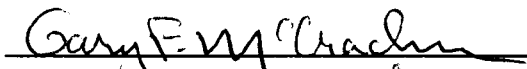
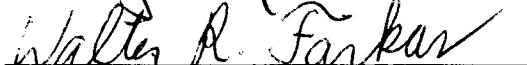
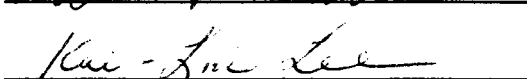


To the Graduate Council:

I am submitting herewith a thesis written by Susan G. Nadig entitled "Evaluating Potential Alteration of Genetic Diversity in Populations of Redbreast Sunfish (*Lepomis auritus*) using RAPD Assay." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.


Marshall Adams, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:


Associate Vice Chancellor and
Dean of The Graduate School

**EVALUATING POTENTIAL ALTERATION OF GENETIC DIVERSITY
IN POPULATIONS OF REDBREAST SUNFISH
(*LEPOMIS AURITUS*) USING RAPD ASSAY**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Susan G. Nadig
May 1996

Dedication

To my parents who have given me the best of themselves
in unfaltering love and support.

Acknowledgements

There are many people to whom I wish to extend my thanks. I am grateful to all those persons involved in field collections particularly Scott Niemela, Dennis Crumby, and Don Harris. I have benefitted greatly from the opportunity given to me by Dr. Marshall Adams and Dr. Kai Lin Lee to work on this project. Appreciation is extended to the Environmental Sciences Division of Oak Ridge National Laboratory, where this research was performed, for the use of their facilities and equipment. The numerous people I have met while working at Oak Ridge National Laboratory have provided help and support and generously shared their knowledge for which I am most grateful. I am especially thankful to Dr. Chris Theodorakis for his assistance and the use of his programs, to Dr. John Beauchamp for his assistance in data analysis, to Dr. Gerald Tuskan and Lee Gunter for their help with the RAPDPLOT programs, and to Dr. Jane Mural for her assistance and invaluable advice.

I thank my committee members, Dr. Marshall Adams, Dr. Kai Lin Lee, Dr. Walter Farkas, and Dr. Gary McCracken, for their consultation and criticism.

I am particularly grateful to Dr. Kai Lin Lee for his instruction and support from the onset of this research from which I have benefitted so much more than I could ever write here. His passion for science will inspire me always.

I am most thankful to my parents who have supported me both financially and emotionally as I undertook this project.

Abstract

Bioindicators of pollutant exposure can be more sensitive and ecologically relevant than simply measuring levels of pollutants in the environment and are applied here as tools for assessing stress on aquatic organisms. DNA polymorphisms, generated using the Randomly Amplified Polymorphic DNA (RAPD) technique, are used as biomarkers to assess genetic diversity and genetic distance among populations of redbreast sunfish (*Lepomis auritus*) residing in a contaminated stream (East Fork Poplar Creek -- EFPC) and populations from reference streams. The RAPD technique uses the polymerase chain reaction (PCR) with short oligonucleotide primers under reduced stringency to produce DNA fragments which when analyzed by gel electrophoresis form banding patterns similar to DNA fingerprints. A total of 13 primers were used which produced forty-five polymorphic bands. Using the RAPD technique, genetic diversity and genetic distance were not found to be greatly different among populations within EFPC. No significant difference was seen between EFPC and the reference sites with respect to the genetic diversity of these populations. However, populations within EFPC were consistently less genetically distant from each other than they were from each of the reference sites. Differences in genetic distance between populations may be due to selection effects of EFPC pollutants. These data are discussed in relation to implications of RAPD assays, recent remediation efforts within EFPC, and future biomonitoring programs.

