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New trends in biological monitoring: application of biomarkers to genetic ecotoxicology

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Abbreviations: BaP – benzo[a]pyrene; DNA – deoxyribonucleic acid; ORNL – Oak Ridge National Laboratory; PCR – polymerase chain reaction; RAPD – randomly amplified polymorphic DNA; USDOE – US Department of Energy

Introduction

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. Organismal responses at the genetic level to exposure to environmental genotoxicants have been well documented. 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. 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. An elucidation of the molecular mechanisms involved with these responses, as well as an assessment of the changes that may occur to the genetic material, will provide an understanding of the potential for deleterious consequences at higher levels of biological organization. Moreover, modern procedures of molecular biology offer the hope that alterations and changes to genetic material can be readily detected.

Genetic toxicology vs genetic ecotoxicology

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 genotoxicants will provide a stronger scientific basis for the assessment of risk of exposure.

Genetic toxicological studies

The Biological Markers Group in the Environmental Sciences Division at the Oak Ridge National Labo-

ratory (ORNL) have included genotoxicity studies as part of their activities concerned with the biological monitoring of environmental pollution. Several examples of problems concerning genotoxic agents in the environment and the approaches/techniques used to address these problems are presented. Our past studies have been concerned with documenting exposure of environmental species to genotoxic agents via the detection of DNA structural damage (genetic toxicology). 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 genotoxicants. 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 genotoxicants at organizational levels beyond the individual (genetic ecotoxicology), 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.

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

Sample	Tissue	BaP Adduct formation
St. Lawrence Estuary		
#1	Brain	206
#2	Brain	94
#3	Brain	69
Mackenzie Estuary		
#1-#4	Brain	ND
#1-#4	Liver	ND

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.

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 molecules occur under normal conditions but exposure to genotoxicants 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 genotoxicants 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 US 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 biologi-

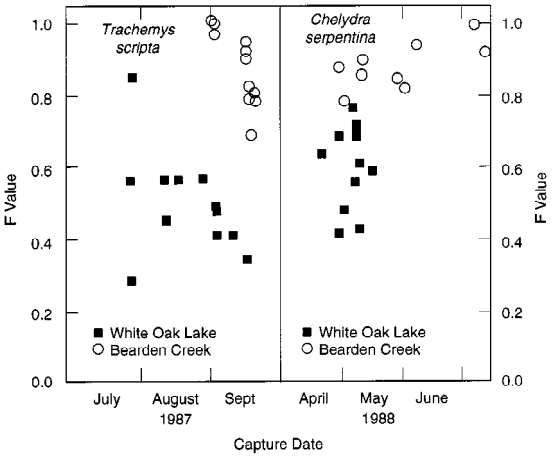

Figure 1. Fraction of double stranded (*F* value) DNA in liver samples of *Trachemys scripta* and *Chelydra serpentina* collected from the Oak Ridge Reservation (taken from reference [31] with permission).

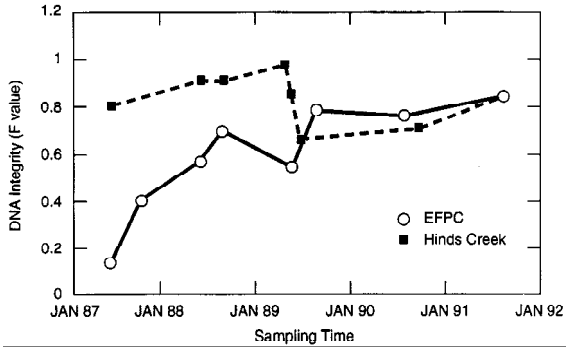

Figure 2. Temporal status of double stranded (*F* value) DNA in liver samples of sunfish from East Fork Poplar Creek (contaminated stream) and Hinds Creek (reference stream) over a four year period (taken from [31] with permission).

cal sentinels for environmental genotoxicants 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 theses 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 attenuate 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

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

Cultivar	Biomass	UVB Absorbing Compounds	DNA Damage (Dimers)	DNA Damage (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.

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 genotoxicants 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 and which in turn 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 (290–320nm) 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].

Genetic ecotoxicological studies

Introduction

In October of 1993, the National Institute of Environmental Sciences in the USA sponsored a conference on 'Genetic and molecular ecotoxicology', an endeavor that was prompted by the realization that little is known about the potentially deleterious effects of environmental pollution at the ecosystem level [20]. The goal of the conference was to identify a framework for the future of genetic and molecular ecotoxicology. The purpose of this paper is to discuss, in general terms, research efforts at the genetic level that may be appropriate within this framework.

