
Industrial Pollutants		
Polychlorinated biphenyls	pine needles	Loganathan et al. 1998a
	sediment	Loganathan et al. 1998b
	freshwater mussels	Loganathan et al. 1998b
	amphibians	Seaford et al. 2000

Butyltins	sediment	Loganathan et al. 2000
(Mono, di-, tri-)	freshwater mussels	
Agricultural Pollutants		
DDT and metabolites	pine needles	Loganathan et al. 1998a
Chlordane compounds	sediment	Loganathan et al. 1998b
Hexachlorocyclohexane	freshwater mussels	Loganathan et al. 1998b
	amphibians	Seaford et al. 2000
Hydrogen ions (pH)	pond water	Whiteman et al. unpubl.
Nitrogen	pond water	Whiteman et al. unpubl.
Phosphate	pond water	Whiteman et al. unpubl.

Previous work by the PIs in western Kentucky has provided evidence of these pollutants and, in some cases, their bioaccumulation. For example, westernmost Kentucky is endowed with a variety of industries and state-of-the-art agricultural operations. A report by the Kentucky Environmental Protection Cabinet (KEPCR 1997) documented high levels (>9.68 million lbs toxic release inventory (TRI)) of toxic pollutants released by the industries of this region. Recent biomonitoring studies have revealed that pine needles (an excellent bioindicator of atmospheric organic pollutants), sediments, and mussel tissues from this region contain significant levels of inorganic ions, as well as detectable concentrations of persistent, bioaccumulative and toxic (PBTs) compounds. These include organochlorine (chlorinated pesticides and polychlorinated biphenyls) and organometallic (butyltin) compounds and their metabolites (Loganathan et al. 1998a, 1998b, 1999, 2000). The presence of organochlorine and organometallic parent compounds such as 4,4'-DDT and tributyltin (TBT) respectively in sediments and mussel tissues indicates recent input into the environment (Loganathan et al. 1998a, 1998b). Furthermore, recent studies on extractable organohalogens (EOX) in sediment and mussel tissues have revealed the presence of additional unknown organochlorine residues in this region (Loganathan et al. 2001). Suspected sources for these toxic contaminants include: non-point sources, industrial or agricultural origin, septic tank effluents, atmospheric transport and shipping activities. Toxic pollutants such as polychlorinated biphenyls (PCBs), chlorinated pesticides, polynuclear aromatic hydrocarbons (PAHs) and butyltin compounds are of particular concern because they are known or suspected of causing cancer and/or other health effects such as endocrine disruption in a wide variety of animals in freshwater and marine ecosystems (Loganathan et al. 1994, Jobling et al. 1998, Giesy and Snyder 1997, Khim et al. 1999, Loganathan et al. 2000). In particular, amphibian development and metamorphosis is hormonally regulated and may be especially vulnerable to the effects of endocrine disrupting chemicals (Patlack 1996, Diana and Beasley 1998).

General Amphibian Sampling and Measurements of Fluctuating Asymmetry (FA)

Ponds and surrounding habitats will be sampled for the various species and life stages listed in Table 1. At least 40 ponds with varying potential stress levels (pristine, agricultural, and industrial sites) and which contain breeding populations of target species will be chosen for study. Ponds will be sampled for adults during breeding events (Table 1) and for larvae several times a year. Forty-six potential study sites have already been strategically located throughout western Kentucky (Fig. 2) to take advantage of land use practices (forested, industrial, and agricultural) as well as prevailing winds (i.e., downwind of major industrial centers). Many of these sites were used in previous studies, and other sites that are immediately adjacent to industrialized areas have been located. Slimy salamanders, which breed terrestrially, will be collected in nearby forested areas using standard herpetological capture techniques.

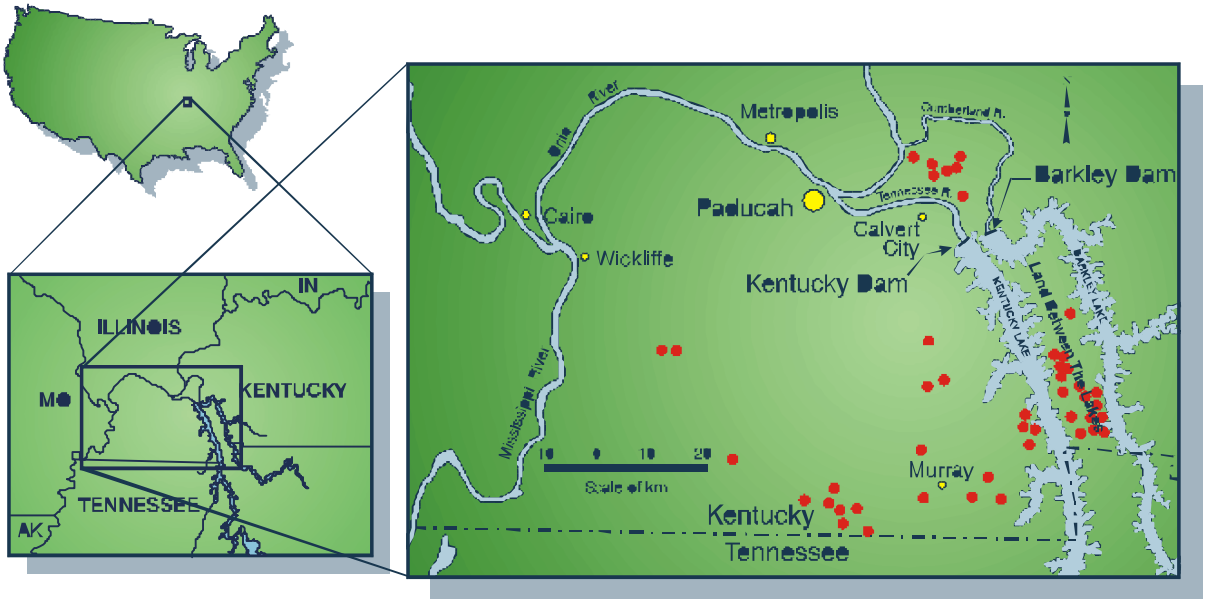

Fig. 2: Locations of 46 sampling sites in the Purchase region of western Kentucky; each dot represents a single pond. Note the strategic locations of sites in forested areas (Land Between the Lakes), agricultural areas (west and north of Murray), and those downwind of industrial areas (east of Paducah, Calvert City, and Murray).

Amphibians will be captured using dip nets, minnow traps, and seines. At least 25 larvae and 10-12 adults (males, because they are typically much more abundant than females) will be sampled from each population. Previous work has shown that these sample sizes will provide excellent power (> 0.8) for statistical analysis (Whiteman et al. unpubl. data). After capture, individuals will be transported in buckets back to Murray State for digital imaging. Each individual will be anesthetized using tricaine methylchloride (MS-222), sexed (adults only) and measured for snout-vent length (SVL; to nearest mm) with a ruler and mass (g) with a balance. Each individual will then be photographed with a Pixera Professional digital camera which is connected to a PC (already available). We utilize digital imagery because it reduces measurement error considerably when compared to other techniques (Fig. 3). In addition, digital imagery allows us to easily take multiple measurements at a later time, rather than immediately while the animal is in the laboratory. After imaging is complete, most animals will be submerged in aged water to revive them from sedation and released back to their pond/area of capture. A subsample of captured animals will be preserved for analysis of bioaccumulated contaminants (see below).

Measurements of FA will concentrate on morphological structures directly related to amphibian fitness, particularly measures associated with the head, mouth, and torso which have already been shown to exhibit significant FA in other species (Table 3; Fig. 1, 3; Whiteman et al., unpubl. data). Each individual will be measured three separate times in order to statistically analyze measurement error (Palmer 1994)

Fig. 3: Left-side digital image of a larval tiger salamander, *Ambystoma tigrinum*. Arrows show the position of characters used in morphometric analysis. These traits are (left to right): nares, corner of the jaw, posterior edge of front limb, anterior edge of hind limb.