TABLE OF CONTENTS

I.	INTRODUCTION.....	1
	Environmental Stress Effects.....	1
	Genetic Diversity as a Biomarker.....	4
	Molecular Measures of Genetic Diversity.....	7
	The RAPD Technique.....	12
	Study Sites.....	14
	EFPC Pollutants.....	18
	Research Objectives.....	20
II.	MATERIALS AND METHODS.....	22
	Specimen Collection.....	22
	DNA Extraction.....	22
	RAPD Analysis.....	25
	Analytical Procedures.....	28
III.	RESULTS.....	32
	RAPD Analysis.....	32
	Band Frequency.....	34
	Band Sharing Index.....	37

	Nucleon Diversity Index.....	39
	Roger's Genetic Distance.....	42
	RAPDPLOT Neighbor Joining Analysis.....	42
IV.	DISCUSSION.....	49
	The RAPD Technique.....	49
	Genetic Diversity in EFPC Populations.....	51
	Negative Results in Toxicological Studies.....	57
V.	CONCLUSIONS.....	59
VI.	FUTURE DIRECTIONS.....	61
	REFERENCES.....	62
	VITA.....	75

LIST OF TABLES

TABLE

1.	Decamer oligonucleotides used as random primers, their sequences and variability.....	33
2.	Band frequencies of polymorphic bands.....	35
3.	Results from one way weighted ANOVA by primer for Band Sharing Index.....	38
4.	Results of one way weighted ANOVA for nucleon diversity index.....	40
5.	Roger's genetic distance for RAPD genotypes.....	43
6.	Number of individuals from each site comprising clusters in phenogram.....	46

LIST OF FIGURES

FIGURE

1.	Principle of the polymerase chain reaction based RAPD technique.....	13
2.	Map of East Fork Poplar Creek sampling sites.....	23
3.	Map of Oak Ridge area showing locations of watersheds sampled.....	24
4.	Phenogram generated using neighbor joining method.....	45
5.	Phenogram generated using bands with increased frequency in impacted sites.....	47
6.	Phenogram generated using banding pattern information from OPA15	48

I. Introduction

Environmental Stress Effects

Stressors within an organism's environment may be natural (temperature fluctuations, diminished food supply, salinity change), anthropogenic (overfishing, toxic chemicals or agents, disturbance of natural habitat) or a combination of both which may have cumulative or synergistic effects on exposed organisms (Adams et al., 1992). These stressors can effect the organism at different levels of biological organization. An organism's primary response to stressors is usually at the biochemical or molecular level which may ultimately effect normal functioning of cellular components. Genetic material, enzymes, or cellular membranes may be affected by environmental stressors. Although these initial responses occur rapidly, there may be a long latent period before the resulting alterations are promulgated upward through higher levels of biological organization, potentially inducing structural and functional changes at these higher levels. These induced responses may impair respiration, immune response, hormone production, osmoregulation, or general metabolism, adversely effecting the organism's ability to survive and grow and, most importantly, reproduce. Chronic stress may impair or prevent performance of physiological systems, restrict growth, impair reproduction, leave organisms prone to disease, and reduce the capacity of fish to respond to subsequent stress (Chagnon and Guttman, 1989). Ultimately, the often irrevocable and deleterious effects of such stressors are observed at the population and community levels (Adams et al., 1992; Adams, 1990; Adams et al., 1990).

Because of the rapid response of biological systems to stressors at the lower levels of biological organization, measurement of particular biological endpoints of exposed organisms can be useful as short-term indicators (biomarkers) of the impact of such stress from toxic chemicals (McCarthy and Shugart, 1990; Shugart, 1990). Early detection of exposure to environmental stressors can forewarn investigators of the potential for harmful consequences of stress-induced changes before more deleterious repercussions are seen at the population and ecosystem levels (Adams, 1990; Adams et al., 1990; Jimenez et al., 1990).

Large numbers of anthropogenic chemicals occur in the natural environment where they may impact organisms in a variety of aquatic ecosystems. Lipophilic contaminants are easily absorbed and accumulated in exposed organisms and usually must undergo metabolism to form more hydrophilic compounds in order to be excreted. Metabolism of various xenobiotics is accomplished through a series of enzymatic biotransformation reactions. A major route of biotransformation of lipophilic compounds is via the cytochrome P-450 monooxygenase system. This system is composed of various forms of cytochrome P450 and NADPH-P450 reductase. The cytochrome P450/NADPH-P450 reductase complex oxidizes lipophilic compounds to form a more hydrophilic and, therefore, more readily excretable metabolite. However, in the biotransformation process incomplete detoxification or bioactivation of certain chemicals can lead to highly reactive metabolites which are more toxic than the parent compound (Sipes and Gandolfi, 1991).

