INTEGRATIVE TECHNIQUES FOR STUDYING THE EFFECTS OF MULTIPLE STRESSORS ON AMPHIBIANS

By

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Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1994

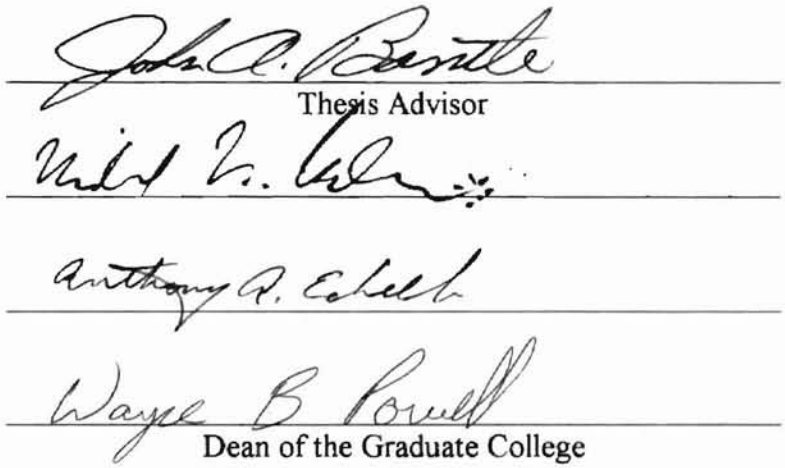
Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1998

INTEGRATIVE TECHNIQUES FOR STUDYING THE

EFFECTS OF MULTIPLE STRESSORS

ON AMPHIBIANS

Thesis Approved:



Thesis Advisor

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ACKNOWLEDGEMENTS

I would like to thank my major advisor, Dr. John Bantle for all his help and guidance. He has been a great guy to work for and I have learned much from him. Secondly, I would like to thank my "unofficial advisor" and friend Paul A. Shipman, from whom I have probably learned more than anyone while here. Dr. Stanley Fox gets my thanks for his advice and my admiration for his scientific abilities. I would also like to thank: Dr. Mike Palmer and Dr. Anthony Echelle for being on my committee and for their critical reviews of my work; Dr. Mahesh Rao for his assistance with figure construction, data processing and analysis; Dr. James Dumont, Thomas Jones, and Mendi Hull for laboratory work; Dr. Mark Payton for statistical advice; Scott Christenson and Jeffrey Lucius with the USGS; David Grimsley and Tim Hughes with the Oklahoma Climatology survey; Joy Yoshioka for her assistance in and out of the field; and all the undergraduate and graduate volunteers who participated in this project. We would also thank A. L. Chacey for allowing us to use his property as a reference site. Special thanks goes to my buddy Angie for her unwavering confidence and support, and my family for all their help and encouragement. Funding was provided by the National Science Foundation, Oklahoma EPSCoR (contract number EPS9550478).

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USE OF FETAX IN AN INTEGRATED APPROACH FOR STUDYING AMPHIBIAN DECLINES

Project Overview

This chapter discusses the use of FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) in environmental monitoring and proposes an integrated, multispecies approach as a way to better understand the problem of amphibian declines. This approach would utilize controlled laboratory studies such as FETAX, field studies, and well-designed multi-tiered mesocosm experiments to minimize the stochastic variables present in natural and impacted systems. The Norman landfill project is an attempt to use this type of approach to understand effects of various physical, biological, and environmental stressors on amphibians across multiple levels of biological organization.

The experimental procedures described in Chapter II can be thought of as the first tier of experiments in this approach. FETAX was used initially to assess the extent of contamination in both the groundwater and surface water at the site. We were able to correlate water toxicity to weather parameters to further identify potential risks to amphibians. Chapter II also mentions the use of native anuran species *in situ* to further validate results obtained from FETAX assays and supports the applicability of these results when extrapolating across species and from the laboratory to the field.

Introduction

There have been reported declines in amphibian populations worldwide (Blaustein and Wake, 1990; Phillips, 1990; Wake, 1991; Crump et al., 1992; Blaustein, et al., 1994a; Blaustein et al., 1994b; Blaustein et al., 1994c). There have been numerous explanations for these declines, including, but not limited to, natural population fluctuations caused in part by climatological change (Pechman and Wilbur, 1994; Pounds and Crump, 1994; Beebee, 1995), increased ultraviolet light due to anthropogenically caused ozone depletion (Blaustein et al., 1994c, Blaustein et al., 1995), acidification (Dunson et al., 1992), introduction of exotic species (Hammerson, 1982), environmental xenobiotics (Carey and Bryant, 1995; Fort et al., 1995), habitat loss (Wyman, 1991; Blaustein and Wake, 1990), and pathogens (Bradford, 1991; Carey, 1993; Blaustein et al., 1994b). Because these are global problems and a great number of different species are being affected, each with different life history strategies and niches, there are undoubtedly many causes for the declines. It is probable that all these factors and more play some role either independently or synergistically at various times and places.

Amphibians are an extremely important component of many natural ecosystems. They are an integral part of food webs (Burton and Likens, 1975a; Lynche, 1979), and can be a significant component of community biomass (Burton and Likens, 1975a,b; Debendictus, 1974). Amphibians are of benefit to humans as well by consuming insects which damage crops. They also have the potential to be of great importance to the pharmaceutical industry. Possibly the greatest benefit to humans could stem from their

potential use in environmental monitoring. Of course this is also of great consequence for the environment and the animals themselves as well.

Amphibians are particularly suited for toxicological assays because of a number of unique attributes. Their biphasic life cycle allows them to be exposed to pollutants both in the water and on the land. The eggs are unprotected and exposed to water, sediments, and sunlight. The larvae are generally herbivorous while the adults are mainly carnivorous. They have thin moist skin which is permeable to many toxins (Blaustein and Wake, 1995). The individuals of many species are sedentary and are thus good indicators of localized influences. They have a relatively short lifespan and can therefore be monitored over many generations (Birge and Just, 1975). Many species have a wide distribution and are abundant and easy to capture. They are bioaccumulators of many xenobiotics (Browne and Dumont, 1979; Canton and Slooff, 1982a,b; Cooke, 1970, 1974; Hall and Kolbe, 1980; Ireland, 1977; Licht, 1976; Licht et al., 1976; Pauvel and Price, 1986). The eggs and larvae are easily collected from the field for use in laboratory and *in situ* studies (Power et al., 1989).

However, because of the r-selected nature of most amphibians, it is difficult to extrapolate from eggs and larvae to population or community effects. The inability to extrapolate from one level of organization to the next and the difficulty of establishing cause and effect relationships are major problems facing ecotoxicologists (Munkittrick and McCarty, 1995). Such limitations suggest that a more integrative approach should be taken.

FETAX Reviewed

FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) is a 96-hour, whole-embryo assay for developmental toxicants. This assay uses embryos of the South African clawed frog, *Xenopus laevis*. Measurement endpoints include a 96-hour LC50 (embryo death), 96-hour EC50 (embryo malformation), and the minimum concentration to inhibit growth (MCIG) (Bantle, 1995).

When applied at levels approaching general cellular toxicity any material can act as a teratogen (Karnofsky, 1965). Many teratogens can exert effects at levels much lower than those required to produce cellular or systemic effects. Therefore, a system was needed to describe the true teratogenic hazard of materials being tested. The FETAX assay uses a *teratogenic index* which is the 96-hour LC50 divided by the 96-hour EC50 (Bantle, 1995). This allows an estimate of developmental hazards of materials in question. Many other measurement endpoints are possible including hatchability, behavior, swimming ability, and pigmentation. These endpoints, as well as the basic FETAX methodology, are detailed in the New Standard Guide (Bantle and Sabourin, 1995) and the Atlas of Abnormalities (Bantle et al., 1990a).

