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ENVIRONMENTAL GENOTOXICITY:

PROBING THE UNDERLYING MECHANISMS

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ENVIRONMENTAL GENOTOXICITY

Key words:

Environmental pollution; DNA adducts; DNA strand breaks; DNA markers;
Genotoxicity

Abbreviations:

BaP, benzo[a]pyrene; DNA, deoxyribonucleic acid; ORNL, Oak Ridge National Laboratory; PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA; USDOE, U.S. Department of Energy

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ABSTRACT

Environmental pollution is a complex issue because of the diversity of anthropogenic agents, both chemical and physical, that have been detected and catalogued. The consequences to biota from exposure to genotoxic agents present an additional problem because of the potential for these agents to produce adverse change at the cellular and organismal levels. Past studies in genetic toxicology at the Oak Ridge National Laboratory have focused on structural damage to the DNA of environmental species that may occur after exposure to genotoxic agents and the use of this information to document exposure and to monitor remediation. In an effort to predict effects at the population, community and ecosystem levels, current studies in genetic ecotoxicology are attempting to characterize the biological mechanisms at the gene level that regulate and limit the response of an individual organism to genotoxic factors in their environment.

Pollution of the environment has become a major concern of society. Perhaps one of the more serious concerns is the potential for exposure to substances that are genotoxic. This problem arises because some of these pollutants are carcinogens and mutagens with the capacity to affect both the structural integrity of DNA and the fidelity of its biological expression (1).

Genetic toxicology is an area of science in which the interaction of DNA-damaging agents with the cell's genetic material is studied in relation to subsequent effect(s) on the health of the organism. Structural changes to the integrity of DNA caused by DNA-damaging agents are useful endpoints for assessing exposure to hazardous environmental pollutants on human health (2,3) and biota (4,5). The organism functions as an integrator of exposure, accounting for abiotic and physiological factors that modulate the dose of toxicant taken up, and the resulting magnitude of the change in DNA structure provides an estimate of the severity of exposure, hopefully in time to take preventive or remedial measures.

Genetic ecotoxicology is an approach that applies the principles and techniques of genetic toxicology to assess the potential effects of environmental pollution, in the form of genotoxic agents, on the health of the ecosystem. To this end, recent advances in toxicology, clinical medicine, and molecular genetics will foster a better understanding of the biological, chemical, and physical processes that accompany exposure to genotoxic agents. Because the techniques and methods unique to these disciplines are extremely sensitive and specific it is anticipated that their implementation into studies concerned with the mechanism of action of genotoxins will provide a stronger scientific basis for

the assessment of risk of exposure.

For these and other reasons, the Biological Markers Group in the Environmental Sciences Division at the Oak Ridge National Laboratory (ORNL) have included genotoxicity studies as part of their activities concerned with the biological monitoring of environmental pollution. The remainder of this report will provide examples of problems concerning genotoxic agents in the environment and the approaches/techniques used to address these problems. In most instances our studies have been concerned with documenting exposure of environmental species to genotoxic agents via the detection of DNA structural damage. DNA was analyzed for specific modifications such as chemical adducts (covalent attachment of a specific chemical to DNA) and photoproducts (dimerization of bases due to ultraviolet light) or generalized structural damage (i.e., DNA strand breakage) that is induced from exposure to any of a number of genotoxins. Each example contains a brief description about the environmental issue/concern being addressed, the approach used (i.e., species sampled and methodology employed to detect DNA damage), and results obtained. Finally, in an effort to define the potential consequence of exposure to genotoxins at organizational levels beyond the individual, two new approaches are described that utilize current techniques of molecular biology.

DNA Adducts in Beluga Whales

Exposure of an organism to a genotoxic chemical may result in the formation of a covalently-attached intermediate to the organism's DNA (adduct). Thus, detection of

adducts provides a way of documenting exposure. This approach was used to examine DNA from beluga whales of the St. Lawrence estuary to determine whether exposure to benzo[a]pyrene (BaP), a potent environmental carcinogen and the suspected etiological agent for the high incidence of cancer in these animals (6), had occurred. Data on BaP adducts (7) in the DNA of brain tissue from stranded beluga whales from the St. Lawrence estuary and in the DNA of brain and liver tissues from whales from the Mackenzie estuary are shown in Table 1. Detection of BaP adducts of the whale DNA was by HPLC/fluorescence analysis (8), a technique that measures only adducts that form between the DNA and the ultimate carcinogenic form of BaP. Values obtained from the St. Lawrence belugas approach those found in animals, both terrestrial and aquatic, exposed under laboratory conditions to carcinogenic doses of BaP. No detectable adducts were noted in the DNA of whales from the Mackenzie estuary.