Environmental genotoxins, which can act directly or indirectly on the organism, may be lethal or cause sublethal genetic alterations in either somatic or germ cell lines. These genotoxins may act as a selection force to eliminate sensitive genotypes within an exposed population. Invariably, there are individual differences in response to environmental stress which may correlate to the differences in genotypes within a population (Chagnon and Guttman, 1989). Certain genotypes within a population may be able to survive the insult of a particular stress event while other genotypes succumb (Nevo et al., 1986). When this occurs, natural selection for resistant genotypes causes a shift in the genotypic frequencies (Diamond et al., 1989) and an overall narrowing of genetic diversity of the exposed population (Chagnon and Guttman, 1989).

In addition to possible lethal effects, environmental genotoxins may cause alterations to the structure and function of genetic material in some detrimental way (ie. DNA alterations) (Sipes and Gandolfi, 1991). While the majority of chemically induced alterations to DNA in somatic cells are repaired, some are either not repaired or repaired incorrectly and these alterations may be fixed and transmitted to daughter cells (Shugart et al., 1992). Such alterations in the somatic cells may lead to the development of cancer within the exposed individual but can not be passed to further generations. However, mutation in the germ cells may be passed on to future generations and over time may potentially alter the genetic structure at the population level (Sipes and Gandolfi, 1991). Genetic damage caused by these environmental genotoxins is manifested in any number of a series of changes at

various levels in the organism: impaired enzyme function, production of initiators of cytotoxic injuries, impaired immune response, impaired general metabolism, inhibited growth, and decreased fecundity (Depledge, 1994).

Genetic Diversity as a Biomarker

The detection and measurement of various altered biological endpoints may be utilized as biomarkers of exposure to genotoxic agents in the environment (Fore et al., 1995; Shugart et al., 1992). Research suggests that several genetic measures are sensitive to anthropogenic stress and, therefore, have potential use as bioindicators of water quality and health of aquatic populations (Fore et al., 1995; Shugart et al., 1992; Gillespie and Guttman, 1989). Measurement of these selected biological variables permits early detection of environmental perturbation(s) before they cause measurable effects at the community and ecosystem levels. Furthermore, because the physiological condition of the animal integrates the effects of all of its environmental stressors, measurement of these bioindicators may give a more clear and holistic estimation of the condition of the environment (Adams et al., 1990). It has been proposed that biomarkers are more sensitive and ecologically relevant than simply measuring levels of contaminants in the environment (Theodorakis et al., 1992).

The ability of a species to respond to stress largely depends on its genetic variability (Kopp et al., 1994; Thorpe et al., 1981); small genotypic differences in individuals appear to strongly influence susceptibility to stressors (Depledge, 1994).

Within a few years, exposure to anthropogenic chemicals can impose new selection pressures on and cause rapid changes in the genetic structure of a population (Thorpe et al., 1981). A population's genetic diversity may deteriorate due to exposure to mixtures of pollutants (Gillespie and Guttman, 1989; Strittholt et al., 1988) which creates a selection force against sensitive genotypes. Decreased diversity is associated with increased inbreeding which may increase homozygosity of individuals within a population, which increases the probability of expression of harmful recessive alleles and decreases both individual fitness and the population's fitness to survive further environmental perturbation (Depledge, 1994; Diamond et al., 1989; Thorpe et al., 1981). While a population's diminished genetic diversity may be sufficient for it to survive in its present environmental conditions, future changes in environmental conditions such as occurrence of other stressors may jeopardize the survival of that population. Therefore, the degree of genetic plasticity a population maintains may be evidence of its capacity to survive future environmental alterations (Chagnon and Guttman, 1989; Gillespie and Guttman, 1989).