It is especially difficult to demonstrate the effect of environmental stressors, including genotoxicants, at the ecosystem level, where population and communities are studied because the responses observed there are latent and so far removed from the initial event(s) of exposure that causality is often almost impossible to establish. A way to approach this problem is to ask mechanistic questions about how an organism relates to its environment. Ecosystems result from the dynamic interactions of living and inert matter where the living material acclimates and adapts to environmental change. These processes are physiological and have a genetic basis, therefore understanding changes at the genetic level (DNA) should help define the more complex changes at the ecosystem level. An important consideration has been the accelerated advancement of new techniques in molecular biology that may help describe and define in great detail the changes anticipated at the genetic level.

Understanding changes at the genetic level

A broader view of the consequences of exposure to genotoxicants is needed to address complex problems of environmental pollution. The genetic apparatus of an organism can interact with genotoxicants in a variety of ways and an understanding of the cellular mechanisms involved in these interactions provide the researcher the opportunity to predict and possibly prevent contaminant-induced genetic damage in exposed populations.

Genotoxicants can alter the structural integrity of the DNA; cause mutations and subsequent heritable effects; or even cause non-mutagenic effects. Conversely, the organism may perceive the genotoxicant and attempt to eliminate the agent or repair modifications to its DNA. Figure 3 [21] briefly summarizes some of the potential mechanisms that occur. First, the organism may perceive the genotoxicant and modify its physiology (pathway #1, Figure 3), as in induction of the P4501A1 detoxication system [22]. If the genotoxic agent (i.e., radiation or chemical mutagen) directly impinges on the DNA, the organism may perceive this damage and attempt repair (pathway #2, Figure 3; Shugart, et al., [9]). Mutational events not corrected allow genotoxic stress to progress within the organism (pathways #3 and #4, Figure 3). The flow of genotoxic stress within a somatic cell is depicted in Figure 4 [23] and the mechanisms involved have been reviewed [24, 25], however, several salient points need to be reiterated. Effects to the cell such as the occurrence of chromosomal aberrations, oncogene activation and protein dysfunction are not usually caused by the direct interaction of the genotoxicant with DNA but rather are the result of faulty repair of DNA damage and the subsequent occurrence of mutations (Figure 4). Cellular processes regulating these events are very complex and for which there is presently only a rudimentary understanding. These processes are affected differently in different species and may depend upon, for example, the type or class of genotoxic agent and the reactivity of its metabolite(s), the capacity of the cell to repair DNA damage, and the ability of the cell to recognize and suppress the multiplication of cells with aberrant properties [25]. Effects expressed in somatic cells can be detrimental to the exposed individual, whereas mutational events in germ cells may affect subsequent generations. Extrapolation of observations made at the somatic cell level of biological organization to predict effects at the germ cell level of biological organization is difficult. This is due to the inherent difference in sensitivity of these types of cells to genotoxicants [26]. Furthermore, establishing a causal relationship between a genotoxic agent in the environment and a deleterious effect in subsequent generations of that organism is also highly unlikely because individuals carrying harmful mutations are eliminated from the population due to a strong selection against less fit and less well-adapted individuals [27].

Finally, a class of genetic effects resulting from exposure to environmental pollutants exist that are not