DNA Strand Breaks in Turtles and Sunfish

Exposure to genotoxic agents may cause, in addition to or concomitant with adduct formation, other types of damage to the DNA molecule. Strand breakage in the DNA molecule occur under normal conditions but exposure to genotoxins can increase the amount. Recent reports (4,9) have detailed the various types of structural changes that may occur to DNA under normal cellular conditions as well as after exposure to chemical and physical genotoxins that may potentiate strand breakage. For example, ionizing radiation can cause strand breakage directly, whereas other physical agents such as UV light or genotoxic chemicals can cause alterations to the DNA molecule that are

candidates for repair (e.g., photoproducts, adducts, modified bases, etc.) and thus for the occurrence of strand breaks (9).

Early in 1987, the detection of excessive strand breakage in the DNA of several aquatic species was implemented as a biological monitor for environmental genotoxicity as a part of the Biological Monitoring and Abatement Program for the U.S. Department of Energy (USDOE) Reservation in Oak Ridge, Tennessee. DNA strand breakage as an endpoint of genotoxicant insult was used for two important reasons. First, it is compatible with routine monitoring as the analysis (alkaline unwinding assay) for this type of damage is easy to perform (10) and cost effective; and second, the assay provides a measure of DNA strand breaks arising from several contaminant-mediated processes (9). Examples with two different aquatic species will suffice to demonstrate the suitability of the approach.

Two species of turtles, the common snapping turtle (*Chelydra serpentina*) and the pond slider (*Trachemys scripta*) were compared for their usefulness as biological sentinels for environmental genotoxins in White Oak Lake on the USDOE Reservation (11). White Oak Lake is a settling basin for low-level radioactive and nonradioactive wastes generated at ORNL since 1943 and supports a high diversity of turtle species with *T. scripta* the most abundant and *C. serpentina* as the second most abundant. Cesium-137, cobalt-60, strontium-90, and tritium contribute most of the radioactivity to the lake. Species-specific data collected on DNA strand breakage in turtles captured in White Oak Lake were compared to Bearden Creek embayment, a reference site with similar biota but with no known history of contamination by hazardous chemicals. Over the entire

course of the study, genotoxic stress was evident in both species taken from White Oak Lake. This is graphically represented in Figure 1, in which individual *F* values are plotted in relation to when and where the turtles were captured. *F* values are a measure of the relative double-strandedness of a particular DNA preparation which in turn can be related to the number of strand breaks present. *F* values are determined under *in vitro* conditions by the alkaline unwinding assay (10) where the rate of conversion of the DNA from double-stranded to single-stranded structures is proportional to the number of strand breaks present. Thus large *F* values are indicative of DNA with few strand breaks. The *F* values for both species of turtles reveal a significant ($p < 0.001$) site effect and indicate that the DNA in these species have higher levels of strand breaks than the same species from the reference site. It should be noted that Bickham et al. (12) also detected DNA damage by flow cytometric analysis in turtles occupying seepage basins containing radioactive contaminants.

Analyzing for strand breaks in the DNA of sunfish has been employed as a biological marker for environmental genotoxicity as part of the Biological Monitoring and Abatement Program at East Fork Poplar Creek (13). This creek is the receiving stream for industrial effluent from the USDOE reservation in Oak Ridge, TN. Water and sediments downstream contain metals, organic chemicals, and radionuclides discharged over many years of operation (13).

DNA strand break data (*F* values), measured in sunfish from the head waters of the creek (near the USDOE reservation) and at Hinds Creek (reference stream) over a period of four years are presented in Figure 2. Two points are clear: a) DNA structural

integrity of the sunfish from the reference stream is high and relatively constant (large *F* value); and b) DNA structural integrity of the sunfish from East Fork Poplar Creek improved during the study period to reach levels similar to those for Hinds Creek. In all probability, the large genotoxic response observed in sunfish from East Fork Poplar Creek during the years 1987 and 1988 (small *F* value) was related to the release of chemicals from the USDOE reservation. Diminution of this response in subsequent years may be due to the remedial activities that occurred on the USDOE reservation to attenuated the release of pollutants. Included in these activities were the capping of existing settling basins, the creation of a new settling basin, and the treatment of waste water before discharge. However the possibility that there has been an adaptive response over time by the resident population of sunfish to their environment can not be excluded (see subsequent discussion on population genetics).