Because it provides greater genetic plasticity for populations to respond to a greater variety and magnitude of environmental perturbations (selection forces) and increases its chances of surviving future stressors, a high degree of genetic diversity may be important to the survival and adaptability of fish populations exposed to pollutant stressors. Greater genetic variability is adaptive or potentially adaptive (Chagnon and Guttman, 1989; Woods et al., 1989). The genetic diversity

of a population may, therefore, temper the stress related effects of pollutants and provide a mechanism of tolerance. Genetically diverse populations have an advantage because they have a larger number of resistant individuals with a higher probability of surviving stress from environmental alterations (Woods et al., 1990; Chagnon and Guttman, 1989; Gillespie and Guttman, 1989). Genotypes which survive may represent those most tolerant of environmental stressors (Kopp et al., 1994).

Numerous field and laboratory studies have been conducted to investigate pollutant effects on various aquatic species, and several of these studies indicate a reduction of genetic diversity due to anthropogenic factors. For example, Chagnon and Guttman (1989) used acute exposures to copper and cadmium to test metal sensitivity of allozyme variants in a polymorphic population of mosquitofish (*Gambusia affinis*) by measuring the survival of individuals with different genotypes. They found copper caused selective mortality on individuals with specific genotypes. This pattern was also seen for cadmium; however, different alleles and different genotypes were found to be sensitive. This strongly directed selection may cause decreased genetic variability within exposed populations, and the authors propose that changes in the frequency of sensitive variants in natural populations may be useful as biomarkers of environmental pollution. Nevo et al. (1986) also found a positive correlation between increased survivorship when confronted with the stress of multiple inorganic and organic pollutants and increased genetic diversity as measured by levels of population heterozygosity. In general,

multilocus heterozygotes displayed a higher relative fitness and pollutant resistance than did homozygotes. Gillespie and Guttman (1989) investigated the genetic structure of populations of central stonerollers (*Campostoma anomalum*) in a contaminated and a reference stream and found a difference in frequency of particular genotypes between the populations attributable to pollutant exposure. Therefore, they report that shifts in allele and genotype frequencies in central stonerollers are sensitive bioindicators of environmental contamination. Strittholt et al. (1988) documented the impact of years of pollutant exposure (specifically mercury, zinc and copper) on populations of yellow perch (*Perca flavescens*) in Lake Erie and other Ohio populations. Extremely low levels of genetic variability in exposed populations in comparison to reference populations was attributed to pollutant exposure. With this reduction of genetic variability, yellow perch in Lake Erie may be unable to adjust to further environmental perturbations, may become more vulnerable to additional environmental stressors, and may ultimately disappear from this ecosystem. Diamond et al. (1989) found time to death (TTD) for mosquitofish (*Gambusia affinis*) exposed to mercury positively correlated to degree of genetic polymorphism at specific loci illustrating selection for individuals possessing pollutant resistant genotypes.

Molecular Measures of Genetic Diversity

Assessment of genetic diversity of populations may be useful as a tool for biological monitoring of environmental pollution. Various molecular markers have

been used to assess genetic diversity including polymorphisms in proteins and DNA. Electrophoretic analysis of allozyme variants has been used by numerous researchers to assess genetic diversity; however, new techniques exploit DNA polymorphisms as molecular markers for this analysis. Allozyme analysis is relatively straightforward, inexpensive, and easy. Additionally, allozyme markers are codominant, meaning that heterozygotes are distinguishable from homozygotes, which is necessary for estimating allele frequencies in population genetic studies (Weising et al., 1995). Allozyme markers represent coding portions of DNA which may be relevant to an organism's fitness in its present environment. Because selection acts on phenotypes, in biomonitoring studies in which selection is an important factor, allozyme markers representing transcribed gene products, may be suitable biomarkers. However, allozyme analysis has limited usefulness in that it lacks sensitivity. Specifically, it does not permit the detection of minor genetic variations as would be necessary when analyzing closely related populations. Alteration of the amino acid sequence only indirectly reflects a change in the nucleotide sequence. Additionally, allozyme markers are not always indicative of changes at the DNA level; substitution of bases in the nucleotide sequence do not necessarily alter the amino acid composition. Furthermore, a change in the amino acid sequence of a particular protein may not change the electrophoretic mobility (related to the size and the charge of the molecule) of that protein such that no difference in banding pattern would occur producing an comigratory allele indistinguishable from the original protein (Woods et al., 1990).