Dumont and co-workers (Dumont et al., 1979; Dumont et al., 1983) were the first to define the basic protocol and name the assay. After much validation work (Bantle and Courchesne, 1985; Sabourin et al., 1985; Dawson and Bantle, 1987a; Sabourin and Faulk, 1987; Bantle and Dawson, 1988; Dawson et al., 1988a; Bantle et al., 1989a, b; Dawson et al., 1989; Fort et al., 1989; Bantle et al., 1990b; DeYoung et al., 1991; Rayburn et al.,

1991a, b) the American Society for Testing and Materials formed an Aquatic Toxicology Task Force for the purpose of defining the best protocol for the FETAX assay. A New Standard Guide (Bantle and Sabourin, 1995) defined the standardized basic FETAX protocol. The Atlas of Abnormalities (Bantle et al., 1990a) can be used as an accessory aide to learning the assay, identifying malformations, and staging embryos. Before standardization of FETAX, many studies were conducted using various species of amphibians and various xenobiotics (Cabejszed and Wojcik, 1968; Cooke 1972; Bancroft and Praulad, 1973; Chang et al., 1974; Anderson and Praulad, 1976; Abbasi and Soni, 1984), but because of differing protocols and variables results between experiments were not comparable.

The use of *Xenopus laevis* for the standard assay versus species native to North America has many advantages. First, much is known about its normal development, biology, physiology, and biochemistry (Deuchar, 1972; Dewchar, 1975; Niewkoop and Faber, 1975). This aides our understanding of mechanistic studies and in the development of biomarkers (Bantle, 1995). Second, the animals can be readily induced to breed throughout the year by injecting human chorionic gonadotropin into the dorsal lymph sacs. Third, a single mating can produce enough eggs to run the typical assay with excellent statistical results. Fourth, the adults eat dead food, are totally aquatic, can be housed in aquaria, and are disease resistant. Fifth, the larvae are transparent allowing for easy observation of internal malformations (Bantle, 1995).

Developmental assays are often more sensitive indicators of certain contaminants. The ability of some xenobiotics to exert effects during specific lifestages necessitates the

need for any developmental assay to include all sensitive developmental stages. During FETAX `the embryo is exposed continuously over the four days to toxicant (with fresh changes of toxicant occurring every 24 hours to ensure that it does not degrade and lose its teratogenic effects). During the four days all primary organs develop except for limb formation (Bantle, 1995).

FETAX results can be compared to mammalian toxicity assays upon the addition of a *metabolic activation system* (MAS) which simulates the metabolism of the test material as though it were in a whole animal system. A 1:1 combination of Aroclor 1254 and isoniazid induced rat liver microsomes plus generator system is used for the MAS (Bantle, 1995). Standard FETAX solution contains a fairly high concentration of Mg2+. This may confound assays for metal toxicity because magnesium can reduce the toxicity of other metals by competition for active binding sites (Miller and Landesman, 1978). The addition of buffers into FETAX solution led to unacceptable rates of mortality and malformation. Therefore, none were included in the standard solution. FETAX solution thus has a low buffering capacity.

The FETAX assay is particularly well suited for complex mixtures such as industrial effluents (Dawson and Bantle, 1987b; Dawson et al., 1991a, b). It has successfully been used to analyze surface water (Dawson et al., 1984), groundwater (Bantle et al., 1989b), and sediment extracts (Dawson et al., 1988b). It has also been modified so that it is applicable for use as an *in-situ* biomonitoring technique using plastic mesh mesocosms (Linder et al., 1990). The assay is reliable, extremely sensitive to many xenobiotics, rapid, and cost effective. It is also flexible enough to allow modifications.

There are differences between species and between lifestages in degree of sensitivity to environmental pollutants. Because FETAX is a standardized assay, very similar protocols can be applied to native species along with *Xenopus* in tests of a material. This allows cross-comparisons between species that would not be possible without the standardization. If mechanism of toxicity is known, which may be made easier due to the large amount already known about *Xenopus*, it may be possible to define causal relationships between toxin and measurement end point (mortality, malformation, growth, behavior, locomotion, feeding ability). The importance of establishing cause and effect relationships will be discussed later.

Discussion

Temporal variation in ecosystem characteristics can periodically produce periods of intense selective pressure which can lead to population declines (Corn et al., 1989). There is a need for long term field studies (Peterson et al., 1992; Pechmann et al., 1994) to better elucidate causes of these population fluctuations. Such fluctuations are difficult to monitor in amphibians because of the large number of influencing factors and their potential interactions. Long term field studies should include a variety of sampling techniques that adequately sample egg mortality, larval survivorship, and recruitment. These life-history variables are crucial to our ability to understand factors affecting

fluctuations in adult populations (Rowe and Dunson, 1994).

Ecotoxicologists have a number of different tools at their disposal for use in biomonitoring. For years traditional toxicologists have tried to measure dose-responses of different species to a variety of compounds. The development and use of biomarkers as a means of determining exposure also has been of great importance. Biomarkers work well in determining causal relationships between abiotic assaults on individuals and their responses (Munkittrick and McCarty, 1995). However, the assault on many ecosystems today from so many different sources, along with the inherent complexity of these systems, has made extrapolation from one organizational level to the next higher level practically impossible. Ecotoxicologists with an ecology background have tried to measure effects at higher levels of organization and relate these effects to possible stressors at lower levels of organization (Munkittrick and McCarty, 1995). This has been difficult because of the inability to control for the diversity of independent variables in this type of study. There are simply too many biotic and abiotic factors that can elicit similar responses. While standardized laboratory assays are much better at controlling such factors, they suffer from oversimplification. An intermediate strategy is the use of *in situ* enclosures, mesocosms, and microcosms. They can be designed so as to control for different variables in each situation. This allows a continuum of design to obtain results that no other system alone can achieve.

The preferred approach is a long term, multispecies, multitiered system using both laboratory and field studies. In such an approach, the field studies would incorporate mesocosms in an experimental design that sequentially increases the number of

independent variables. This sequential design would allow assessment of interactions and consequences of added variables. It is unrealistic to think that all the complexities of a natural system could ever be actually modeled using this approach. However, it does bridge many of the gaps that exist in current experimental designs. The measurement end-points at each organizational level should be designed to allow direct causal analysis of stessors at each level or at lower levels.

The complexity of amphibian life cycles presents a major problem in trying to discover the reasons for their declines. The plan outlined above could provide researchers with a new strategy for solving many of these problems and a better approach to establishing reasons for, and solutions to these declines.

Conclusions

Amphibians are well suited for environmental monitoring. There is already a need for monitoring populations of these animals due to their reported declines. A great deal of work already has been done using *Xenopus laevis* as the test organism. Because of this, much is already known about its biology, physiology, and development. FETAX, a standardized developmental assay utilizes the embryos of this animal. The assay has been shown to be sensitive, reliable, and flexible enough to be used for biomonitoring. Use of this assay, combined with field and mesocosm studies may aid in extrapolating effects and

responses between levels of organization. This may aid researchers examining the reasons for the reported declines.

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Chapter II

GROUND AND SURFACE WATER DEVELOPMENTAL TOXICITY AT A MUNICIPAL LANDFILL: DESCRIPTION AND WEATHER RELATED VARIATION

Introduction

There is a growing concern over contamination of groundwater aquifers. One of the greater problems facing scientists and regulatory agencies is that groundwater reservoirs are by their very nature much more difficult to monitor and remediate than surface sites (United States EPA, 1984). Landfills have been identified as one of the major threats to groundwater resources (United States EPA, 1984). It is often not until wells or surface waters become significantly contaminated that a problem is identified. With approximately one-half of United States residents relying on groundwater as their potable water supply (United States EPA, 1984), the need to preserve the integrity of this resource is obvious. Groundwater reservoirs can contaminate surface waters and directly affect amphibians and other wildlife using these surface waters. Contamination with xenobiotics has been shown to adversely impact amphibian populations (Fashigbauer, 1957; Hazelwood, 1969; Paulov, 1977; Cooke, 1983) and has been cited as one of many possible causes for the reported worldwide decline in amphibian populations (Wake and Morowitz, 1990; Carey and Bryant, 1995).

Landfills have been the principal disposal method for both industrial and domestic

waste. There is a growing list of landfill sites that are known to be leaching contaminants into underlying aquifers (Reinhard *et al.*, 1984). Before the widespread use of pollution abatement measures such as waterproof liners, improper waste disposal methods were frequently used with little regard for the potential adverse impacts these practices might have on the environment. Also, too little is known about the health effects of exposure to contaminants, particularly complex mixtures such as those often found in landfill leachate. Toxicological assays such as FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) (Dumont *et al.*, 1983), combined with hydrological studies, can be used to help determine the potential impacts of these contaminants to both humans and other organisms. FETAX is a 96-hr whole embryo assay for developmental toxicants that uses the embryos of the South African clawed frog, *Xenopus laevis*, and is thus particularly useful in studies dealing with impacts on amphibians. Additionally, the ability of *Xenopus* to breed year-round allows studies dealing with long-term temporal changes in toxicity.