Table 3: Proposed Measurements for Each Species and Life Stage

Measurement	Acronym	Description
Eye to Nares	EN	distance from the eye to the nares
Eye to Jaw	EJ	distance from the eye to corner of the jaw
Eye to Hind Limb	EHL	distance from the eye to the anterior edge of the hind limb
Nares to Jaw	NJ	distance from the nares to corner of the jaw
Nares to Hind Limb	NHL	distance from the nares to the anterior edge of the hind limb
Jaw to Front Limb	JFL	distance from the corner of the jaw to the posterior edge of the front limb
Front Limb to Hind Limb	FLHL	distance from the posterior edge of the front limb to the anterior edge of the hind limb

Larval amphibian densities will be determined using a .75m x .75m drop box (Whiteman et al., in review), and adult population sizes will be estimated using mark-recapture (Whiteman et al. 1994, 1996). Temperature, pH, conductivity, dissolved oxygen, ORP, and turbidity will be measured with a YSI portable meter (requested here), alkalinity will be measured with a digital titrator (already available) and orthophosphates and nitrate/nitrite will be measured using a Lachet Nutrient Analyzer at Murray State's Hancock Biological Station (HBS).

Chemical Analysis of Amphibian Tissues

Amphibian tussues from a subset of FA sites (10-20 per species) will be analyzed for possible contaminants All organic, organometallic and trace element analyses in amphibian tissue samples will be performed at the Murray State University Chemical Services Laboratory (MSU-CSL). MSU-CSL participated in the NIST/NOAA/NS&T/EPA-EMAP Intercomparison Exercise Program and analyzed trace organics and inorganic contaminants in sediment and tissue samples during 1998 and 1999. MSU-CSL maintained high accuracy and the values obtained are comparable with assigned values. CSL's participation in the QA program was sponsored by NOAA, and CSL will continue to participate in the QA program during the course of the proposed project.

Outline of Analytical Methods

Appendix 2 presents a list of target analytes (chemical stressors) to be measured in amphibian tissues as well as water and soil samples from amphibian sampling locations. The analytes were chosen based on general contamination of western Kentucky by industrial and agricultural operations. This list includes contaminants already reported in environmental and biological samples from this region (Table 2) and several new compounds such as polynuclear aromatic hydrocarbons (PAHs), phenyltins, and agricultural pesticides and fungicides that are known and/or suspected as serious environmental stressors for aquatic and terrestrial biota (Appendix 1).

Amphibian tissue samples will be analyzed following approved procedures (Kannan et al. 1997, Loganathan et al. 1995, 1999, Maruya et al. 1997, U.S. EPA 1993). Amphibian species will be collected and preserved from each of the > 40 sample sites (Fig. 2) according to standardized procedures. Numbers of amphibians utilized from each sample site will depend on life stage (larva, adult) and species. For example, because we require approximately 25 g (wet weight) of tissue for sample analysis, we will require numerous larvae to complete one larval sample per site. This will limit our larval contamination samples to one per site per species for most amphibian species. In contrast, because adult amphibians can often provide enough tissue within a single individual, we will be able to include multiple-individual contamination measurements of each species within each study site and target specific tissues within an individual for analysis (i.e., muscle, reproductive tissue, etc. The mean of adult individual measurements within a site will be used for comparison with larval samples. Although larval and adult samples will thus have different underlying sample distributions, these techniques should provide excellent power (> 0.8) for statistical comparisons across land use areas and for comparisons with FA.

Amphibian tissue will be placed in pre-cleaned I-Chem sample jars and handled by trained personnel at all times to minimize contamination. All glassware and dissecting materials utilized at this time will be solvent cleaned prior to use. For organic contaminant analysis, the sample will be cut into small pieces (depending on the size, a pooled sample will be used) and freeze dried in a Labconco FreeZone Freeze Dry System (Model 77535). About 3-5 g of freeze dried tissue will be cut into small pieces and Soxhlet extracted using a 3:1 mixture of methylene chloride and acetone. The extract will be K-D concentrated and cleaned using silica gel column chromatography. An aliquot of the extract will be used for PCB congeners and chlorinated pesticides, and another aliquot will be used for PAH analysis. The PCB fraction will be further cleaned using concentrated sulfuric acid. PCB congeners will be analyzed using a Shimadzu Model GC-17A gas chromatograph (GC) with Shimadzu Model AOC-17 autoinjector. A capillary column DB-5 (30mX0.25 mm i.d. X 0.25 µ film thickness) and a ^{63}Ni electron capture detector (ECD) will be used for analyte separation and detection respectively. NIST (National Institute of Standards Technology) SRM 2262 PCB calibration standard and SRM 2261 chlorinated pesticide standards will be used for instrument calibrations, calibration verification, generation of response factors and quantitations. 4,4'-dibromooctafluorobiphenyl will be used as a surrogate standard. Confirmation analysis will be performed with a DB-17 capillary column and/or GC-MS (Hewlett-Packard (HP)-5890 Series II and HP5989A Mass Engine).

PAHs will be quantified by capillary gas chromatography-mass spectrometry utilizing full scan and selected ion monitoring (SIM) modes. An apolar DB-5 column mounted in a Hewlett Packard 5890 Series II gas chromatograph and interfaced to a Hewlett-Packard 5989A mass spectrometer/ data system will be used. It is anticipated that in water and amphibian tissue samples the concentrations of PAHs will be very low (parts per trillion to parts per billion level). Therefore, these sample extracts will be analyzed using a High Performance Liquid

Chromatograph (HPLC) interfaced with a fluorescence detector, which is requested in this proposal. The HPLC with fluorescence detector system is very sensitive to PAHs and will allow us to meet required detection limits. The HPLC and GC-MS will be calibrated over the appropriate PAH standard concentration range through construction of calibration curves. Response factors will be developed and used for calculation of concentrations in the samples and recoveries of internal standards. The GC-MS system also will be used for confirmation of PCBs, pesticides, and quantification of non-fluorescent PAHs.

Butyltin derivatives will be analyzed following procedures described elsewhere (Kannan et al. 1997, Loganathan et al. 1999, Maruya et al. 1997). Briefly, 3-5 g of freeze dried amphibian tissue sample will be acidified and extracted with 70 ml of 0.1% tropolone-acetone, and the solvent will be transferred to 100 ml of 0.1 % tropolone-benzene in a separatory funnel. Moisture in the organic extract will be removed using 35 g of anhydrous sodium sulfate and then the concentrated extract will be propylated with n-propylmagnesium bromide (ca. 2 mol/L in THF solution, Tokyo Kasei Kogyo Ltd., Japan) as a Grignard reagent. The derivatized extract will be passed through a 6 g Florisil packed wet column for cleanup. The eluate from the Florisil column will be rotary evaporated to 5 ml and injected into a gas chromatograph.

Chromatographic separation will be performed using a Shimadzu Gas Chromatograph ((Model GC-17A) equipped with flame photometric detector (FPD) and 30m x 0.25mm i.d. DB-1 capillary column coated with 0.25 micron film thickness. The column oven temperature will be programmed from 80^{o}C (1 min. hold) to 160^{o}C at a rate of 15^{o}C /min. and then at a rate of 5^{o}C /min. to a final temperature of 260^{o}C with a 5 min. final hold time. Injector and detector temperatures will be held at 200 and 270^{o}C, respectively. The flame photometer will be operated using a hydrogen-air-nitrogen flame and will be equipped with a 610 nm band-pass filter that is selective for tin-containing compounds. Butyltin trichloride, dibutyltin dichloride and tributyltin chloride of known amounts (100 ng) will be spiked in to tropolone-acetone and passed through the whole analytical procedure, and used as an external standard. Only freshly derivatized external standards prepared along with every set of eight samples will be used to determine the concentrations. A procedural blank will be analyzed with every set of eight samples to check for interfering compounds and to correct sample values if necessary.