It is often difficult to relate effects observed in the field to the contaminants themselves or their source found in the environment because of the influence of non-contaminant mediated factors. In such instances, laboratory studies may sometimes be important for establishing a chain of causality. For example, sunfish were exposed in the laboratory to sediment from East Fork Poplar Creek for a period of 16 weeks to determine whether this was the major source of genotoxins for the native population of sunfish (14). Sediment-exposed sunfish showed a time dependent increase in the level of strand breakage of their DNA. Also, other biological responses of toxicological relevance were measured and correlated with the genotoxic response (e.g., stress proteins and detoxication enzyme induction, metabolite in the bile, change in chromosomal

proteins, etc.). Such information can be used not only to verify the source of environmental contamination (sediment in this case), but also to define cellular mechanisms that respond to genotoxic stress that may lead to a better understanding of the consequences of genotoxic exposure. During the course of this laboratory investigation several different techniques for measuring strand breaks in DNA were compared. As a result, strand break analyses in DNA from non mammalian environmental species such as fish, birds, and amphibians, are now being supplemented in our laboratory by agarose gel electrophoresis, an analytical technique that can provide quantitative data on both double- and single-strand breaks present in the DNA molecule (15).

UVB-induced Photoproducts in DNA of Plants

In addition to its application to chemical contamination, genetic toxicology can also address concerns about possible adverse effects of enhanced ultraviolet-B (UVB) radiation (290 to 320nm) on the growth, reproduction and survival of plants. Decreasing stratospheric ozone levels will result in an increase in net UVB radiation at the earth's surface. For example, a 10% decrease in stratospheric ozone could result in a 20% increase in UV penetration at 305 nm and a 250% increase at 290 nm (16). The large proportional increase in the shorter wavelength region (below 300 nm) of the UVB spectrum is of concern because of its ability to disrupt physiological function and the likely induction of DNA damage in the form of pyrimidine dimers. Although UV radiation below 300 nm is extremely difficult to measure as it makes up only 1% of the

UV that reaches the surface of the earth, this portion of the UV spectrum has been postulated to have had a major impact on the evolution of life on the planet (17).

A UVB exposure and monitoring system (18) was established at ORNL to deliver specific but adjustable levels of UVB radiation in order to investigate the effects of this type of radiation on plants and other biota in the environment. Preliminary results (19) using this exposure system over a 2-month period with two cultivars of soybean exposed to elevated UVB (32% above ambient) are summarized in Table 2. Changes in biomass and UV-absorbing compounds (secondary metabolites that attenuate ultraviolet light within plant tissue) were documented. One cultivar (Forrest) was found to be sensitive to elevated UVB as demonstrated by a decrease in biomass and UV-absorbing compounds while the resistant one (Essex) showed no change in biomass but an increase in UV-absorbing compounds. In addition, it was observed that total DNA damage (strand breaks and pyrimidine dimers) was 4.6 times greater in the sensitive cultivar vs the resistant one (19).

New Research Initiatives At ORNL

New research initiatives in genetic ecotoxicology are underway at ORNL to examine changes at the gene level that may be responsible for an organisms response to genotoxins. These investigations are based on two important assumptions: a) that there may be a genetic basis for this response, and b) that techniques of molecular biology are available with the sensitivity and specificity to address questions about organism-toxicant interactions at the gene level. Two new initiatives are briefly discussed

to illustrate the direction of our research.

Transgenic Fish - A transgenic fish (Japanese medaka, *Oryzias latipes*) has been produced containing the *lacZ* reporter gene through electroporation of medaka eggs at the 4-cell stage of development. Currently, backcross matings to wild type medaka have begun to detect integration of a single transgene and to establish inheritance in a Mendelian fashion. The transgenic fish will be used to determine the mutagenic potential of aquatic environments. For example, the *lacZ* cam be retrieved from the transgenic fish after exposure and analyzed for change in mutational frequency. Also the organism can be used to test for tissue susceptibility to genotoxic/mutagenic compounds or their metabolites, and to detect specific DNA base changes caused by genotoxic agents.