FETAX has successfully been used to test toxicity of complex mixtures such as industrial effluents (Dumont and Schultz, 1980; Dumont, *et al.*, 1983; Dawson and Bantle, 1987; Dawson, *et al.*, 1991a; Dawson, *et al.*, 1991b), surface water (Dawson, *et al.*, 1984), groundwater (Bantle, *et al.*, 1989), and sediment extracts (Dawson, *et al.*, 1988). This assay can also be modified such that it is applicable for use as an *in situ* biomonitoring technique using plastic enclosures (Linder, *et al.*, 1990). Because FETAX is a standardized assay, similar protocols using native species can be applied to test the same material. This is important due to potential differences between species in sensitivity to pollutants (Hall and Swineford, 1980, 1981).

Surface water contamination may play a significant role as a population stressor because amphibians are dependent on water for reproduction. Rainfall events may alternately dilute toxicity or increase it if rate of transport increases the flow of contaminants to the surface. Therefore, the purpose of this study was to evaluate the developmental toxicity of ground and surface waters near the closed municipal landfill at Norman, Oklahoma. Movement of toxic materials from groundwater to surface water where they could impact amphibians was of particular concern. Additionally, we examined the relationship of temporal changes in surface water toxicity at the landfill to changes in weather parameters.

Materials and Methods

Study Site Description

The landfill is located south of the city of Norman in central Oklahoma on alluvium deposited by the Canadian River (Fig. 1). The landfill was operational from 1922 - 1985 with no restrictions on the type of material deposited. It received waste prior to the use of pollution abatement measures such as waterproof liners. In 1985 the landfill was closed, capped with clay, and vegetated (Callender *et al.*, 1993). In 1994 the landfill site was selected to be the focus of the Toxic Substances Hydrology Program of the United States Geological Survey (USGS), Water Resources Division (Lucius and Bisdorf, 1995). Other

researchers have identified more than 40 semi-volatile and non-volatile compounds (Dunlop *et al.*, 1976) as well as 35 volatile compounds (Scott Christenson, USGS, personal communication) (Table 1) in the groundwater downgradient from the landfill. Many of these chemicals are known xenobiotics and carcinogens. This groundwater was also found to have low levels of dissolved oxygen and elevated concentrations of hydrogen sulfide and methane (Gibson and Suflita, 1986; Beeman and Suflita, 1987; Beeman and Suflita, 1990). Additionally, we have performed a Toxicity Identification Evaluation (TIE) on groundwater samples. Preliminary results indicated elevated toxicity may result, in part, from high concentrations of metals. A small stream, and associated riparian habitat is adjacent to the landfill. The stream flows into the Canadian River. Treated sewage effluent from the city of Norman is discharged directly into this stream.

We also analyzed surface water samples from a reference location approximately 8 kilometers northwest of the landfill. This site is located in riparian habitat similar to that of the Norman landfill and is also on alluvium deposited by the Canadian River. The reference site also has a small stream that flows through the site and into the Canadian River (Fig. 2).

Sample Collection and Analysis

On 16-17 November, 1995, and 21 January, 1996, groundwater samples were taken from a network of USGS wells adjacent to, and downgradient from, the landfill (Fig. 3). The groundwater is in a shallow, unconfined, alluvial aquifer that extends under the landfill (Callender *et al.*, 1993). Syringes (60 ml) with latex surgical tubing attached

were used to draw water from the wells. All water samples were taken from the top 0.5 m of the water column. The water from each well was placed into 250-ml amber bottles with Teflon-lined lids. These bottles were completely filled with water leaving no head space. The syringes were thoroughly rinsed with distilled water between wells and replaced after every 6-8 wells. The latex tubing was replaced after each well. Surface water samples were also taken from various locations along the small stream adjacent to the landfill and from the stream at the reference site. All water samples were cooled to 4° C the day they were taken and stored at this temperature until testing.

Upon obtaining results for this portion of the study, we then established seven permanent surface water sampling locations (NL1-NL7) at the landfill along with one groundwater sampling location (Well 0) (Figure 1). Four permanent surface water sampling locations (AL1-AL4) were established at the reference site (Fig. 2). From June 1996 through May 1997 samples were taken from these locations. Our goal was to correlate surface water toxicity at each of the seven landfill surface water sampling locations to changing weather parameters. Both embryo mortality and malformation data were examined, but only mortality data are presented here because malformations did not significantly correlate with any weather parameter.

Weather data were gathered every five minutes by an automated Mesonet weather station installed at the landfill site (Crawford *et al.*, 1992). Weather parameters considered in this study included air temperature, relative humidity, solar radiation, net solar radiation, and rainfall. Correlation analyses (Pearson *r*) were used to examine the relationship between variation in toxicity and each of these weather parameters. Since

conditions immediately preceding the sampling would presumably have more impact than the earlier conditions at the site, we grouped our weather data into two time intervals, days 1-3 preceding sampling and days 4-7 preceding sampling.

All water samples were analyzed at 100% concentration for developmental toxicity using the standardized FETAX assay (Bantle *et al.*, 1990; Bantle, 1995; Bantle and Sabourin, 1991). Experiments where controls exceeded the 10% acceptable limits for mortality or malformation were repeated if possible. In no instance was data used when controls showed greater than 16% mortality or greater than 14% malformation. Values for pH were always between 6.0 and 9.0. Cosolvents to dissolve hydrophobic contaminants were not used in this study. Water quality parameters including temperature, pH, conductivity, salinity, turbidity, and dissolved oxygen were collected using *YSI 6000* (YSI Incorporated, Yellow Springs, OH) multiparameter water quality sondes installed in the streams at both locations.

Results

The data from the *YSI* water quality meters are summarized to show that ambient water quality could sustain normal growth and development of amphibians in the absence of xenobiotics (Table 2). Values for pH ranged from 6.45 to 8.79 at the landfill and 6.35 to 9.23 at the reference site. Conductivity at the reference site ranged from 0.48 to 1.91

mS/cm. The conductivity range at the landfill was between 0.00 to 1.87 mS/cm. Salinity values ranged from 0.23 to 0.96 parts per thousand (ppt) at the reference site and 0.00 to 1.31 ppt at the landfill. Dumont (unpublished) showed that *Xenopus* embryos developed and grew normally in artificial seawater (*Instant Ocean*®) at concentrations up to 1% (10 ppt). Ammonia content for the landfill water was between 0.20 and 3.40 mg/L. Turbidity fluctuated from 0.00 to 867.0 and 0.90 to 785.9 NTU at the landfill and reference sites, respectively. These data suggest that the standard water quality variables (exclusive of toxicants) were within acceptable ranges for FETAX and probably did not affect native populations of amphibians nor the growth and development of test embryos.

Results of the FETAX developmental toxicity tests on the ground and surface water samples taken from the landfill site are shown in Tables 3 and 4. The spatial distribution of the samples taken November 16-17 in relation to the landfill site and the FETAX results are depicted in Fig. 3. Groundwater samples were highly toxic in the area between the landfill and the stream, indicating a plume of toxicants from the landfill. The toxicity diminished with increasing distance from the landfill. Nearly one half of the groundwater samples taken during these time periods caused 100% mortality. Many surviving embryos of the remainder of the samples were moderately to severely malformed (Table 3). Five of the groundwater samples caused 100% of the surviving embryos to be malformed. Ten of the samples caused malformations in 50 to 100% of the survivors and the remaining samples caused malformations in greater than 12% of the survivors. Finally, all samples except two caused a significant reduction in growth of exposed embryos.