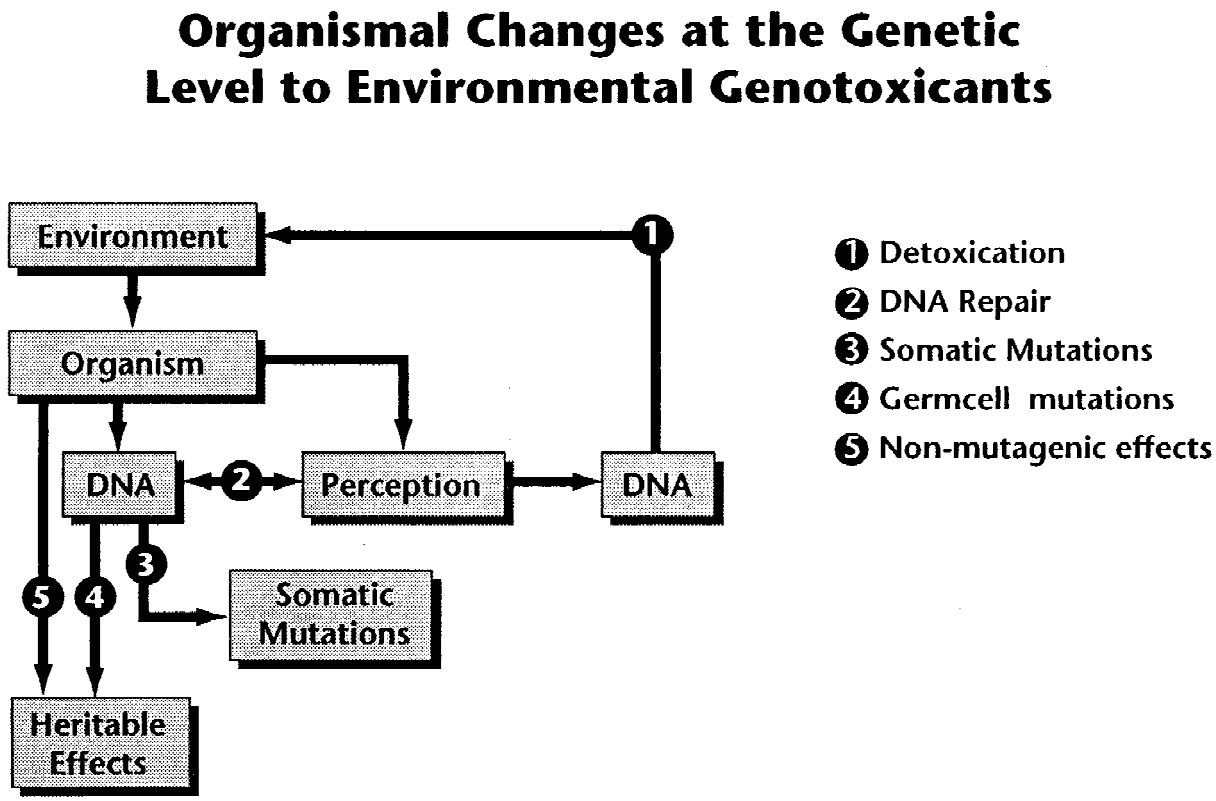

Figure 3. Organismal changes at the genetic level to environmental genotoxicants (taken from reference [32] with permission).

necessarily due to alterations of the DNA molecule (pathway #5, Figure 3). Rather they are the result of organisms adapting to a polluted environment. Subsequent ecological phenomenon such as bottlenecks and inbreeding could result in changes in allele frequencies of populations [28].

Genotypic diversity

Ideally, genetic ecotoxicology will begin to address such outcomes of exposure to environmental genotoxicants as disease, decreased reproductive success and altered genotypic diversity. All of these outcomes are important to the survival of species, however the remainder of this discussion will focus on genotypic diversity.

Studies on the effects of exposure on gene pools, genetic variability and Darwinian fitness are sparse [29], however the principles underlying research of effects of genotoxicants on genotypic diversity are not new. In a heterozygous population, there are likely to be certain genotypes that are more sensitive to genotoxic exposure than others. This is especially so if the population is heterozygous at loci that are both critical to fitness and susceptible to toxicant-induced structural alterations. Genotoxic exposure can act as a selective force by eliminating sensitive genotypes, or reducing the number of offspring that they contribute to the next generation. The result can be a reduction in the total genetic variation within the population or a shift in genotypic frequencies.

Current research at the Oak Ridge National Laboratory (see below) addresses these principles and 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 [30].

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 organism's response to genotoxicants. These investigations are based on two important assumptions: (a) that there may be a genetic basis for this response, and (b) that