DNA markers offer the most direct route toward measurement of genetic diversity. DNA markers, which represent an unlimited number of detectable polymorphisms, reveal differences directly at the DNA level in both coding and noncoding regions. DNA is relatively stable and may be stored for extended periods. A variety of techniques for analyzing DNA polymorphisms have been developed (Weising et al., 1995) based on polymorphisms of mitochondrial or nuclear DNA (Hallerman and Beckman, 1988).

Polymorphisms in DNA markers can be analyzed by several means including restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA, RFLPs of nuclear DNA, and the fairly new technique of polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD). The appeal of mtDNA analysis is the ease of purification of mtDNA compared to nuclear DNA. Conventional mtDNA analysis uses restriction endonucleases to cleave mtDNA at various selected recognition sites (Hallerman and Beckman, 1988). The resulting restriction fragments are separated by gel electrophoresis and restriction fragment length polymorphisms (RFLPs) due to the gain or loss of restriction enzyme recognition sequences are viewed as different sized DNA fragments (Weiss et al., 1995; Ferris and Berg, 1987). The advantages of mtDNA analysis include the relatively small molecular weight of mtDNA with highly conserved gene order among vertebrates, maternal inheritance of mtDNA, genes are closely packed with no introns meaning essentially all mtDNA except the D loop represents coding sequences, and mtDNA evolves five to ten times as rapidly as nuclear DNA. This

analysis is limited by the need to know the sequence of interest before selecting the proper restriction endonuclease. Additionally, a limited number of restriction sites are available within the mtDNA (Ferris and Berg, 1987).

RFLPs of nuclear DNA use restriction enzymes to digest DNA molecules at specific sequences generating a reproducible set of fragments of specific lengths which are separated by gel electrophoresis. RFLPs are viewed by hybridization of a specific probe to homologous DNA sequences (Hallerman and Beckman, 1988). Point mutations, insertions, and deletions in the DNA sequence result in an altered pattern of restriction fragments causing discernable polymorphisms between different genotypes (Weiss et al., 1995). RFLP markers have the advantage of being codominant. RFLPs are limited in that the sequence of interest must be known in order to cleave DNA in the proper region (Hallerman and Beckman, 1988) and a limited number of gels can be assessed as a limited number of restriction endonucleases are available. Additionally, RFLP assays require large amounts of relatively pure DNA, are fairly laborious and expensive, and often use radioisotopes for detecting polymorphisms (Dawson et al., 1993; Chalmers et al., 1992).

RAPDs, developed by Williams et al. (1990) and Welsh and McClelland [AP-PCR] (1990), utilize PCR with arbitrary short primers. RAPD assays are relatively quick and easy to perform. The appeal of the RAPD technique is the simplicity of the procedure and its requirements for only small quantities of DNA. Even degraded DNA can be used for RAPDs because only a small amount of target DNA must still be intact to produce copies of the target sequence (Arnheim et al., 1990; Saiki,

1990). No prior knowledge of the genome being analyzed is necessary, and a large number of commercially available primers can be used. The RAPD technique randomly samples the genome detecting variation in both coding and noncoding regions (Bachman, 1994; Lynch and Milligan, 1994; Liu and Funier, 1993).

However, RAPDs do have several limitations. One is that impurities may contaminate samples and produce altered banding patterns (Bachman, 1994). Secondly, nearly all (95%) RAPD markers are dominant limiting the ability to distinguish heterozygotes (marker/null) from homozygotes (marker/marker) with respect to a dominant RAPD marker (Lynch and Milligan, 1994; Williams et al., 1990). Therefore, it is difficult to calculate allele frequencies using RAPD data. The RAPD technique randomly samples the genome and the resulting RAPD markers represent both coding and noncoding portions of DNA, the majority being from noncoding regions. Markers from noncoding regions are unlikely to have selective significance. Finally, there is no assurance of homology of RAPD markers (Bachman, 1994; Lynch and Milligan, 1994; Liu and Funier, 1993); that is to say, that comigratory products, products migrating together during gel electrophoresis due to identical electrophoretic mobility, may contain internal sequence variations or be length alleles. However, homology can be verified using Southern blot technique. When reaction conditions are rigidly standardized, careful attention is given to avoiding cross-contamination of samples, and reactions are run in duplicate, reliable and consistent banding patterns can be produced (Hadrys et al., 1992).