Surface water samples from the landfill site often showed higher than normal

toxicity (Table 4) with mortality values ranging from 0 to 100%. More than one-fourth of the samples tested produced greater than 20% mortality. Toxicity was particularly high at location NL 4, which is a seep where groundwater surfaces (Fig. 1). Samples from this location always caused 100% mortality. Malformation rates ranged from 0 to 95% at the other sampling locations. Nearly one fourth of these samples had a malformation rate greater than 20% and most samples caused a significant reduction in growth of embryos (Table 4). Control 96-hr *Xenopus* embryos had well formed muscular and nervous systems and organs including eyes, gut, and heart (Fig. 4a). Examples of malformations caused by samples from the landfill site are illustrated in Figs. 4b and 4c. These embryos were exposed for 96 hrs to a 20% and 30% concentration of water from location NL 4. The malformed embryos were shorter than the controls and, although the eyes appeared normally developed, the gut was improperly coiled. This was more pronounced in the embryo exposed to the 30% concentration of water (Fig. 4c). This embryo also showed a reduced development of the head and face. The most distinctive malformation observed in these embryos was the dorsal curvature of the tail, which suggests abnormal development of the notochord. All of these responses increase in severity with increasing concentrations of contaminated water. The dorsal curvature of the tails has been observed in at least one other instance, that of exposure to aqueous extracts of crude shale oil (Dumont, unpublished; Bantle *et al.*, 1990).

Surface water samples taken from the reference site showed higher than normal toxicity in several cases with mortality values ranging from 0 to 100%. However, the 100% mortality observed with sample AL 4 taken 13 May, 1997 was possibly due to

factors other than toxicity because no previous samples from that location were toxic. Occasionally, bacteria or fungi can cause high mortality in samples. Other than this sample, the greatest observed mortality was 37%. Only 15% of all samples from the reference site had mortality greater than 20%. Malformation at the reference site ranged from 0 to 41%, with only one sample showing malformations greater than 20%. Less than one-half of the samples caused a significant reduction in growth of embryos (Table 4).

Surface water samples collected from June 1996 through May 1997 indicated a temporal variation in surface water developmental toxicity at the landfill site. Correlation analyses on this series of water samples indicated relationships between toxicity and weather parameters (Table 5). The most obvious observation was that location NL 4 displayed a complete lack of correlation with any weather parameter. Samples from this location always caused 100% mortality of exposed embryos regardless of weather conditions. For this reason, results for this location are not shown in Table 5. Another notable observation was that location NL 6 did not display the same general trends in relation to the weather variables as the other locations. Water samples from NL 6 were taken directly from the Norman sewage effluent discharge and were thus representative of a completely different source for contaminants.

Several trends were observed across the remaining sample locations. There was a general trend for mortality to be negatively correlated with cumulative rainfall for days 1-3 preceding sampling, and one location, NL 3, showed a statistically significant negative correlation. There was also a trend for mortality to be negatively correlated with average relative humidity for days 1-3 preceding sampling, with two locations, NL 5 and NL 7,

showing significant negative correlations. Mortality in general was positively correlated with average solar radiation for days 1-3 preceding sampling with two locations, NL 1 and NL 3, showing significant positive correlations. Mortality also tended to be positively correlated with net solar radiation for days 1-3 preceding sampling with one location, NL 3, showing a significant positive correlation. There was no observable trend across sampling locations for mortality to be correlated with average air temperature. However, mortality of samples from NL 6 was negatively correlated with average air temperature of days 1-3 preceding sampling. No significant correlations were observed between mortality and weather parameters for days 4-7 preceding sampling.

We next averaged mortality from all the sampling locations and used the averages in another set of correlation analyses to examine the relationships between toxicity and weather parameters for the landfill site as a whole (Table 5). For reasons previously mentioned, locations NL 4 and NL 6 were not included in this analysis. Mean mortality was negatively correlated with both cumulative rainfall and average relative humidity for days 1-3 preceding sampling. Mean mortality was positively correlated with both average solar radiation and net solar radiation for days 1-3 preceding sampling. Mean mortality was not correlated with average air temperature nor with any weather parameter during the 4-7 days preceding sampling.

Discussion

FETAX results indicated an area of groundwater downgradient from the landfill was contaminated. Toxicity diminished with increasing distance from the landfill. Therefore, we concluded that the contamination was due to leachate from the landfill. This conclusion is further supported by an electromagnetic survey conducted by the USGS to determine the extent of the leachate plume. The survey assessed the apparent conductivity of the alluvium, which is determined by several factors including the porosity, pore space saturation, and the conductivity of the water or leachate in these pore spaces. The survey indicated an area of higher conductivity downgradient of the landfill which was interpreted as being caused by leachate flowing from the landfill and mixing with the water in the alluvium (Lucius and Bisdorf, 1995; Figure 5). Additionally, the surface water at the landfill site is almost certainly receiving contaminants from the leachate plume due to the shallow nature of the aquifer.

We have shown that weather parameters influence toxicity of surface waters at the landfill site. The significant negative correlation between rainfall and mean mortality probably reflects dilution. The negative correlation between relative humidity and mean mortality may be a secondary effect due to the strong correlation between rainfall and relative humidity. Another possibility, however, is that low relative humidity increases evaporation thereby concentrating toxicants. The positive correlation between average mortality and both average solar radiation and net solar radiation could be explained in several ways. One possibility is that solar radiation is low during rain events when

toxicants are being diluted, whereas during periods of high solar radiation evaporation increases, concentrating toxicants. Another possibility is that photochemical reactions caused by solar radiation are increasing toxicity by converting less toxic compounds to more toxic derivatives. We are currently conducting experiments designed to determine if ultraviolet radiation modifies the toxicity of water.

The weather conditions during the three days immediately preceding sampling had a greater effect on toxicity of surface waters than did earlier weather conditions. For days 4-7 preceding the sample date, mean mortality was not correlated with weather parameters, whereas, for days 1-3 all weather parameters except average air temperature were significantly correlated with mortality.

The surface water sampling locations at the landfill site differ in hydrological conditions, which affect the toxicity at these locations (see Fig. 1). Location NL 4 is a seep where the groundwater surfaces and merges with the stream and with water from the sewage discharge. This sampling location appears to be the only surface location where emerging groundwater was not significantly diluted by surface water. Because samples from NL 4 always induced 100% mortality, FETAX results from this location were not included in the correlation analyses for the landfill site as a whole. Future experiments using dilutions of samples from NL 4 will determine the LC-50. The LC-50 data will then be correlated with weather parameters to determine if the toxicity was affected.

Samples from NL 6, which were taken directly from the sewage discharge, were not contaminated by the groundwater. Therefore, mortality results from this location were not included in the correlation analyses for the landfill site as a whole. We suspect that the

negative correlation between mortality and average air temperature at this location is related to the efficiency of the sewage treatment plant. The plant may be more efficient at removing toxicants during warmer weather.

The remaining surface water sampling locations could be receiving contaminants from the shallow groundwater near the landfill (S. Christenson, USGS, personal communication). As the plume of toxicants moving from the landfill contacts the stream, changes in surface water toxicity would be expected. However, the contamination appears to be diluted by surface waters.

FETAX indicated the surface water samples taken from the reference site were generally less toxic than the landfill site. However, there were occasions when the reference samples showed elevated toxicity. It should be noted that land use upstream of the reference site is agricultural and agrichemicals may be affecting toxicity.

The evaluation of water toxicity described in this paper is part of a larger study of the effects of contamination on amphibians at the landfill. One *in situ* study is using mesh enclosures containing embryos of native anuran species to corroborate the results of the FETAX evaluation. Preliminary results suggest that some locations cannot support growth and development of amphibians. Long-term amphibian biomonitoring studies are in progress, which will provide data on population fluctuations and activity patterns associated with climate changes. Preliminary analysis indicates that climatic variables impacting amphibian activity and breeding success can be identified. Amphibians had very low breeding success in the spring of 1996. We were unable to locate any eggs or larvae. We attributed this to the extremely dry conditions during this period. During the spring of

1997, weather conditions were better and amphibians bred in temporary pools in the sandy areas between the streams and the river at both locations (Figs. 1 and 2). Eggs and larvae of *Bufo woodhousii, Rana blairi, and Rana catesbeiana* were found in these pools in large numbers. Larvae of *R. blairi* and *R. catesbeiana* were also observed in the streams at both sites. The temporary pools were not present during the spring of 1996. Other researchers have also observed correlations between recruitment failures and drought (Pechman et al., 1991). Such non-equilibrium population dynamics typify amphibians and makes population declines difficult to distinguish from natural population fluctuations (Berven, 1990; Pechman et al., 1991; Blaustein, 1994; Pechman and Wilbur,1994). Subsequent long-term studies are needed to elucidate the effects of the numerous factors affecting amphibian populations.