Trace elements in amphibian tissue samples will be analyzed following the procedures described by the U.S. EPA (1993) with some modifications. The freeze dried tissue samples will be stored in a desiccator for 24 h prior to digestion. About 0.1 g dried sample will be placed into a digestion bomb Teflon cup and 2.5 ml of trace metal grade nitric acid will be added. The lid will be placed on the cup and the covered cup will be placed inside the bomb. The bomb will be microwaved for 25 seconds, and then cooled in a hood for 30 min. prior to dilution. After cooling, the samples will be diluted to 25 ml using 18MÍ Millipore deionized water. Elemental concentrations in the sample will be quantitated using a Perkin Elmer Plasma II Inductively Coupled Atomic Emission Spectrometer. CRM-TMDW (Certified Reference Material- Trace Metals in Drinking Water) will be analyzed as part of quality assurance and quality control requirements.

Chemical Analysis of Water, Sediment, and Soil Samples

One liter water samples will be collected from each study pond. The water sample will be extracted with methylene chloride and analyzed for PCBs, pesticides etc. using a GC-ECD and PAHs using a HPLC with fluorescence detector (Maruya et al. 1997, Schantz et al. 1990). For

butyltin and phenyltin, the sample will be extracted using 0.2% tropolone in methylene chloride and analyzed using GC-FPD (U.S. EPA 1993). Sediment and soil samples (40g collected from each site) will be Soxhlet extracted using 3:1 mixture of methylene chloride and acetone for 16 hrs for PCBs, pesticides, and PAHs. Butyltin and phenyltin extraction in soil and sediments will be performed as described for amphibian tissues. The sample extracts will be concentrated, cleaned up using column chromatographic techniques, and analyzed as described for amphibian tissues.

Data Analysis

We will analyze FA across populations utilizing the methods of Palmer (1994) to assess departures from FA (such as directional asymmetry) and statistically evaluate measurement error. FA levels will be analyzed for relationships with tissue contaminant concentrations, water chemistry, land use practices, and density/adult population size for each species and life stage using MANOVA (multivariate analysis of variance) as well as correlational techniques.

Expected Results and Benefits

We generally predict that FA will increase with increased tissue contamination, decreased water quality, increased land use degradation, and in populations with high densities (particularly in larvae) and/or very small adult population sizes. We believe that by analyzing FA across multiple stressors of both human and natural origin, we will have the ability to tease apart their effects, and better understand the degree to which FA acts as a bio-indicator of environmental degradation. In sum, we expect that understanding the relationship between developmental stability and natural and anthropogenic stressors will provide the following benefits:

1. ***Developmental stability will be useful as a measure of amphibian population health, revealing which populations are stressed before the stressors cause negative effects***.

2. ***Developmental stability in amphibians will be a useful proxy for anthropogenic impacts on surrounding ecosystems.***

3. ***Developmental stability in amphibians will act as a bio-indicator for human health, because many of the stressors affecting amphibians also cause harm in humans.***

4. ***We will evaluate which species/life history stages/trophic levels are the most accurate indicators with regard to anthropogenic stress.***

Once these methodologies have been developed, researchers will be able to choose the species/trophic level/life history stage which is most useful for a particular stressor, and use FA measures as a proxy for more expensive contaminant or water chemistry analysis. In those populations with substantial FA, researchers can conduct further studies to identify the contaminants or other stressors affecting development. Thus, these methods will allow a more efficient use of time and resources, by providing a screening process for environmental sampling. The results of this research will thus be valuable for the assessment and management of amphibian populations, the surrounding ecosystem, and human health.

General Project Information

FACILITIES:

HHW (PI) Laboratory: Murray State University has generously provided the PI with newly renovated laboratory and office space, a Macintosh Power PC with color monitor for digital imaging, a Percival Scientific Environmental Chamber (I-36VL) for holding of amphibians, a Leica dissecting microscope, and miscellaneous lab and field supplies including chest waders, nets, buckets, minnow traps, and a seine. Previous grants have allowed the purchase of a Pixera Professional digital camera and software needed for digital imaging.

MSU's Chemical Services Laboratory (CSL): BGL has access to nearly 2000 sq. ft. of recently renovated space located in three laboratories. Modern equipment utilized in CSL includes a GC/MS/LC instrument array featuring the Hewlett Packard "MS Engine" as a key component, and electronically linked to various data bases. This unit is equipped with an dual autoinjector and presampler-desorber. CSL also has a Shimadzu GC with an autoinjector and EC detector system, a Shimadzu GC with a flame photometric detector, a modern freeze-dryer, a stand-alone water purification system, numerous dedicated refrigerators and freezers, and necropsy equipment. Hoods, rotary evaporators, balances and other needed equipment items are available. CSL has the most modern and complete holding of environmental glassware in Kentucky, along with needed solvents and standards. Previous successful grants have allowed the CSL staff to stockpile replacement vacuum pumps and instrument replacement parts. CSL staff also make full advantage of several inductively coupled plasma and graphite furnace atomic absorption spectrophotometers (ICPAA & GFAA), which are also computer-interfaced systems, for various trace metal measurements. Each laboratory has at least one additional computer, with full internet access.

MSU's Hancock Biological Station (HBS): The Hancock Biological Station, located only 16 miles from the MSU campus, has a complete water chemistry lab including a Lachet Nutrient Analyzer with associated trained technicians that will utilized for inorganic chemistry analyses.

PERSONNEL

This proposal represents a unique collaborative mix of expertise in conservation biology, aquatic ecology, toxicology, and analytical chemistry.

Howard H. Whiteman (PI): HHW has extensive experience in amphibian biology and conservation as well as aquatic ecology. HHW will be directly involved with the location of study sites, the capture, imagery, and measurement of amphibians, the collection and preparation of tissue and water samples for analysis, statistical analyses, and manuscript preparation. To meet these goals, HHW will utilize a graduate student and an undergraduate student.

Bommanna G. Loganathan (Co-PI): BGL has extensive experience in analyzing organic and inorganic chemical contaminants in environmental and biological samples. BGL has successfully completed five major environmental trace analysis projects funded by the U.S. Environmental Protection Agency and the U.S. Army Corps of Engineers. BGL will be directly involved in the sample handling and chemical analysis with graduate and undergraduate students and data interpretation.

PROJECT SCHEDULE

Year	Months	Activities
2001-02	**Sept-Nov**	**Begin field sampling of sites** **Begin contaminant analysis once tissues have been acquired.**
	Dec-Jan	**Analysis of digital images, continued contaminant analysis.**
	Feb-Aug	**Continued field sampling, contaminant and image analysis, and data analysis.**
	Mar-April	**Presentation of results at regional meetings by students.**
2002-03	**Sept-Nov**	**Continued field sampling, contaminant and image analysis, and data analysis.**
	Dec-Jan	**Continued contaminant, image, and data analysis.** **Begin manuscript preparation.**
	Feb-May	**Continued field sampling, contaminant and image analysis, data analysis.**
	Mar-April	**Presentation of results at regional meetings by students.**
	May-Aug	**Complete contaminant and image analysis, data analysis.** **Complete first manuscript.** **Presentation of results at national meetings.**
2003-2004	**Sept-Dec**	**Complete second manuscript; continued work on others if necessary.**

Appendix 1: Selected Pollutants and Associated Chemistry with Known Negative Effects on Amphibians