Population genetics - The effect of environmental contamination on population genetics of aquatic species is under investigation. This research is based on the hypothesis that there will be a selective advantage to variants in the population that are genetically predisposed to cope with toxicants. For example, we have been examining a series of retention ponds heavily contaminated with radionuclides, but which have support a resident population of mosquitofish (*Gambusia affinis*) for the past 20 years.

In a recent study (unpublished data) we found that there was an inverse correlation between DNA strand breakage and fecundity of fish from the contaminated ponds. This has implications for higher-order ecological effects, as well as for contaminant-induced selection of resistant phenotypes. Current investigations have provided evidence that genetic diversity is increased in the population of fish occupying

the radionuclide-contaminated sites relative to reference sites. These findings are supported both by allozyme analysis - through determination of average heterozygosity and percent polymorphisms, and by the RAPD (randomly amplified polymorphic DNA) technique - by determining average similarities of banding patterns between individuals within populations. In addition it has been found that certain banding patterns are more prevalent in the contaminated sites than in the reference sites. Individuals which display these banding patterns at one of the contaminated sites have a higher fecundity and lower degree of strand breakage than do individuals with the less common banding patterns. This type of pattern is also observed with allozyme analysis - heterozygotes, especially at the nucleoside phosphorylase locus, are more common in the contaminated sites. Within the contaminated sites, heterozygotes have a higher fecundity and lower degree of strand breakage than do homozygotes. Long term laboratory exposures where environmental variables can be more rigidly controlled are underway in an effort to establish relationships between genotype, DNA strand breakage, and fecundity.

Discussion

This report 1) summarizes several past attempts at ORNL to detect genotoxic insult in environmental species exposed to pollution, and 2) outlines current investigations to predict or define the potential consequences at higher levels of organization (e.g., population). The former studies examined DNA for structural modifications indicative of damage caused by a genotoxic agent (adduct, strand breakage, and photoproduct). The data was then applied to a particular environmental problem. For example, with the

beluga whale, the data helped stimulate the debate on how to manage a threatened species in a polluted environment (7). At the USDOE reservation in Oak Ridge, Tn, the data have been used to define hazardous environments (turtle studies) or to monitor the effectiveness of activities associated with remediation (sunfish studies).

Even though genetic toxicological investigations are important for the documentation of exposure, they often fail to provide the information necessary to establish why the insult occurred or the outcome. Ancillary data can help ameliorate this situation by defining other cellular mechanisms associated with or linked to the genotoxic response. For example, the difference noted in the amount of UVB-type damage to the DNA of two soybean cultivars could be explained to some extent by the increase in UV-absorbing compounds in one cultivar but not the other (19). Nevertheless, none of these observations explains the effect of UVB exposure on biomass in these plants.

Therefore, new approaches are needed that answer questions of ecological significance. Populations and communities within an ecosystem result from dynamic interactions where biota acclimate and adapt to environmental change. These processes have a genetic basis; therefore, understanding change at the genetic level should help identify the more complex changes at higher levels. Application of experimental tools currently in use in molecular biology and other related disciplines should help in our understanding of key biological mechanisms that regulate and limit the response of organisms to stresses in their environment. This is a fruitful area for new genetic ecotoxicological research, as it offers an opportunity to rapidly advance our knowledge and understanding of the effect of environmental pollution (20).

REFERENCES

1. Wogan GN, Gorelick NJ. Chemical and biochemical dosimetry to exposure to genotoxic chemicals. *Environ Health Perspec* 62:5-18 (1985).
2. Kohn HW. The significance of DNA-damaging assays in toxicity and carcinogenicity assessment. *Ann NY Acad Sci* 407:106-118 (1983).
3. Committee on Biological Markers of the National Research Council: Biological markers in environmental health research. *Environ Health Perspec* 74:3-9 (1987).
4. Shugart LR. Biological monitoring: testing for genotoxicity. In: *Biological Markers of Environmental Contaminants*, (McCarthy J. and Shugart L. eds). Boca Raton, FL:Lewis Publishers Inc, 1990;205-216.
5. Shugart LR, McCarthy JF, and Halbrook RS. Biological markers of environmental and ecological contamination: an overview. *Risk Analysis* 12:353-360 (1992).
6. Martineau D, Lagace A, Beland P, Higgins R, Armstrong D, Shugart LR. Pathology of stranded beluga whales (*Delphinapterus leucas*) from the St. Lawrence Estuary, Quebec, Canada. *J Comp Path* 98:287-311 (1988).
7. Shugart LR. Detection and quantitation of benzo[a]pyrene-DNA adducts in brain and liver tissues of beluga whales (*Delphinapterus leucas*) from the St. Lawrence and Mackenzie estuaries. In: *Proceeding of the international forum for the future of the beluga*. Quebec:Presses de l'Universite du Quebec, 1990;219-223.
8. Shugart LR, Holland J, Rahn R. Dosimetry of PAH carcinogenesis: covalent