Conclusions

Results of this study indicated a plume of toxicants exuding from the landfill and mixing downgradient with the groundwater. The groundwater between the landfill and the Canadian River is contaminated, with toxicity diminishing as distance from the landfill increases. The small stream which runs adjacent to the landfill also showed high toxicity, probably as a result of interaction between the stream and the shallow alluvial aquifer. Water samples from the landfill induced mortality, malformations, and growth inhibition in

test embryos. Samples from the reference site were generally less toxic.

Surface water developmental toxicity varied through time. Some toxicity at the landfill site was significantly correlated with measurable weather parameters. The variable toxicity of the surface-water indicated the need to sample numerous times during the year and suggested that the hazard to amphibians may be dependent on surface water toxicity during the breeding season when eggs and larvae would be exposed.

TABLE 1
Compounds Identified in Water Samples Collected in September, 1993, by the USGS, Using Gas Chromatography / Mass Spectrometry (EPA Method 8240)

benzene	1-methyl-4-propylbenzene
toluene	1,3-dimethyl-5-ethylbenzene
ethylbenzene	1,2-diethylbenzene
m,p-xylene	1-methyl-2-propylbenzene
isopropylbenzene	1,4-dimethyl-2-ethylbenzene
n-propylbenzene	1,3-dimethyl-4-ethylbenzene
1-ethyl-3-methylbenzene	1,2-dimethyl-4-ethylbenzene
1-ethyl-4-methylbenzene	1,3-dimethyl-2-ethylbenzene
1,3,5-trimethylbenzene	1,2-dimethyl-3-ethylbenzene
1-ethyl-2-methylbenzene	1,2,4,5-tetramethylbenzene
1,2,4-trimethylbenzene	1,2,3,5-tetramethylbenzene
2-methylpropylbenzene	1,2,3,4-tetramethylbenzene
1,2,3-trimethylbenzene	chloroform
1-methyl-3-isopropylbenzene	1,1,1-trichloroethane
1-methyl-4-isopropylbenzene	vinyl chloride
1,3-diethylbenzene	trichloroethane
1-methyl3-propylbenzene	1,4-dichlorobenzene
o-xylene	

TABLE 2

Monthly Statistics of Water Quality Data for the Landfill (NL) and Reference (AL) Sites.

			pH	Conductivity (mS/cm)	Salinity (ppt)	Water Temperature (°C)	Ammonia N (mg/L)	Turbidty (NTU)
Jun '96	AL	Min	7.14	0.66	0.32	21.14	†	1.10
		Max	8.66	1.91	0.96	32.72	†	89.60
		Mean	7.72	1.33	0.66	27.38	†	6.30
	NL	Min	6.78	0.00	0.00	17.49	0.40	0.00
		Max	8.79	1.87	0.94	39.44	1.50	867.00
		Mean	7.78	0.64	0.31	27.19	0.99	60.71
Aug '96	AL	Min	6.35	0.63	0.30	23.71	†	2.50
		Max	8.43	0.97	0.47	31.48	†	146.30
		Mean	7.30	0.86	0.42	27.26	†	13.17
	NL	Min	6.45	0.04	0.02	19.95	BD	0.00
		Max	8.15	2.53	1.31	28.65	BD	820.00
		Mean	6.89	1.53	0.77	22.54	BD	39.45
Mar '97	AL	Min	7.72	0.75	0.37	8.75	*	5.50
		Max	9.23	1.43	0.72	20.41	*	33.20
		Mean	7.97	1.13	0.56	13.72	*	9.96
	NL	Min	7.47	0.89	0.44	7.72	0.30	0.00
		Max	8.10	0.97	0.48	10.84	1.20	183.20
		Mean	7.79	0.95	0.47	9.15	0.52	7.32
Apr '97	AL	Min	7.41	0.66	0.32	12.97	†	0.90
		Max	8.86	1.35	0.67	21.98	†	785.90
		Mean	7.85	1.04	0.51	16.81	†	178.51
	NL	Min	7.02	0.31	0.15	7.61	0.30	5.00
		Max	7.94	1.01	0.50	21.48	3.40	34.00
		Mean	7.39	0.78	0.38	14.91	1.14	12.29
May '9	AL	Min	7.52	0.48	0.23	16.07	†	3.10
		Max	8.36	1.41	0.70	28.51	†	362.30
		Mean	7.87	1.05	0.52	22.02	†	21.95
	NL	Min	6.90	0.80	0.39	16.24	0.20	0.00
		Max	7.27	1.21	0.60	23.28	1.60	99.40
		Mean	7.10	1.11	0.55	20.42	1.28	3.10

† Ammonia not measured at reference site.

* Electrode failed during this time period.

TABLE 3

Results of FETAX Toxicity Tests on Groundwater Samples Taken from the Landfill Site.

SITE*	SAMPLE DATE	MORTALITY (%)	MALFORMATION (%)	GROWTH INHIBITION (% OF CONTROL)
W 0	18-Aug-96	100	†	†
	31-Jan-97	100	†	†
	29-Mar-97	100	†	†
	28-Apr-97	100	†	†
W 28	16 -17 Nov 95	18	12.4	98.9
W 31	16 -17 Nov 95	4	75	83.1**
W 32	16 -17 Nov 95	28	100	72.9**
W 35	16 -17 Nov 95	100	†	†
	21-Jan-96	100	†	†
W 36	16 -17 Nov 95	100	†	†
W 37	16 -17 Nov 95	10	17.8	93.6**
W 38	16 -17 Nov 95	100	†	†
W 39	16 -17 Nov 95	100	†	†
	21-Jan-96	100	†	†
W 40	16 -17 Nov 95	100	†	†
	21-Jan-96	100	†	†
W 41	16 -17 Nov 95	16	35.4	91.6**
	21-Jan-96	4	52.6	99.3
W 42	16 -17 Nov 95	40	100	69.4**
	21-Jan-96	56	100	83.3**
W 43	16 -17 Nov 95	100	†	†
	21-Jan-96	100	†	†
W 44	16 -17 Nov 95	100	†	†
	21-Jan-96	100	†	†
W 45	16 -17 Nov 95	100	†	†
W 46	16 -17 Nov 95	14	68	85.0**
	21-Jan-96	0	48	91.2**
W 47	16 -17 Nov 95	100	†	†
	21-Jan-96	100	†	†
W 48	16 -17 Nov 95	16	73.4	92.2**
	21-Jan-96	24	92.3	83.6**
W 49	16 -17 Nov 95	38	100	83.5**
	21-Jan-96	30	23.8	94.3**
W 50	16 -17 Nov 95	12	56.8	93.0**
	21-Jan-96	4	25	93.1**
W 51	16 -17 Nov 95	100	†	†
	21-Jan-96	100	†	†
W 54	16 -17 Nov 95	2	67.3	90.2**
	21-Jan-96	22	100	79.8**
W 55M	16 -17 Nov 95	10	67	93.8**
	21-Jan-96	10	40.4	90.3**
W 57	16 -17 Nov 95	4	59	86.3**
W 58	16 -17 Nov 95	24	68.9	90.3**

* For samples taken from November 16-17 1995 the location and results are shown in Figure 3.

** Significantly different from controls ($P<0.05$) using the t-test for grouped observations.

† No malformation/growth inhibition data due to 100% mortality.

TABLE 4

Results of FETAX Toxicity Tests on Surface Water Samples Taken from the Landfill (NL) and Reference Sites (AL).