	Species	Effect	References
Industrial Pollutants			
Mixed	*Xenopus laevis*	endocrine disruption deformities	Garber et al. 2000
PCBs	*Bufo americanus*	increased mortality	Birge and Cassidy 1983
	Bufo woodhousii fowleri	increased mortality	
	Rana temoraria	deformities reduced body mass	Gutleb et al. 1999
	Xenopus laevis	deformities reduced body mass increased larval period	Gutleb et al. 1999
benzene	*Ambystoma mexicanum*	increased mortality	Sloof and Baerselman 1980
	Xenopus laevis	increased mortality	Sloof et al. 1983
	Rana pipiens	increased mortality	Birge and Cassidy 1983
mercury	*Xenopus laevis*	increased mortality	Dumpert and Zietz 1984
	Rana pipiens	deformities	Dial 1976
coal ash	*Rana catesbeiana*	deformities increased metabolic rate	Rowe et al. 1998a, Hopkins et al. 2000 Rowe et al. 1998b
hydrogen ions	various (review)	increased mortality	Pierce 1985, 1993
(pH)	*Rana sylvatica*	reduced mass increased larval period	Rowe et al. 1992
	Ambystoma tigrinum	increased mortality reduced embryonic period reduced size at hatching	Whiteman et al. 1995
Aluminium	*Hyla cinerea*	increased mortality reduced body size reduced swimming speed increased susceptibility to predators	Jung and Jagoe 1995
	Ambystoma macrodactylum *Pseudacris regilla*	reduced body size reduced embryonic period	Bradford et al. 1994
Agricultural Pollutants			
General Effluent	*Rana aurora aurora*	increased mortality inhibited metamorphosis	Loveridge et al. 2000
Herbicides			
Acetochlor	*Rana pipiens*	accelerated metamorphosis	Cheek et al. 1999
Atrazine	*Hyla versicolor*	reduced size at metamorphosis	Diana et al. 2000
Paraquat	*Rana berlandieri*	increased mortality	Dial and Bauer 1984
	Rana pipiens	increased mortality	
Diquat	*Rana pipiens*	increased mortality	Dial and Dial 1987
Pesticides			
various	various	increased mortality temporary paralysis	Berrill et al. 1997
various	*Rana clamitans*	deformities increased susceptibility to disease	Bonin et al. 1997
Permethrin	*Rana clamitans*	deformities behavioral abnormalities	Berrill et al. 1993
Carbamate	*Rana perezi*	deformities	Honrubia et al. 1993
	Rana temporaria	increased mortality deformities	Rzehak et al. 1977
Fenitrothion	*Microhyla ornata*	deformities behavioral abnormalities reduced growth	Pawar and Katdare 1994
Malathion	*Rana pipiens*	increased mortality	Kaplan and Glaczenski 1965
DDT	*Rana catesbeiana*	increased mortality	Mulla 1963
Lindane	*Xenopus laevis*	increased mortality	Marchal-Segault&Ramande 1981
Phenyltins	*Ambystoma barbouri*	reduced growth/development	Lynn et al. 2000
Chlorpyrifos	*Rana utricularia*	deformities behavioral abnormalities	Sheffield 2000
Nutrients			
Ammonium	various anurans (review)	increased mortality	Schuytema and Nebeker 1999
	Pseudacris regilla	reduced body size	
	Xenopus laevis	reduced body size	
	various	increased mortality deformities reduced body size reduced activity	Hecnar 1995

Nitrate	various anurans (review)	deformities increased mortality reduced growth rate behavioral abnormalities	Rouse et al. 1999
	Rana pipiens	reduced growth rate	Allran and Karasov 2000

Appendix 2: List of target analytes for amphibian biomonitoring study.

Compound	Compound (continued)
PCB Congeners	**Chlorinated Pesticides**
2,4'-D_2CB (8)	hexachlorobenzene
2,2',5-T_3CB (18)	lindane (gamma-HCH)
2,4,5- T_3CB (29)	beta HCH
2,2',4,6- T_4CB (50)	cis-Chlordane
2,4,4'- T_3CB (28)	trans-Nonachlor
2,2',5,5'- T_4CB (52)	2,4'-DDE
2,2',4,6,6'- P_5CB (104)	4,4'-DDE
2,2',3,5' -T_4CB (44)	2,4'-DDD
2,3,4,4'- T_4CB (66)/2,2',3,5',6- P_5CB (95)	4,4'-DDD
2,2'4,5,5'- P_5CB (101)/2,2'3,4',5- P_5CB (90)	2,4'-DDT
2,2'3,4,5'- P_5CB (87)	4,4'-DDT
3,3',4,4'- T_4CB (77)	heptachlor
2,2'4,4',5,6'- H_6CB (154)	heptachlor epoxide
2,3',4,4',5- P_5CB (118)	mirex
2,2',3,4',5,6,6'- H_7CB (188)	endosulfan-I
2,2',4,4',5,5'- H_6CB (153)	endosulfan-II
2,3,3',4,4',- P_5CB (105)/2,2'3,3'4.6'- H_6CB (132)	aldrin
2,2',3,4,4',5'- H_6CB (138)/2,3,3',4',5,6- H_6CB (163)/	dieldrin
2,3,3',4',5,6- H_6CB (164)	endrin
3,3',4,4',5- P_5CB (126)	dicofol
2,2',3,4',5,5',6- H_7CB (187)/2,2',3,4,4',5,6'- H_7CB	chlorpyrifos
(182)/2,3,3',4,5,5'- H_6CB (159)	
2,2',3,3',4,4'- H_6CB (128)	**Polynuclear Aromatic**
2,2',3,3',4,5',6,6'-O_8CB (201)	**Hydrocarbons (PAHs)**
2,2',3,4,4',5,5'- H_7CB (180)	(* internal standards)
2,2',3,4,4',5,5'- H_7CB (180)	Naphthalene
2,2',3,3',4,4',5-H_7CB(170)/2,3,3',4,4',5,6- H_7CB(190)	2-Methylnaphthalene
2,2',3,3',4,4',5,6-O_8CB(195)	1-Methylnaphthalene
2,2',3,3',4,4',5,5',6-N_9CB(206)	Acenaphthalene
decachlorobiphenyl	*Acenaphthalene-d_8
	Trimethylnaphthalene
Butyltins	Fluorene
Mono-, di, and tributyltins	Phenanthrene
Phenyltins	*Phenanthrene- d_{10}
Mono-, di, and triphenyltins	Anthracene
	1-Methylphenanthrene
Trace Metals	*Pyrene-d_{10}
(Primary interest group)	Fluoranthene
Manganese	Benzo(a)anthracene
Mercury	Chrysene
Cadmium	Benzo(b&k)fluoranthene
Lead	Benzo(a)pyrene
Selenium	Benzo(e)pyrene
(Secondary interest group)	Perylene
Nickel	*Perylene- d_{12}
Zinc	Dibenzo(a,h)anthracene
Iron	Benzo(g,h,i)perylene
Copper	*Benzo(g,h,i)perylene- d_{12}

Strontium	Indeno(2,3,4-cd)pyrene

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Whiteman, H. H., S. A. Wissinger, and A. J. Bohonak. 1994. Seasonal movement patterns in a subalpine population of the tiger salamander, *Ambystoma tigrinum nebulosum***.** Canadian Journal of Zoology 72:1780-1787.

Whiteman, H. H., R. D. Howard, and K. A. Whitten. 1995. Effects of pH on embryo tolerance and adult behavior in the tiger salamander, *Ambystoma tigrinum tigrinum*. Canadian Journal of Zoology 73:1529-1537.

Whiteman, H. H., S. A. Wissinger, and W. S. Brown. 1996. Growth and foraging consequences of facultative paedomorphosis: a test of alternative selection mechanisms. Evolutionary Ecology 10:433-446.

Whiteman, H. H., J. S. Sheen, E. Johnston, A. Van Duesen, R. Cargille, and T. Sacco. In review. Heterospecific prey and trophic polyphenism in larval tiger salamanders, *Ambystoma tigrinum nebulosum*. J. Animal Ecology.