binding of BaP to mouse epidermal DNA. *Carcinogenesis* 4:195-198 (1983).

9. Shugart LR, Bickham J, Jackim G, McMahon G, Ridley W, Stein J, Steiner S. DNA alterations. In: *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*, (Huggett R, Kimerle R, Mehrle P, Bergman H. eds). Boca Raton, FL:Lewis Publishers Inc, 1992;127-153.

10. Shugart LR. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. *Aquatic Tox* 13:43-52 (1988).

11. Meyers-Schone L, Shugart LR, Beauchamp JJ, Walton BT. Comparison of two freshwater turtle species as monitors of radionuclide and chemical contamination: DNA damage and residue analysis. *Environ Tox Chem* 12:1487-1496 (1993)

12. Lamb T, Bickham JW, Gibbons JW, Smolen MJ, McDowell S. Genetic damage in a population of slider turtles (*Trachemys scripta*) inhabiting a radioactive reservoir. *Arch Environ Contam Tox* 20:138-142 (1991).

13. Shugart LR. DNA damage as an indicator of pollutant-induced genotoxicity. In: *13th Symposium on Aquatic Toxicology and Risk Assessment: Sublethal Indicators of Toxic Stress*, (Landis WG, van der Schalie WH. eds). Philadelphia, PA:ASTM, 1990;348-355.

14. Theodorakis CW, D'Surley SJ, Bickham JW, Lyne TB, Bradley BP, Hawkins WE, Farkas WL, McCarthy JF, Shugart LR. Sequential expression of biomarkers in bluegill sunfish exposed to contaminated sediment. *Ecotoxicology* 1:45-73 (1992).

15. Theodorakis CW, D'Surley SJ, Shugart LR. Detection of genotoxic insult as DNA strand breaks in fish blood cells by agarose gel electrophoresis. *Environ Tox*

Chem 13:in press (1994).

16. Cicerone RJ. Changes in stratospheric ozone. *Science* 237:35-42 (1987)
17. Caldwell MM. Plant life and ultraviolet radiation: some perspectives in the history of the earth's UV climate. *BioScience* 29:520-525 (1970).
18. McEvers JA, Hileman MS, Edwards NT. Air pollution effects field research facility: 3. UV-B exposure and monitoring system. ORNL/TM-11607 (1993).
19. D'Surley SJ, Tschaplinski TJ, Edwards NT, Shugart LR. Biological responses of two soybean cultivars exposed to enhanced UVB radiation. *Environ Exp Botany* 33:347-356 (1993).
20. Shugart LR. State of the art-ecological biomarkers. In: *Use of Biomarkers in Assessing Health and Environmental Impacts of Chemical Pollutants*, (Travis CC. ed). New York:Plenum Press, 1993;237-245.

Table 1. Detection of benzo[a]pyrene adducts in DNA of beluga whales

Sample	Tissue	BaP Adduct Formation
<hr/>		
St. Lawrence Estuary		
#1	Brain	206
#2	Brain	94
#3	Brain	69
Mackenzie Estuary		
#1-#4	Brain	ND
#1-#4	Liver	ND
<hr/>		

Analysis for BaP adducts to DNA were as described in reference 8, and data expressed as nanograms of BaP tetrol I-1 per gram of DNA. ND - none detected.