MORTALITY (%)														
Date	NL1	NL 1A	NL2	NL3	NL4	NL5	NL6	NL7	NL8	NL9	AL1	AL2	AL3	AL4
16 -17 Nov 95	10	6	18	NT	100	0	NT	44	2	4	20	2	16	NT
21-Jan-96	4	22	18	NT	100	12	NT	NT	18	NT	NT	NT	NT	NT
6/16/1996[a]	20	NT	15	NT	100	NT	8	12	NT	NT	NT	4	10	6
8/18/1996[a]	4	NT	NT	13	100	NT	4	6	NT	NT	5	10	17	NT
1/31/1997[a]	21	NT	20	15	100	25	25	39	NT	NT	22	37	11	6
27-Feb-97	6	NT	6	16	NT	6	8	12	NT	NT	12	8	18	0
29-Mar-97	8	NT	NT	32	100	24	12	34	NT	NT	12	16	10	12
12-Apr-97	12	NT	2	6	NT	6	6	8	NT	NT	8	4	6	8
27-Apr-97	2	NT	6	6	NT	2	14	8	NT	NT	4	26	6	4
13-May-97	32	NT	7	36	100	6	4	8	NT	NT	6	8	8	100
MALFORMATION (%)														
Date	NL1	NL 1A	NL2	NL3	NL4	NL5	NL6	NL7	NL8	NL9	AL1	AL2	AL3	AL4
16 -17 Nov 95	91.70	68.20	19.40	NT	†	26.00	NT	38.80	26.30	12.50	41.00	16.20	4.50	NT
21-Jan-96	41.90	95.70	77.40	NT	†	31.80	NT	NT	22.20	NT	NT	NT	NT	NT
6/16/1996[a]	14.80	NT	11.30	NT	†	NT	17.95	33.15	NT	NT	NT	16.10	18.65	18.25
8/18/1996[a]	3.00	NT	NT	7.05	†	NT	5.00	8.10	NT	NT	4.03	4.93	4.37	NT
1/31/1997[a]	7.73	NT	4.68	33.64	†	11.18	4.47	1.04	NT	NT	1.19	2.94	5.58	2.79
27-Feb-97	2.13	NT	0.00	0.00	NT	4.26	2.17	0.00	NT	NT	0.00	0.00	0.00	4.00
29-Mar-97	6.52	NT	NT	8.82	†	2.63	2.27	15.15	NT	NT	9.09	0.00	2.22	9.30
12-Apr-97	11.36	NT	6.25	4.27	NT	6.55	8.27	6.52	NT	NT	2.17	4.00	4.27	15.09
27-Apr-97	8.60	NT	4.26	4.26	NT	8.16	11.63	4.35	NT	NT	2.08	10.81	10.64	2.08
13-May-97	8.83	NT	2.00	21.88	†	8.00	2.00	10.00	NT	NT	4.26	12.42	17.23	†
GROWTH INHIBITION (% OF CONTROL)														
Date	NL1	NL 1A	NL2	NL3	NL4	NL5	NL6	NL7	NL8	NL9	AL1	AL2	AL3	AL4
16 -17 Nov 95	85.9*	87.7*	90.8*	NT	†	92.0*	NT	92.6*	95.8*	96.9*	91.6*	97.3	99.1	NT
21-Jan-96	92.9*	91.9*	90.7*	NT	†	95.9*	NT	NT	96.1*	NT	NT	NT	NT	NT
6/16/1996[a]	102.4*	NT	102.7[b]	NT	†	NT	98.2	95.6[b]	NT	NT	NT	100.4[c]	101.9[c]	98.3[c]
8/18/1996[a]	99.9	NT	NT	97.9	†	NT	100.0	96.3[b]	NT	NT	98.4	96.9[b]	99.9	NT
1/31/1997[a]	95.9*	NT	98.1[b]	89.0	†	99.3	95.9[b]	93.3	NT	NT	98.5[b]	94.9	95.3	98.4
27-Feb-97	98.2	NT	98.0	100.9	NT	99.1	91.8*	103.4	NT	NT	92.3*	94.7*	93.1*	94.0*
29-Mar-97	89.1*	NT	NT	95.6*	†	100.9	96.7	97.9	NT	NT	96.4	98.3	99.9	96.2
12-Apr-97	98.0	NT	102.0	105.1*	NT	103.2	100.6	95.8	NT	NT	101.6	102.4	104.3	97.6
27-Apr-97	93.9*	NT	95.5*	92.7*	79.6*	94.3*	83.8*	86.6*	NT	NT	93.9*	90.7*	96.1	93.5*
13-May-97	89.1*	NT	96.3*	92.8*	†	95.6*	98.8	89.5*	NT	NT	94.0*	97.1	94.6*	†

* Significantly different from controls ($P<0.05$) using the *t*-test for grouped observations.

[a] Results shown for these dates are averages of two tests.

[b] Only one test was significantly different from control ($P<0.05$).

[c] Result of one test because of error in the other test.

NT, Not Tested.

† No malformation/growth inhibition data due to 100% mortality.

TABLE 5

Correlation Coefficients (Pearson *r*) of Mortality vs. Environmental Variables for Norman Landfill Surface Water Samples Taken from June 1996 to May 1997.

Environmental Variable	Period preceeding sampling	NL1	NL2	NL3	NL5	NL6	NL7	Mean mortality[†]
Cumulative Rain	Days 1-3	-0.61	-0.69	-0.81*	-0.67	-0.07	-0.47	-0.77*
	Days 4-7	-0.40	-0.54	0.08	-0.28	-0.29	-0.12	-0.18
Average Relative Humidity	Days 1-3	-0.22	-0.45	-0.68	-0.91*	-0.52	-0.87*	-0.91*
	Days 4-7	0.36	0.12	0.01	-0.54	-0.44	-0.58	-0.42
Average Solar Radiation	Days 1-3	0.70*	0.38	0.87*	0.48	-0.14	0.28	0.73*
	Days 4-7	-0.01	-0.23	0.02	-0.42	-0.50	-0.51	-0.30
Average Net Radiation	Days 1-3	0.70	0.31	0.89*	0.49	-0.19	0.32	0.77*
	Days 4-7	-0.28	-0.71	0.05	-0.41	-0.36	-0.37	-0.34
Average Air Temperature	Days 1-3	0.26	-0.08	0.55	-0.35	-0.75*	-0.54	0.22
	Days 4-7	0.23	-0.21	0.39	-0.49	-0.69	-0.54	0.32

* Significantly different ($P < 0.05$) using the *t*-test

[†] Correlation coefficients calculated based on average mortality of NL1, NL2, NL3, NL5, and NL7