Wyman, R. L. 1990. What's happening to the amphibians? Conservation Biology 4:350-352.

HOWARD H. WHITEMAN
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howard.whiteman@murraystate.edu

Education:

1994 Ph. D. (Biological Sciences) Purdue University, West Lafayette IN
1988 B. S. (Biology and Psychology double major) Allegheny College, Meadville PA

University Positions:

1997- Assistant Professor, Department of Biological Sciences, Murray State University
1997-98 Adjunct Assistant Professor, Center for Public Issues in Biotechnology, University of Maryland Biotechnology Institute
1994-96 Postdoctoral Research Associate, Savannah River Ecology Laboratory (University of Georgia)
1994-96 Summer Teaching Faculty, Rocky Mountain Biological Laboratory
1992-94 Graduate Research Fellow, Purdue University
1988-92 Graduate Teaching Assistant, Purdue University

Academic Honors:

2000 MSU Sigma Xi Outstanding Teacher Award
1992-94 Purdue Research Foundation Fellowship
1988 Cum Laude graduate
Bugbee Prize in Biology for outstanding senior thesis
Honors Graduate in Psychology
1986-88 Alden Scholar, Allegheny College

Selected Research Grants:

1999-03 NSF Collaborative Research at Undergraduate Institutions (C-RUI). $789,764. "Biogeochemical and ecological processes within a reservoir littoral zone".
1999-00 Kentucky Water Resources Research Institute. $16,656. "Developmental stability as an indicator of amphibian population health and environmental degradation"
Colorado Natural Areas Program. $1280. "Developmental stability as an indicator of amphibian population health: an assessment of tiger salamander populations in Colorado". (renewed 00-01; $2300).
1999 Pittsburgh Zoo Conservation Fund. $1030. "Developmental stability as an indicator of amphibian population health".
1998 Kentucky NSF EPSCoR Research Enhancement Grant. $11,928. "Evolutionary ecology of polyphenism and development of sex-specific genetic markers".
Committee on Institutional Studies and Research Grant, Murray State University. $2500. "Ecology of polymorphism in salamanders".
Kentucky Department of Fish and Wildlife Resources. $69,264. "Vertebrate Distribution Mapping Component of the Kentucky GAP Analysis Project" (with T. Derting).

Selected Publications (***** denotes undergraduates under my direction)

Directly Related to the Proposal (in order of relative importance):

Sheen*****, J. P. and H. H. Whiteman. 1998. Head and body size relationships in polymorphic tiger salamander larvae from Colorado. **Copeia** 1998:1089-1093.

Whiteman, H. H. and R. D. Howard. 1998. Conserving alternative amphibian phenotypes: Is there anybody out there? In: Lannoo, M. J. (ed.), **The Status and Conservation of Midwestern Amphibians**, pgs. 317-324. Iowa University Press, Ames, IA.

Whiteman, H. H., R. D. Howard, and K. A. Whitten*****. 1995. Effects of pH on embryo tolerance and adult behavior in the tiger salamander, *Ambystoma tigrinum tigrinum*. **Canadian Journal of Zoology** 73:1529-1537.

Whiteman, H. H. and S. A. Wissinger. 2001. Multiple hypotheses for population fluctuations: the importance of long-term data sets for amphibian conservation. In: M. L. Lanoo (ed.), **Status and Conservation of U.S. Amphibians**, California University Press (in press).

Wissinger, S. A. and H. H. Whiteman. 1992. Fluctuation in a Rocky Mountain population of salamanders: anthropogenic acidification or natural variation? **Journal of Herpetology** 26:377-391.

Other Significant Publications:

Wissinger, S. A., H. H. Whiteman, G. L. Rouse, G. B. Sparks, and W. S. Brown. 1999. Foraging trade-offs along a predator-permanence gradient in subalpine wetlands. **Ecology**, 80:2102-2116.

Gutrich, J. J. and H. H. Whiteman. 1998. Analysis of the ecological risks associated with genetically engineered marine macroorganisms. In: Zilinskas, R. A. and P. J. Balint (eds.), **Genetically Engineered Marine Organisms: Environmental and Economic Risks and Benefits**. Kluwer Academic Publishers.

Whiteman, H. H. 1997. Maintenance of polymorphism promoted by sex-specific fitness payoffs. **Evolution** 51:2039-2044.

Whiteman, H. H., S. A. Wissinger, and W. S. Brown. 1996. Growth and foraging consequences of facultative paedomorphosis: a test of alternative selection mechanisms. **Evolutionary Ecology** 10:433-446.

Whiteman, H. H. 1994. Evolution of facultative paedomorphosis in salamanders**. Quarterly Review of Biology** 69:205-221.

Other Pertinent Activities:

2000-01 President-elect, MSU Chapter of Sigma Xi

1998- Ecological Society of America Partner, Strategies for Ecology Education, Development, and Sustainability (SEEDS) program

1992-97 Research Mentor, NSF Research Experience for Undergraduates program.

Recent Collaborators (last 48 months, not listed above)**:**

Ian Billick, Rocky Mountain Biological Laboratory
Nancy L. Buschhaus, University of Tennessee-Martin
John D. Krenz, Mankato State University
Peter Niewiarowski, University of Akron
Joseph H. K. Pechmann, University of New Orleans
Edmund Zimmerer, Murray State University

Graduate Students (last five years; total number advised = 4)

Vivien Braslau (M.S., Bard College, 1998)
Jill Kruper (Ph. D., MSU-University of Louisville Cooperative Program)
Monica Pope (M.S., Murray State University)
Amy Benson (M.S., Murray State University)

Former Research Advisors:

Ph. D. Richard D. Howard (Purdue University)
Postdoc J. Whitfield Gibbons (University of Georgia-SREL)

Bommanna G. Loganathan
Department of Chemistry and Center for Reservoir Research (CRR)
Murray State University, Murray, KY 42071
Telephone: (270)-762-3044/2586
E-mail: bommanna.loganathan@murraystate.edu

Education:
1990 Ph.D. Ecotoxicology and Environmental Chemistry, Ehime University, Japan
1986 Ph.D. Marine Microbiology, Annamalai University, India
1979 M.S. Marine Biology, Annamalai University, India
1977 B.S. Zoology, Chemistry, Botany, University of Madras, India.

Professional Experience:
2000- present. Assistant Professor, Department of Chemistry and CRR, Murray State University, Murray, KY 42071.
1997-1999 Post-doctoral fellow, Department of Chemistry and CRR, Murray State University, Murray, KY 42071.
1993-1996 Research Assistant, Skidaway Institute of Oceanography, University System of Georgia, Savannah, GA 41411
1990-1993 Post-doctoral fellow, SUNY College at Buffalo, NY 14222

Selected Professional Services: Ad Hoc reviewer for Research Grant Proposals submitted to Michigan Sea Grant Program, Reviewer Panel for five international journals (1994-present). Member- Kentucky Academy of Science Awards Committee (1998-present)

Memberships in Professional Societies:
Kentucky Academy of Science-2000, Council of Undergraduate Research-2000, Society of Environmental Toxicology and Chemistry (SETAC) –2000.

Selected Grants:
1999-2003 Biogeochemical and ecological processes within a reservoir littoral zone. National Science Foundation. $789,764. Co-PI.
1994-1996 Contaminant levels in Louisianian and West Indian Provinces, U.S. EPA-EMAP-Estuaries $ 1.2 million (P.Is. H. Windom, B.G. Loganathan, R. Smith, R. Lee and J. Ertel)
1992-1994 Evaluation of PCBs and HCB sources in the Babcock Street Sewer District. U.S. EPA. $ 30,044 (P.Is. K. Irvine and B.G. Loganathan)
1990-1993 Field, Laboratory and Engineering Support Buffalo River Mass Balance Project. $280,134 (P.I. H. Sikka, P.I. for Data Contact: B.G. Loganathan).