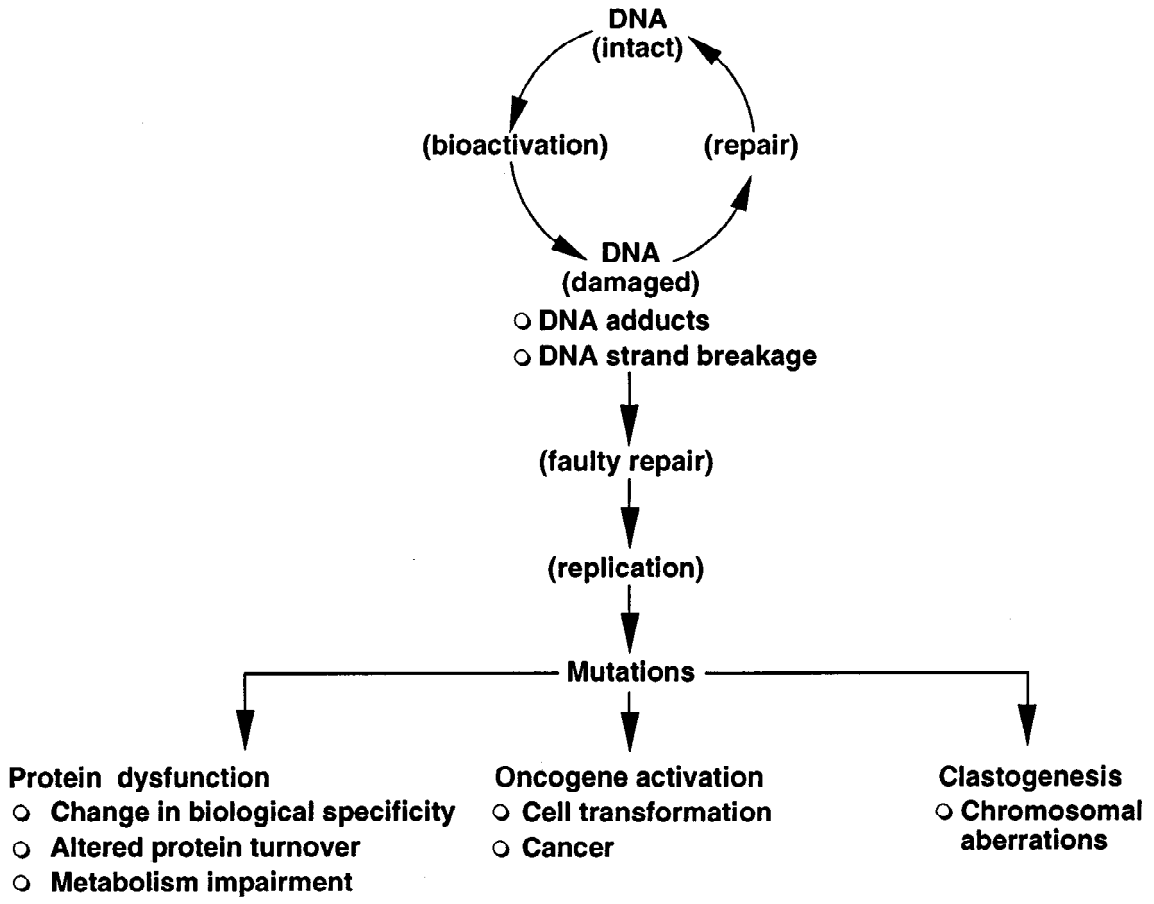

Figure 4. Flow of genotoxic stress in an organism due to DNA damage (taken from reference [32] with permission).

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* can 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 support a resident population of mosquitofish (*Gambusia affinis)* for the past 20 years. In a recent study [30, 31] 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 controled are underway in an effort to establish relationships between genotype, DNA strand breakage, and fecundity.

Discussion/conclusion

We have summarized several past attempts at ORNL to detect genotoxic insult in environmental species exposed to pollution and outlined 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.

It is obvious that new approaches in genetic ecotoxicology will offer the opportunity to address questions of ecological significance of exposure to genotoxicants in the enviornment [32]. Our studies with a population of *G. affinis* introduced into a radionuclide contaminated pond show that acclimation and adaptation to environmental stress occurred. 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 genetic ecotoxicological research, as it offers an opportunity to rapidly advance our knowledge and understanding of the effect of environmental pollution [33]. In this context, biomarker technologies are adding to our maturing concepts [5, 34].

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References

1. Wogan GN, Gorelick NJ. Chemical and biochemical dosimetry to exposure to genotoxic chemicals. Environ Health Perspec 1985; 62: 5–18.
2. Kohn H.W. The significance of DNA-damaging assays in toxicity and carcinogenicity assessment. Ann NY Acad Sci 1983; 407: 106–18.
3. Committee on Biological Markers of the National Research Council:Biological markers in environmental health research. Environ Health Perspec 1987; 74: 3–9.
4. Shugart LR. Biological monitoring: testing for genotoxicity. In: McCarthy J. and Shugart L. (eds). Biological Mark-