Table 2. Change in biological responses of soybean cultivars exposed to elevated UVB radiation

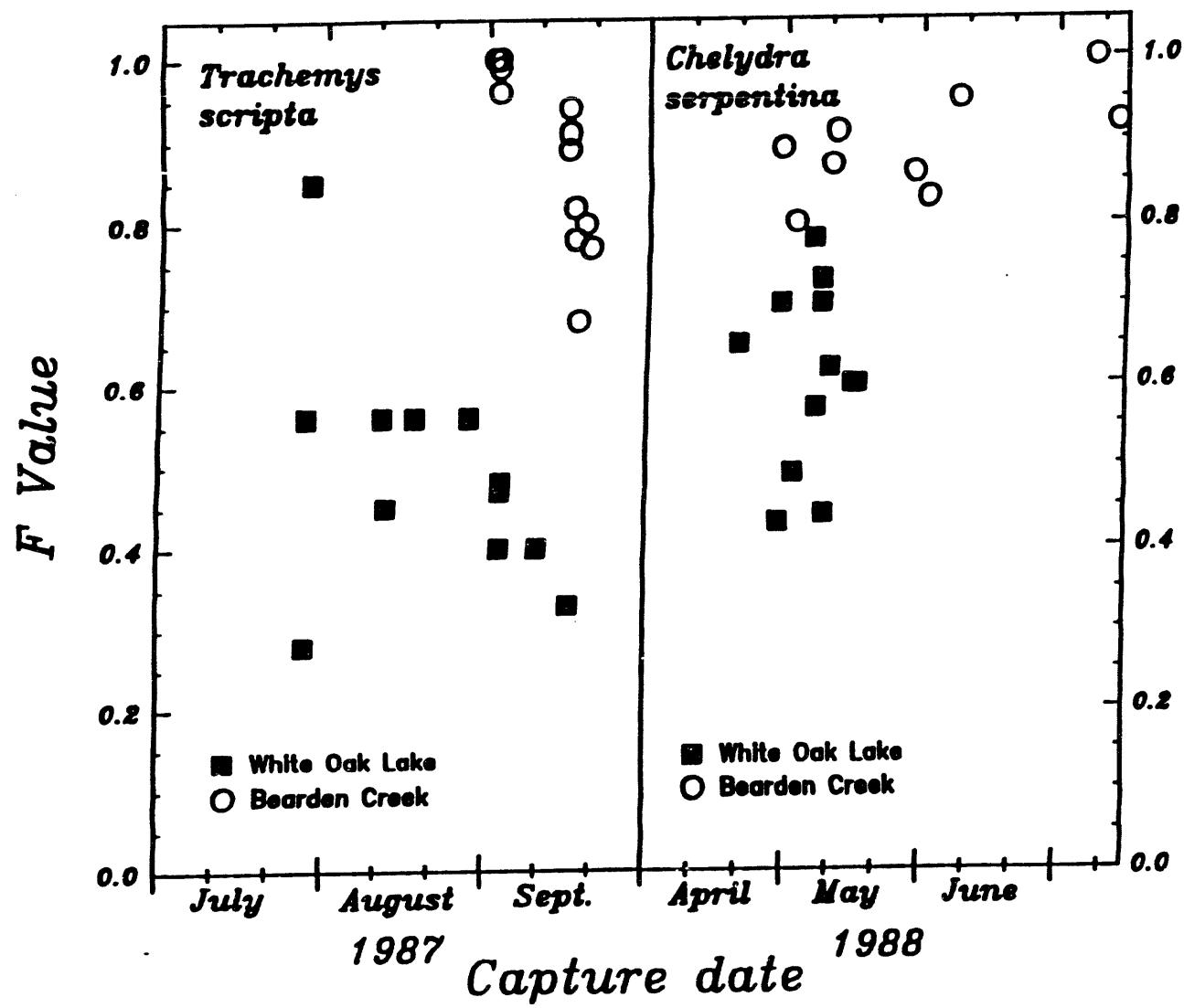
Cultivar	UVB Absorbing		DNA Damage	
	Biomass	Compounds	(Dimers)	(Strand Breaks)
Forrest	Decreased (14%)	Decreased (25%)	Increased	Increase
Essex	No Change	Increased (13%)	No Change	Slight Increase