Publications (Selected from over fifty published articles):
Loganathan, B.G., Kawano, M., Sajwan, K.S. and Owen, D.A. Extractable organohalogens (EOX) in sediment and mussel tissues from the Kentucky Lake and Kentucky Dam Tailwater. *Toxicological Environmental Chemistry.* (In press).
Loganathan, B.G., Kannan, K., Sajwan, K.S. and Owen, D.A. **2000**. Butyltin compounds in freshwater ecosystems. In: *Persistent, Bioaccumulative and Toxic Chemicals I: Fate and Exposure.* (Eds. R.L. Lipnick, J. Hermens, K. Jones and D. Muir). American Chemical Society, Washington, DC. 308pp.
Watanabe, M., Kannan, K., Takahashi, A., Loganathan, B.G., Odell, D.K., Tanabe, S. and Giesy, J.P. **2000**. Polychlorinated biphenyls, organochlorine pesticides, tris(4-chlorophenyl)methane, and tris(4-

chlorophenyl)methanol in livers of small cetaceans stranded along Florida coastal waters, USA. *Environ. Toxicol. Chem.* **19**, 1566-1574.

Whalen, M.M., Loganathan, B.G. and Yamashita, N. **2000**. Effect of *in vitro* exposure to selected endocrine disrupting chemicals on human natural killer (NK) cell function. Dioxin 2000. *Organohalogen Compounds* **40**, 259-261.

Loganathan, B.G., Kannan, K., Senthilkumar, K., Sickel, J. and Owen, D.A. **1999**. Occurrence of butyltin residues in sediment and mussel tissues from the lowermost Tennessee River and Kentucky Lake, U.S.A. *Chemosphere* **39**, .2401-2408.

Whalen, M.W., Loganathan, B.G., Kannan, K. **1999**. Immunotoxicity of environmentally relevant concentrations of butyltins on human natural killer cells *in vitro*. *Environmental Research* **81**, 108-116.

Irvine, K.N. and Loganathan, B.G. 1998. Localized enrichment of PCB levels in street dust due to redistribution by wind. *Water, Air and Soil Pollution*, **105,** 603-615.

Loganathan, B.G., Irvine, K.N., Kannan, K. Pragatheeswaran, V. and Sajwan, K.S. **1997**. Distribution of selected PCB congeners in the Babcock Street Sewer District: A multimedia approach to identify PCB sources in combined sewer overflows (CSOs) discharging to the Buffalo River, New York. *Arch. Environ. Contam. Toxicol*. **33,** 130-140.

Loganathan, B.G., Kannan, K., Watanabe, I., Kawano, M., Irvine, K.N., Kumar, S. and Sikka, H.C. **1995**. Isomer specific determination and toxic evaluation of polychlorinated biphenyls, polychlorinated/brominated dibenzo-*p*-dioxins and dibenzofurans, polybrominated biphenyl ethers, and extractable organic halogen in carp from the Buffalo River, New York. *Environ. Sci. Technol*. **29,** 1832-1838.

Loganathan, B.G. and Kannan, K. **1994**. Global organochlorine contamination: An Overview. *Ambio* **23**, 187-191.

Selected Presentations (*undergraduate students):

Loganathan, B.G. and Seaford, K*. **2000**. Observations on the fate of PCBs using contaminated sediments and pine needles. Poster presented at CUR 2000 (8th National Conference, Council of Undergraduate Research) June 22, 2000. Wooster College, Wooster, OH.

Seaford, K*. and Loganathan, B.G. **2000**. Chlorinated hydrocarbons in Pine needles: Finger print for the history of use. Poster presented at 8th National Conference, CUR 2000, June 22, 2000, Wooster, OH.

Morton, J.D*., Loganathan, B.G. and Owen, D.A. **1999**. Evaluation of bamboo leaves as a monitor of atmospheric chlorinated hydrocarbon pollutants in westernmost Kentucky. Poster (*selected- national level competition*) presented at the CUR Poster Session at Capitol Hill, April 14, 1999.

Quality Assurance and Quality Control Manuals Written

Smith, R. and Loganathan, B.G. 1994. Quality Assurance Project Plan for EMAP- Estuaries. Environmental Monitoring and Assessment Program (EPA-EMAP-E).

Smith, R. and Loganathan, B.G. 1995. Comprehensive Quality Assurance Plan. Florida Department of Environment Protection.

Loganathan, B.G. and Owen, D.A. 1998. Quality Assurance and Quality Control Plan for the project entitled 'Control of sediment and contaminant input into the Clarks River using vegetative filter strips. Department of Environment Protection, Commonwealth of Kentucky.

Current and Pending Support

The following information should be provided for each investigator and other senior personnel. Failure to provide this information may delay consideration of this proposal.

Investigator: **Howard H. Whiteman**	Other agencies (including NSF) to which this proposal has been/will be submitted. **N/A**

Support: X Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **Biogeochemical and ecological processes within a reservoir littoral zone**

Source of Support: **NSF Collaborative Research at Undergraduate Institutions (C-RUI)**

Total Award Amount: $**789,764** Total Award Period Covered: **January 2000-December 2003**

Location of Project: **Murray State University/Hancock Biological Station/Center for Reservoir Research**

Person-Months Per Year Committed to Cal: Acad: 1 Sumr:

Support: X Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **Developmental stability as an indicator of amphibian population health and environmental degradation**

Source of Support: **Kentucky Water Resources Research Institute**

Total Award Amount: $**16,656** Total Award Period Covered: **March 2000-February 2001**

Location of Project: **Murray State University/Hancock Biological Station**

Person-Months Per Year Committed to Cal: Acad: 4 Sumr:

Support: ☐ Current X Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **Developmental stability as an indicator of amphibian population health and environmental degradation degradation (renewal)**

Source of Support: **Kentucky Water Resources Research Institute**

Total Award Amount: $**7,500** Total Award Period Covered: **March 2001-February 2002**

Location of Project: **Murray State University/Hancock Biological Station/Center for Reservoir Research**

Person-Months Per Year Committed to Cal: Acad: 1.5 Sumr:

Support: ☐ Current X Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **Developmental stability in amphibians as a biological indicator of environmental stressors stressors**

Source of Support: **Kentucky NSF EPSCoR**

Total Award Amount: $**11,988** Total Award Period Covered: **March 1, 2001-February 28, 2002**

Location of Project: **Rocky Mountain Biological Laboratory/Murray State University/Hancock Biological Station**

Person-Months Per Year Committed to Cal: Acad: 2 Sumr: 2

Support: ☐ Current X Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **RUI: Collaborative research on mechanisms underlying salamander population fluctuations**

Source of Support: **NSF-DEB**

Total Award Amount: $ **303,876** Total Award Period Covered: **June 2001-May 2006**

Location of Project:

Person-Months Per Year Committed to Cal: Acad: 1 Sumr: 2

*If this project has previously been funded by another agency, please list and furnish information for immediately preceding funding period.

Current and Pending Support

The following information should be provided for each investigator and other senior personnel. Failure to provide this information may delay consideration of this proposal.