The RAPD Technique

In this study, DNA polymorphisms are examined using the RAPD technique. The RAPD technique utilizes the polymerase chain reaction (PCR) to detect DNA polymorphisms which may function as genetic markers. PCR is an *in vitro* enzymatic amplification reaction which produces exponential accumulation of targeted genomic DNA fragment(s) (Arnheim and Erlich, 1992; Erlich et al., 1991; Arnheim et al., 1990; White et al., 1989). Automated thermal cyclers amplify target DNA fragments through repeated cycles of denaturation, annealing, and extension. First DNA is denatured, then two oligonucleotide primers anneal to opposite strands of the target sequence such that their 3'OH ends face each other, and finally DNA polymerase extends the annealed primers through the target region (Bachman, 1994; Erlich and Arnheim, 1992; Arnheim et al., 1990; White et al., 1989; Saiki et al., 1988). The target fragment (s) are replicated and may serve as a template for the next cycle(s). Unlike the conventional PCR process, the RAPD technique utilizes a single short primer of arbitrary nucleotide sequence (Williams et al., 1990) under reduced stringency; a lower annealing temperature is used (Erlich and Arnheim, 1992). See Figure 1 for an outline of the principle of the PCR based RAPD technique. The primer anneals to randomly distributed priming sites on the double stranded DNA enclosing a relatively short stretch of DNA (up to a few thousand nucleotide pairs) and amplifies target DNA sequence(s) in the absence of specific nucleotide sequence information (Bachman, 1994; Erlich and Arnheim, 1992; Hadrys et al., 1992; Williams et al., 1990). The resulting amplification