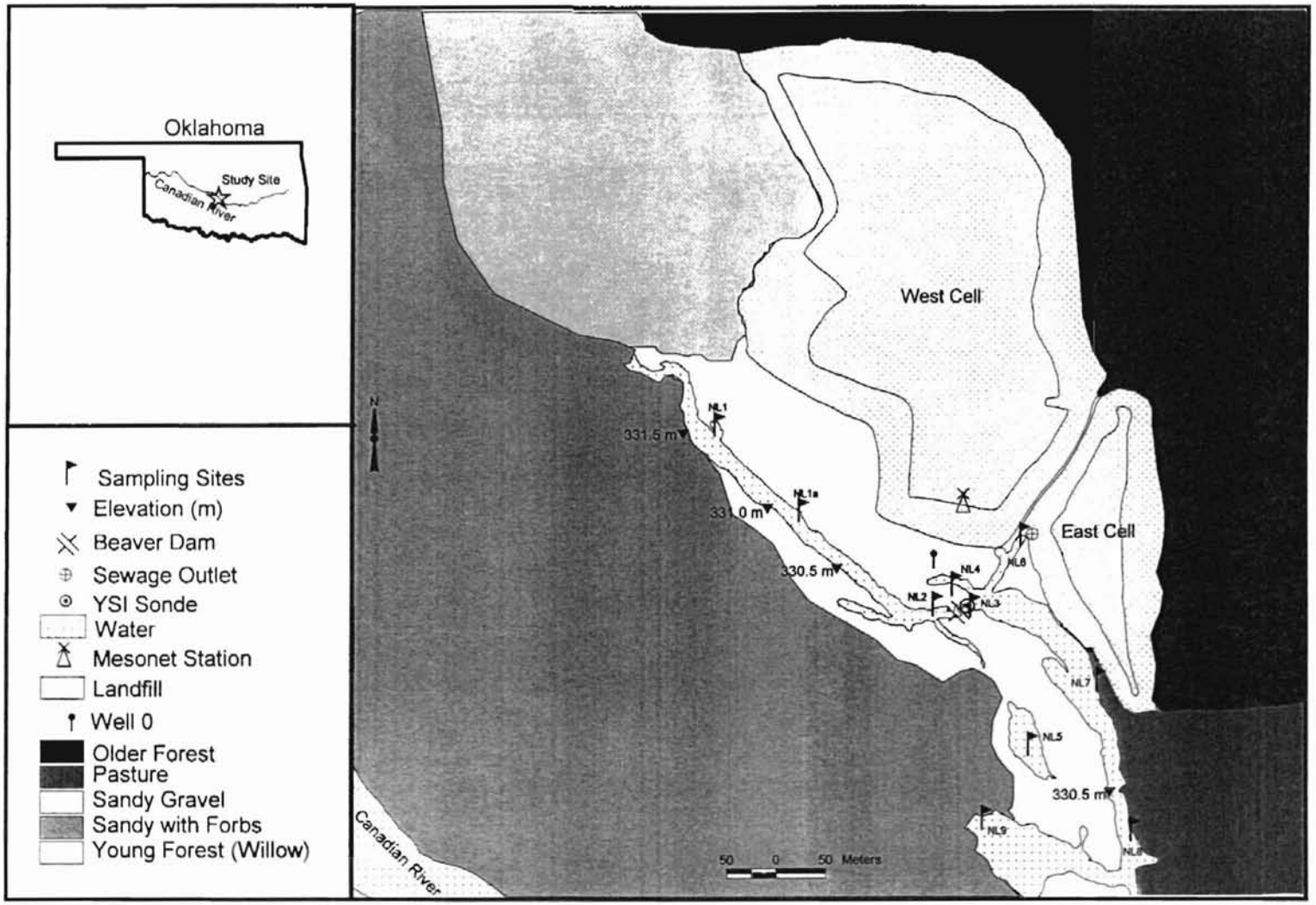

FIG. 1. Map of the Norman landfill research site.

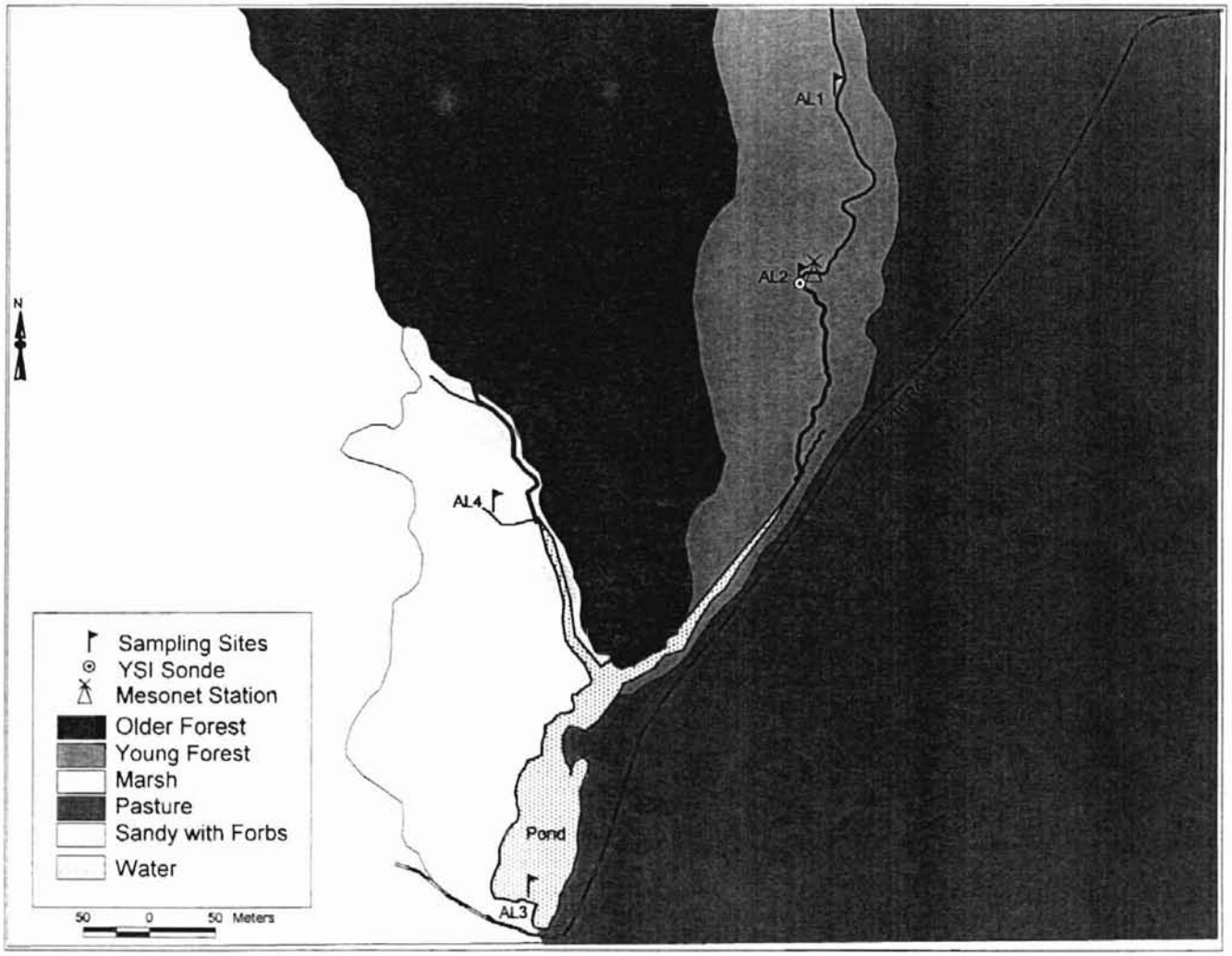

FIG. 2. Map of the reference site located near Norman, OK.