Investigator: Bommanna G. Loganathan	Other agencies (including NSF) to which this proposal has N/A

Support: X Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **Biogeochemical and ecological processes within a reservoir littoral zone**

Source of Support: **NSF Collaborative Research at Undergraduate Institutions (C-RUI)**

Total Award Amount: $**789,764** Total Award Period Covered: **January 2000-December 2003**

Location of Project: **Murray State University/Hancock Biological Station/Center for Reservoir Research**

Person-Months Per Year Committed to the Cal: Acad: Sumr: 1

Support: X Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **Historical trends of persistent organic pollutants in Kentucky Lake and Kentucky Dam tailwaters Kentucky Dam tailwater using sediment cores**

Source of Support: **Kentucky Academy of Sciences**

Total Award Amount: $**12,992** Total Award Period Covered: **January-December 2001**

Location of Project:

Person-Months Per Year Committed to the Cal: Acad: 1 Sumr:

Support: ☐ Current X Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title: **"New Traditions in Chemistry" at Murray State University**

Source of Support: **NSF-CCLI**

Total Award Amount: $**153,979** Total Award Period Covered: **2001-2002**

Location of Project:

Person-Months Per Year Committed to the Cal: Acad: 1 Sumr:

Support: ☐ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title:

Source of Support:

Total Award Amount: $ Total Award Period Covered:

Location of Project:

Person-Months Per Year Committed to the Cal: Acad: Sumr:

Support: ☐ Current ☐ Pending ☐ Submission Planned in Near Future ☐ *Transfer of Support

Project/Proposal Title:

Source of Support:

Total Award Amount: $ Total Award Period Covered:

Location of Project:

Person-Months Per Year Committed to the Cal: Acad: Sumr:

*If this project has previously been funded by another agency, please list and furnish information for

BUDGET

CATEGORIES	Year 1			Year 2			Total		
	EPA	State	Local	EPA	State	Local	EPA	State	Local
a. Personnel									
PI- H. Whiteman									
22.5% of Academic Year			9,444			9,727			19,171
Course Release Time	4,197			4,323			8,520		
Co-PI- B. Loganathan									
25% of Academic Year			10,485			10,800			21,285
Summer Salary	4,194			4,320			8,514		
QA/QC Officer	2,339				2,456		2,339	2,456	
Graduate Students (2)	24,000			24,000			48,000		
Undergraduate RAs (2)	9,216			9,216			18,432		
Total Personnel Costs	**$43,946**		**$19,929**	**$41,859**	**$2,456**	**$20,527**	**$85,805**	**$2,456**	**$40,456**
b. Fringe Benefits									
HHW, @ 32%	1,343		3,022	1,383		3,113	2,726		6,135
BGL, @ 21.5 & 32%*	902		3,355	929		3,456	1,831		6,811
QA/QC Officer, @ 21.5%	503				528		503	528	
Grad Students @ 7.65%	1,836			1,836			3,672		
Graduate Tuition	6,000			6,000			12,000		
Undergrads @ 7.65%	705			705			1,410		
*see budget justification									
Total Fringe Benefits	**$11,289**		**$6,377**	**$10,853**	**$528**	**$6,569**	**$22,142**	**$528**	**$12,946**
c. Travel									
Study Sites from MSU		2,000			2,000			4,000	
Professional meetings					4,000			4,000	
Total Travel Costs		**$2,000**			**$6,000**			**$8,000**	
d. Equipment									
HPLC System with Fluorescence Detector		17,500						17,500	
YSI Water Quality Measurement System		5,500						5,500	
MacIntosh G4		3,500						3,500	
Rotary evaporator		3,000						3,000	
Field Vehicle			$13,500						$13,500
Total Equipment Costs		**$29,500**	**$13,500**					**$29,500**	**$13,500**
e. Supplies									
Field sampling		3,000			3,000			6,000	
Inorganic water chemistry		3,000			3,000			6,000	
Contaminant analysis	17,500	11,750			24,000		17,500	35,750	
Total Supply Costs	**$17,500**	**$17,750**			**$30,000**		**$17,500**	**$47,750**	
f. Contracts									
g. Other									
h. Total Direct Costs (sum of a-g)	**$72,735**	**$49,250**	**$39,806**	**$52,712**	**$38,984**	**$27,096**	**$125,447**	**$88,234**	**$66,902**
I. Indirect Costs (50% of salaries and wages)	**$21,973**		**$9,965**	**$20,929**		**$11,492**	**$42,902**		**$21,457**
j. Total Project Costs (sum of f & g)	**$94,708**	**$49,250**	**$49,771**	**$73,641**	**$38,984**	**$38,588**	**$168,349**	**$87,234**	**$88,359**
k. Total Requested from EPA	**$94,708**			**$73,641**			**$168,349**		

BUDGET JUSTIFICATION

PERSONNEL

Howard H. Whiteman (PI): HHW will spend 35% of his academic year on this project. He currently has a 10 month contract in which he teaches two courses per five-month semester. Thus, we request 1 month academic year salary (**$4,197**) to purchase release time from one non-majors course per year. A 3% cost of living raise is added to this amount in year 2. His summer salary is currently paid by other grants. Approximately 12.5% of his time is made available by the course reduction, with the remaining 22.5% used as local match (22.5% x $**41,973 = $9444** in year 1 and **$9,727** in year 2[assumes a 3% cost of living raise].

Bommanna G. Loganathan (Co-PI): BGL will spend 25% of his academic year and 50% of his summer on this project. His 25% commitment will be used as a local match (25% x $41,940 = **$10,485** in year 1 and **$10,800** in year 2 [assumes a 3% cost of living raise]). He currently has a 10 month salary; thus, we request 1/10 summer salary. A 3% cost of living raise is added in the second year of the project.

QA/QC Officer: A QA/QC Officer is needed to check all data reports produced by the personnel involved in the research and evaluate and sign a QA Audit summary report. Dr. Terry Derting, an associate professor from the Department of Biological Sciences, MSU, will act as our QA/QC officer. She will spend approximately 15 days per year on QA/QC. She currently has a 10 month contract; thus, we request 0.5/10 summer salary. A 3% cost of living raise is added in the second year of this project.

Graduate Students: We request funds to support two graduate students (one involved in amphibian sampling and imagery, one in contaminant analyses) to support this research. Graduate students will be paid a 12 month stipend of **$12,000**, and spend 100% of their time on this project.

Undergraduate Research Assistants: We request funds to support two undergraduate research assistants (one each involved in amphibian sampling and contaminant analyses) per year. Undergraduates will be paid an hourly wage of $8.00 /hr, 12hr/week, across 48 weeks (approx. **$4,608**/year/student).

FRINGE BENEFITS

Actual fringe benefits at Murray State vary depending upon the kind of salary and type of health care used by personnel. Generalized percentages were used for approximate calculations of fringe benefits based on these variables. Thus, summer salary fringes are estimated at 21.5% x gross, academic salary fringes at 32% x gross, and graduate and undergraduate fringes at 7.65% x gross. We have also budgeted tuition funds (**$3,000** per year) for each graduate student.

TRAVEL

We request funds to cover travel costs (mileage) from MSU to surrounding study sites (**$2000/yr**) for amphibian collection and water chemistry analysis. We also request travel funds for professional meetings for the PIs, graduate students, and undergraduate students to present the results of this research during the 2nd year (**$4,000**).

EQUIPMENT

We request funds for a **Variable Wavelength Isocratic HPLC System with Fluorescence Detector** (**$17,500**; BUCK Scientific Instruments, East Norwalk, CT). This system will allow us to measure polynuclear aromatic hydrocarbons, including Benzo-a-pyrene (a highly potent

carcinogen and known endocrine disruptor), Benzo-a-anthracene, Benzo-b-fluoranthene, Benzo-k-fluoranthene,
pyrene and other PAHs at part per billion to part per trillion level. We anticipate that levels of the above mentioned chemicals (stressors) will occur in amphibian tissues at ppb or ppt. The current GC/MS system may not detect such trace levels.

We request funds for a **YSI Water Quality Measurement System** (**$5,500**) to monitor water quality at each sampling site. This system will replace older pH and DO meters that have been used in previous studies. The YSI system is a compact, computerized probe and display that measures water depth, temperature, conductivity, DO, pH, ORP, and turbidity.

We request funds to upgrade the computer currently used for amphibian digital imaging to a faster model with greater storage space (**Macintosh G4** with dual processor and 80GB HD = **$3,500**). We will utilize the current monitor and accessories to minimize costs. Finally, we request funds for a **rotary evaporator** (**$3000**) to concentrate sample extracts at low temperatures.