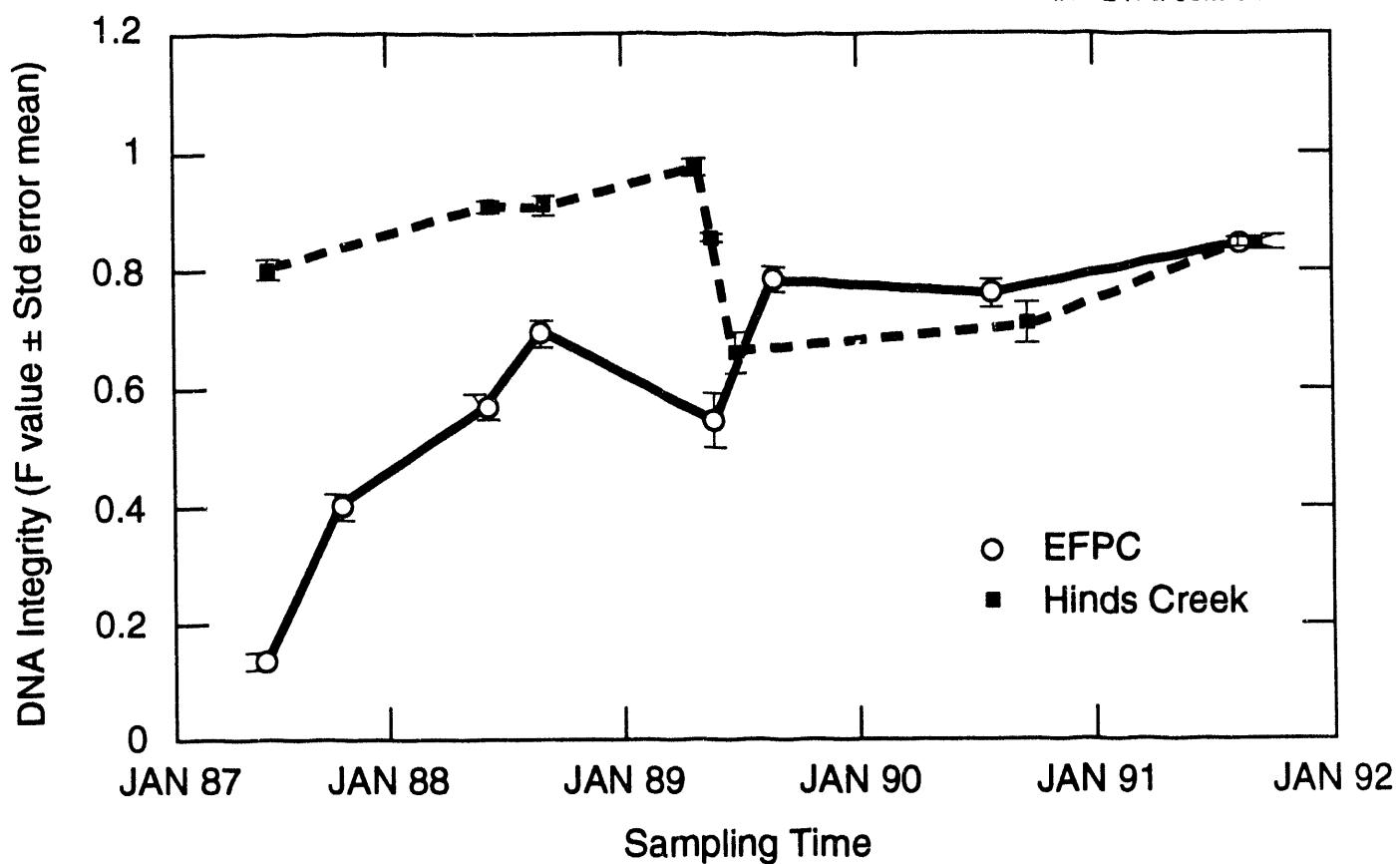
Plants were exposed for a period of two month with the exposure and monitoring system (18) set to deliver 32% above ambient UVB radiation and to simulate daily and seasonal changes in solar irradiance with adjustment for cloud/canopy conditions.

LEGENDS

Figure 1. Fraction of double stranded (F value) DNA in liver samples of *Trachemys scripta* and *Chelydra serpentina* collected from the Oak Ridge Reservation (Taken from reference 10).

Figure 2. Temporal status of double stranded (F value) DNA in liver samples of sunfish from East Fork Poplar Creek (contaminated stream) and Hinds Creek (reference stream) over a four year period.





A vertical stack of four abstract black and white shapes. The top shape is a horizontal rectangle divided into five vertical segments of varying widths. The second shape is a thick horizontal bar. The third shape is a thick diagonal bar extending from the bottom-left to the top-right. The bottom shape is a large, dark, irregular U-shaped or shield-like form containing a white, rounded rectangular area.

High

DATA
MANAGEMENT