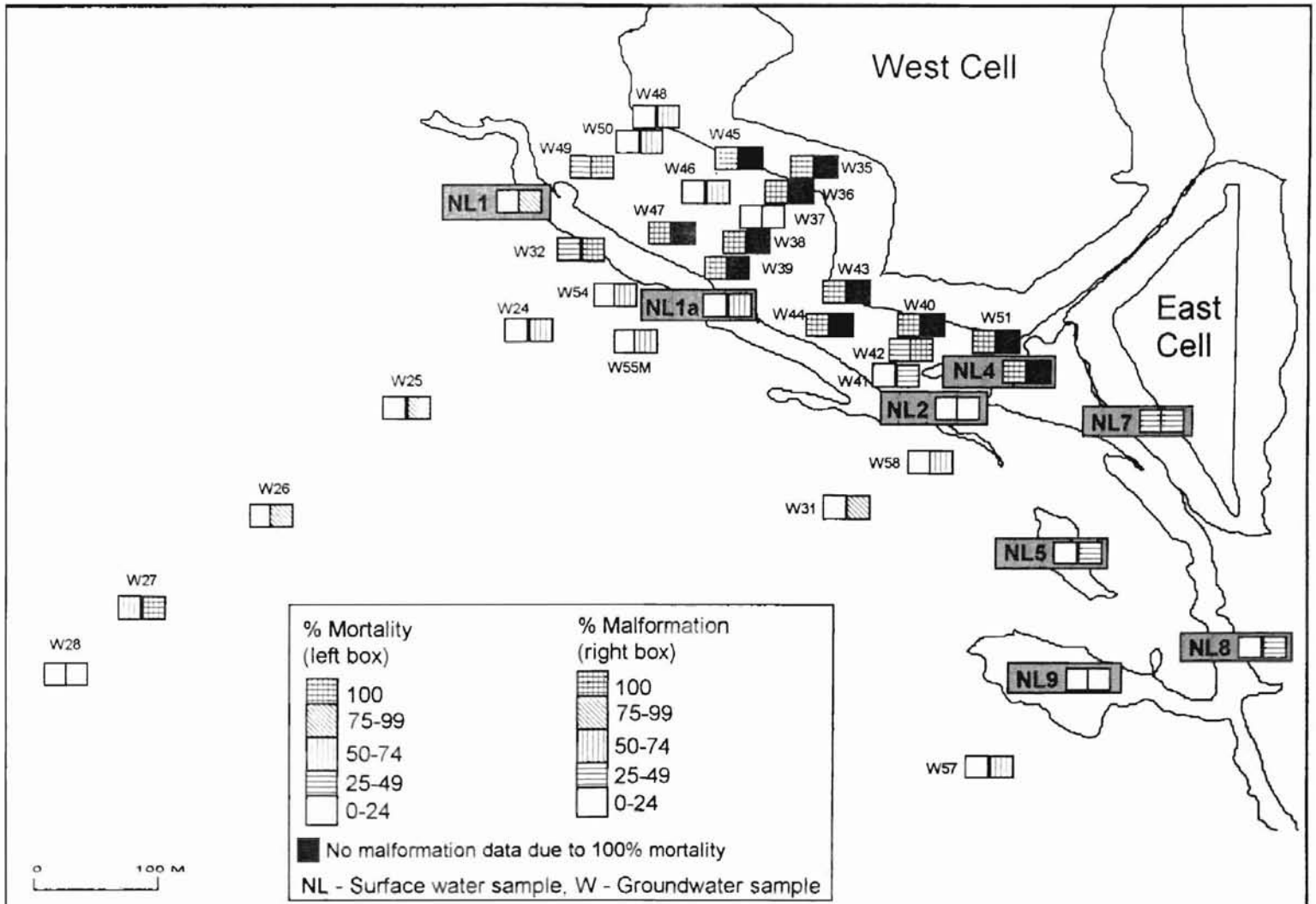

FIG. 3. Results of the FETAX toxicity tests on ground and surface water samples taken from the landfill site (Nov 16-17, 1995).

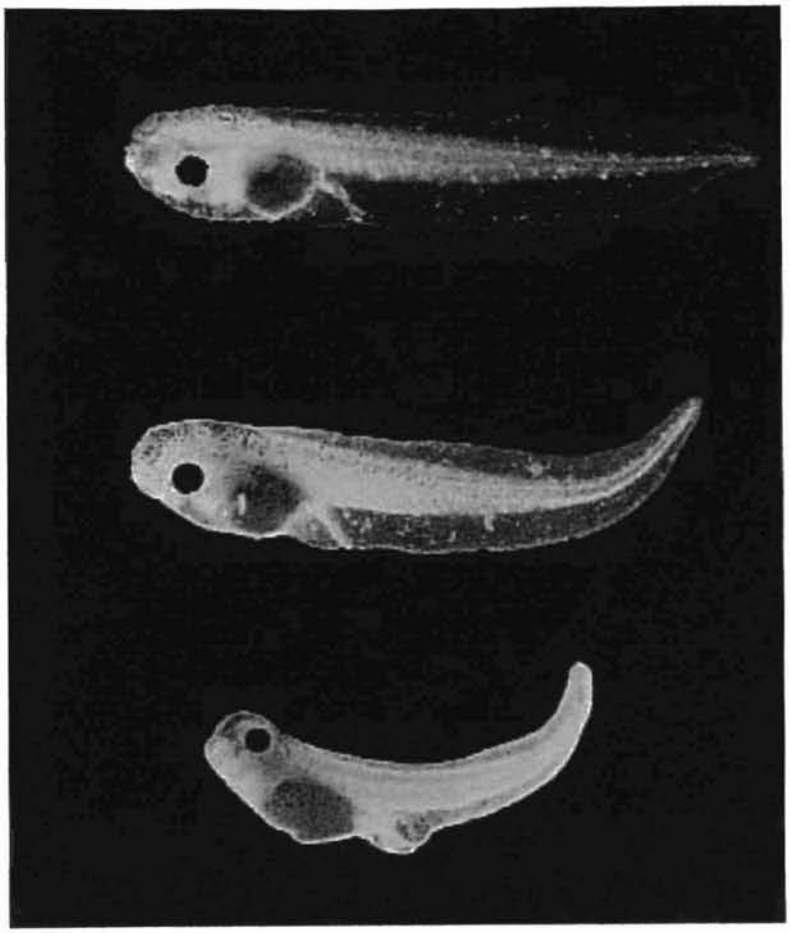

FIG. 4. Effect of water from Norman landfill NL 4 location on *Xenopus* embryo development. (a.) Control 96-h embryo. (b.) Embryo exposed to a 20% concentration of water from sample location NL 4. (c.) Embryo exposed to a 30% concentration of water from sample location NL 4

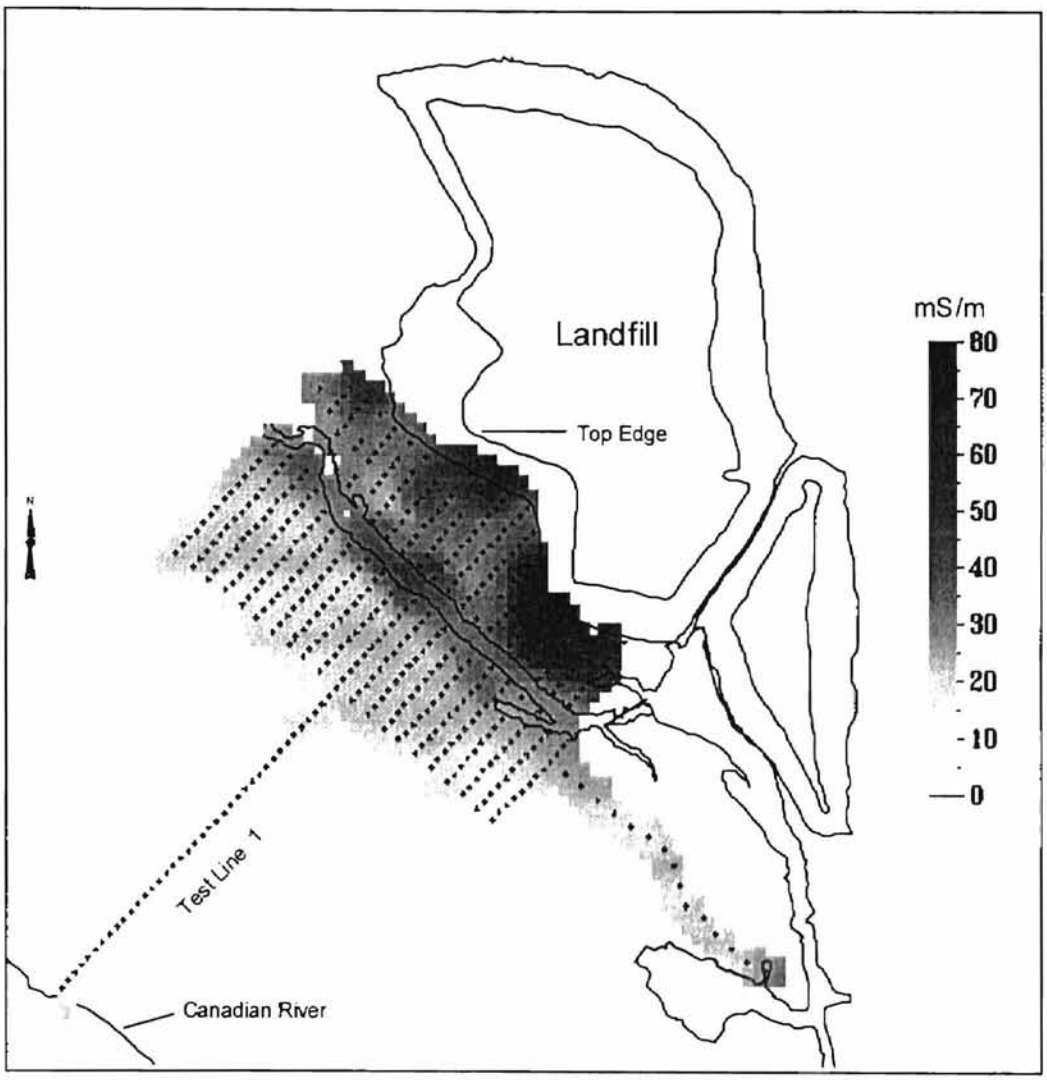

FIG. 5. Image map of EM31-D HMD apparent conductivity for the Norman, OK landfill area (Reproduced, with permission from Lucius and Bisdorf, 1995).

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