As a match to this grant, Murray State University will purchase a field vehicle (**Ford Ranger pickup truck**) for the sole use of this project (**$13,500**). The field vehicle will provide a much needed improvement over the use of personal vehicles, which the PIs and their students have used in the past. The field vehicle will provide ready access to all of the study sites, many of which vary in the quality of nearby roads. It will also be useful for hauling sampling gear, water samples, and amphibians to and from MSU.

SUPPLIES

We request funds for miscellaneous field and digital imagery supplies, including waders, minnow traps, seines, nets, buckets, holding containers, computer disks, re-writable CDs, replacement hard drives and service for environmental chambers ($3000 per year). We request funds for inorganic water chemistry analysis ($3000 per year). For contaminant analysis, we request funds for sample containers ($1200), standards (PCBs, PAHs, butyltins and trace metals; $5000), chemicals and solvents ($3500), glassware ($6000), GC supplies and replacement parts ($5000), equipment service ($4000), computer supplies ($1000), NIST QA participation ($3000) and miscellaneous other supplies ($550). Total contaminant analysis supplies in year one is $29,250. In year two, we request $24,000 for replacement supplies.

INDIRECT COSTS

Murray State utilizes a rate of 50% of gross salaries and wages for indirect costs. Indirect costs associated with MSU cost sharing and unrecoverable indirect costs from the State match in year 2 are utilized as part of the total MSU matching funds.

Quality Assurance Narrative Statement

Murray State University Chemical Services Laboratory (MSU-CSL), an active participant of NIST/NOAA/NS&T/EPA-EMAP QA Program, has developed a quality assurance project plan (QAPjP) to assure that all environmental chemical analysis data generated by the laboratory are scientifically valid and of acceptable quality. Key elements of the quality control protocols followed are described below briefly.

Project Organization and Responsibility: The Project Manager is responsible for management of the project, insuring that all required analyses have been performed and reports prepared. Along with the Laboratory Coordinator, the Project Manager also provides a point of contact. The Laboratory Coordinator is responsible for tracking sample progress and reporting results to the project manager. The Analytical Chemist is responsible for scheduling analyses and procedures for organics and metals and reporting results to the project manager. The Quality Assurance Officer keeps quality assurance records, ascertains whether analyses meet requisite quality assurance parameters, and prepares quality assurance reports.

Sample collection, sample receipt, preservation and holding time: Field personnel are thoroughly trained in proper use of sample gears, handling of samples and sample containers to avoid potential sources of sample contamination. The samples are properly labeled and transported to MSU-CSL in suitable containers and preservatives. Sample custody forms will be signed by each of the personnel handling the samples. Upon receipt, the sample integrity will be investigated by the laboratory coordinator. Any problem with sample integrity will be noted on the chain of custody forms and appropriate actions will be taken. The date of receipt and maximum holding time will be noted in our database with a report containing this information supplied to each analyst. Appropriate holding times have not been established for frozen samples, but general guidelines are 6 to 12 months for samples held at $-20^{o}C$.

Quality Control Procedures for Laboratory Operations: MSU-CSL Standard Operating Procedures (SOP) are updated and used for specified environmental contaminant analysis projects. Prior to analysis of the samples, method detection limit (MDL), an initial calibration curve with $r^2 = 0.99$ will be established for each analyte. Continued performance will be evaluated using control charts (X -charts). There will be three sets of limits superimposed on the chart: 1) The "central line" which is the mean value as reported for the certified reference material, 2) upper and lower "warning limits" representing the 95% confidence limits around the mean value and 3) upper and lower "control limits" representing the 99% confidence limits within which 99% of the measured values should lie. Control charts will be updated by laboratory personnel after the completion of each batch of samples. Based on the result of each measurement parameter on the control sample the following course of action will be taken: 1) If the measured value of the control sample is within the warning limit, all sample data for that parameter since the last acceptable control sample are accepted. 2) If the measurement value for the control sample is outside of the control limits, the analysis is no longer considered to be in statistical control and all data since the last accepted control sample are unacceptable. The instrument will be checked for loss of calibration or other malfunction, corrective action taken and statistical control is then reestablished by the results of three consecutive sets of control sample measurements that are in control as established above. Once statistical control has been

established all samples since the last accepted control sample measurement are reanalyzed. Continued laboratory capability will be assessed by (i) participation in the annual NIST Intercomparison exercise program; (ii) repeated analysis of Certified Reference Material (CRM); (iii) laboratory control samples (LCM); (iv) Calibration checks; (v) analysis of reagent blanks; (vi) blind samples; and (vii) matrix spike and matrix spike duplicate samples.

One CRM will be analyzed with each batch of 25 samples. Control limits of the laboratory values must be within 100 ± 30% of the true values on average for all analytes, and not to exceed 35% of true value for more than 30% of individual analytes (e.g., PCB congeners, chlorinated pesticides, PAHs and butyltins). For inorganic analysis, laboratory values should be within 100 ± 20% of true value for each analyte.

For reagent blanks, no analyte should be detected >3 times the MDL. Recovery of the spiked samples should be within the range of 50% -120% for at least 80% analytes. Field duplicate analysis will be performed on 5% of the total number of samples. Relative percent difference (RPD = $(C_1 - C_2 / C_1 + C_2)/100$) must be <30% .

Surrogate standard spike (spiked prior to extraction of the sample) recovery (4,4'-dibromooctafluorobiphenyl) must be 100 ± 30%. For PAHs, deuterated PAHs (acenaphthene-d_{10}, phenanthrene-d_{10}, pyrene-d_{10}, perelene- d_{12} and benzo(g,h,i)perelyne- d_{12}) will be spiked. For butyltins, tetrabutyltin will be spiked as a surrogate standard.

Injection internal standard will be spiked in the final sample extract prior to injection into the gas chromatograph. For PCBs, PCB-103 (2,2',4,5,6-pentachlorobipheny), a PAH analyte which is not commonly found in tissue samples will be used, and hexyltin will be used as an injection internal standard for butyltins. Control limits on the recovery of injection internal standards will be 100 ± 20%.

Standards used for instrument calibration and generation of response factors must be authentic and purchased from NIST (National Institutes of Standards and Technology) and/or NIST certified standards from scientific companies. All sample injections will be performed using an autoinjector. Modern data systems will be used for data reduction, validation and reporting.

Precision, accuracy, reproducibility, and completeness of the data will be assessed using the QA/QC results. Any data that do not meet the specified requirement will be liable for corrective action.

Safety and Waste Handling: All applicable safety and waste handling rules will be followed including proper labeling and disposal of chemical wastes. All injuries and major spills will be reported as required. All used solvents are considered waste and will be disposed according to the Murray State University guidelines for waste disposal. All solvents which are not immediate used will be stored in a fire proof locker.

Statistical Analysis of FA Data: We will analyze FA across populations utilizing the methods of Palmer (1994). FA is first analyzed using frequency distributions and kurtosis tests to determine if the data exhibit FA, DA (directional asymmetry, when one side is consistently larger that the other across individuals) or other asymmetries. A two-way ANOVA is then use to

determine the effect of measurement error on the data. If measurement error is non-significant, simple linear regression models are run to determine if an FA measures are depended on body size. Based on the results of the distribution, kurtosis and Regression analysis, an appropriate indices of FA is chosen. Such indices are designed to control for departures from FA (i.e., DA) and/or size-dependence. The most simple index is (R-L): however (var R-L) is also commonly used.

Using such indices, FA levels will be analyzed for relationships with tissue contaminant concentrations, water chemistry, land use practices, and density/adult population size for each species and life state using MANOVA (multivariate analysis of variance) as well as correlational techniques.