ers of Environmental Contaminants, Boca Raton, FL: Lewis Publishers Inc, 1990: 205–16.
5. Shugart LR, McCarthy JF, Halbrook RS. Biological markers of environmental and ecological contamination: an overview. Risk Analysis 1992; 12: 353–60.
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 1988; 98: 287–311.
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; 219–23.
8. Shugart LR, Holland J, Rahn R. Dosimetry of PAH carcinogenesis: covalent binding of BaP to mouse epidermal DNA. Carcinogenesis 1983; 4: 195–8.
9. Shugart LR, Bickham J, Jackim G, McMahon G, Ridley W, Stein J, Steiner S. DNA alterations. In: Huggett R, Kimerle R, Mehrle P, Bergman H, (eds). Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress, Boca Raton, FL: Lewis Publishers Inc, 1992: 127–53.
10. Shugart LR. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquatic Tox 1998; 13: 43–52.
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 1993; 12: 1487–96.
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 1991; 20: 138–42.
13. Shugart LR. DNA damage as an indicator of pollutant-induced genotoxicity. In: Landis WG, van der Schalie WH. (eds). 13th Symposium on Aquatic Toxicology and Risk Assessment: Sublethal Indicators of Toxic Stress, Philadelphia, PA:ASTM, 1990: 348–55.
14. Theodorakis CW, D'Surney 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 1992; 1: 45–73.
15. Theodorakis CW, D'Surney SJ, Shugart LR. Detection of genotoxic insult as DNA strand breaks in fish blood cells by agarose gel electrophoresis. Environ. Tox. Chem., 1994; 13: 1023–31.
16. Cicerone RJ. Changes in stratospheric ozone. Science 1987; 237: 35–42.
17. Caldwell MM. Plant life and ultraviolet radiation: some perspectives in the history of the earth's UV climate.BioScience 1970; 29: 520–5.
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'Surney SJ, Tschaplinski TJ, Edwards NT, and Shugart LR. Biological responses of two soybean cultivars exposed to enhanced UVB radiation. Environ Exp Botany 1993; 33: 347–56.
20. Anderson S, Sadinski W, Shugart L, Brussard P, Depledge M, Ford T, Hose J, Stegeman J, Suk W, Wirgin I, Wogan G. Genetic and molecular ecotoxicology: a research framework. Environ. Health Perspec., 1994; 102: 3–8. bibitemThaler DS. The evolution of genetic intelligence. Science 1994; 264: 224–5.
21. Guengerich FP. Cytochrome P450 enzymes. Am. Sci., 1993; 81: 440–7.
22. Shugart LR. Molecular and biochemical responses to toxic agents. In: Newman MC, Jagoe CH, (eds.), Quantitative Ecotoxicology: A Hierarchical Approach Boca Raton, FL: Lewis Publishers Inc., 1995: 133–61.
23. Brusick, D. Principles of Genetic Toxicology, New York, NY: Plenum Press, 1980.
24. Thilly WG, Call KM. Genetic toxicology, In: Klaassen DD, Amdur MO, Doull J, (eds.) Third Edition of Casarett and Doull's Toxicology, New York, NY: Macmillan Publishing Co., 1986: 174–9.
25. Clive D. Genetic toxicology: from theory to practice. Clin. Res. Drug Development, 1987; 1: 11–41.
26. Wurgler FE, Kramers PGN. Environmental effects of genotoxins (eco-genotoxicology). Mutagenesis, 1992; 7: 321–7.
27. Bickham JW, Smolen MJ. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ. Health Perspec., 1994; 102: 25–8.
28. Depledge MH. The rational basis for the use of biomarkers as ecotoxicological tools, In: Fossi MC, Leonzio C. eds. Nondestructive Biomarkers in Vertebrates, Boca Raton, FL: Lewis Publishers Inc., 1992: 271–95.
29. Theodorakis CW, Shugart LR. Genetic Ecotoxicology I: DNA integrity and reproduction in mosquitofish exposed *in situ* to radionuclides. Ecotoxicology, 1996, in press.
30. Shugart LR, Theodorakis C. Environmental genotoxicity: probing the underlying mechanisms, Environ. Health Perspec., 1994; 102: 13–7.
31. Shugart LR, Theodorakis C. Genetic ecotoxicology: The genotypic diversity approach, Comp Biochem Physiol, 1996; 113: 273–276.
32. Shugart LR. State of the art-ecological biomarkers. In: Travis CC. (ed). Use of Biomarkers in Assessing Health and Environmental Impacts of Chemical Pollutants, New York: Plenum Press, 1993: 237–45.
33. McCarthy JF, Shugart LR (eds.) Biomarkers of Environmental Contamination, Boca Raton, FL: Lewis Publishers Inc., 1990.