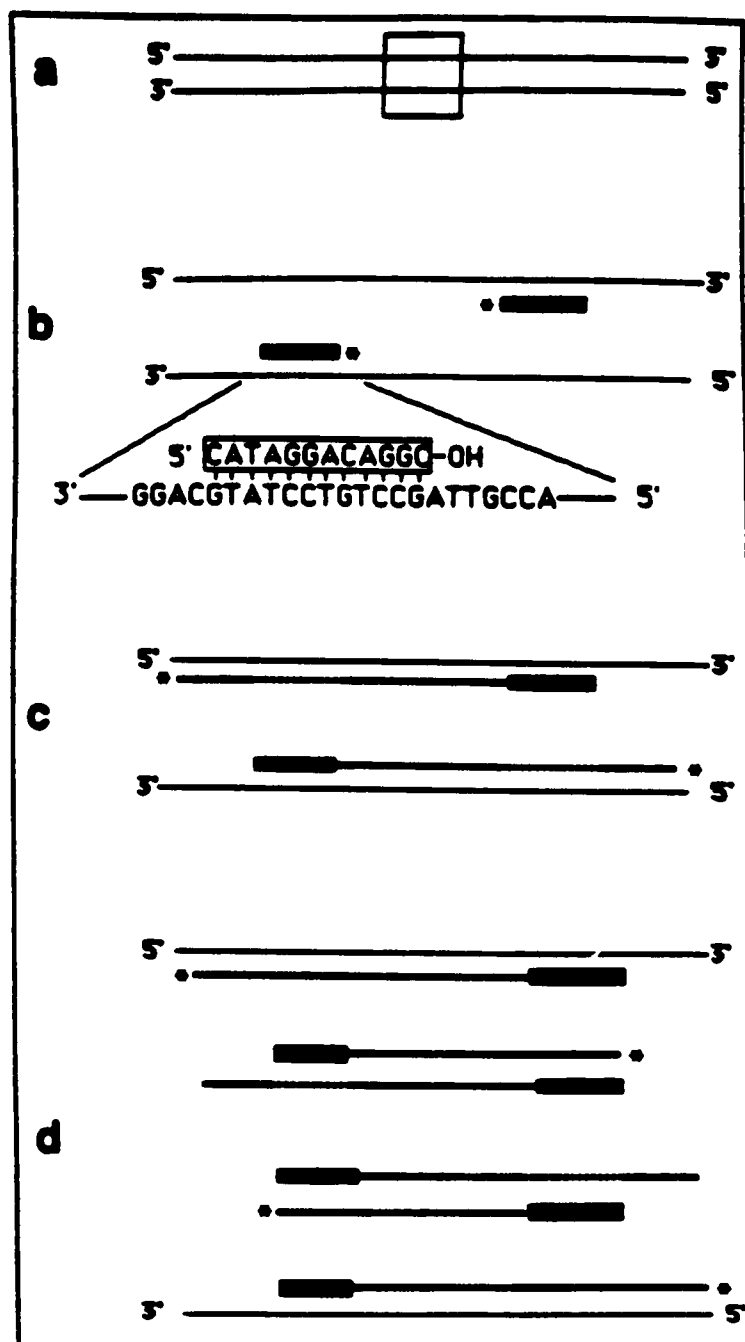


Figure 1. Principle of the polymerase chain reaction based RAPD technique. **a.** The target sequence within a double-stranded DNA molecule is indicated with a box. **b.** After DNA denaturation, the RAPD primer anneals to the sequences flanking the target region. The 3' end of each primer is denoted by an asterisk. Elongation by DNA polymerase proceeds in the 5' to 3' direction. Details of the base pairing between primer (boxed) and DNA strand are given. **c.** DNA polymerase extends the primers. The region complementary to the primer is given by a broken line. **d.** In the second cycle of PCR, each of the four strands of DNA shown in c anneals to a primer, which is subsequently extended (Adapted from Arnheim et al., 1990).

products can be resolved on 1.5% agarose gels by gel electrophoresis and polymorphisms used as genetic markers (Hadrys et al., 1992; Williams et al., 1990).

Study Sites

EFPC is a third order stream and has its headwaters near the Oak Ridge Y-12 plant, a US Department of Energy (DOE) nuclear weapons production facility from which it receives industrial effluent (Theodorakis et al., 1992). The headwaters of EFPC originate above Lake Reality at the Y-12 plant and the stream flows through the city of Oak Ridge and the Oak Ridge DOE reservation before joining Poplar Creek 23.7 km downstream. The stream changes from a first to fourth order over its 23.7 km length. The depth of the stream varies from 2 cm in shallows and 1.7 m in pools and the width ranges between 2-8 m (Gatz and Adams, 1994). Reference streams were selected for their hydrological and physical similarity to EFPC. Brushy Fork and Hinds Creek are similar in flow, depth, width, and physiochemical characteristics but are located in separate watersheds (Jimenez et al., 1990). Brushy Fork joins Poplar Creek several miles upstream from where EFPC enters Poplar Creek. An internal reference could not be sampled as EFPC's headwaters originate at the source of pollution. Gatz and Adams (1994) have documented that there is little net movement of fish in EFPC assuring sampling of the same fish populations each year at each site.

Until November 1988, the headwaters of EFPC originated from the 2.2-ha impoundment called New Hope Pond (NHP). However, in 1988 NHP was drained

and the sediment removed, and the impoundment was filled and capped. A new settling and treatment basin, Lake Reality, has now replaced NHP. From Lake Reality to its confluence with Poplar Creek, EFPC is approximately 23.7 km in length with the section between EFK (East Fork kilometer) 22.7 and EFK 7.7 located off the DOE reservation in the town of Oak Ridge. According to Oak Ridge National Laboratory's (ORNL) First Report on the Y-12 Plant Biological Monitoring and Abatement Program (BMAP) (Hinzman et al., 1993), effluent discharges of 388 L/s from the Y-12 plant at EFK 23.7 and 227 L/s from Oak Ridge Wastewater Treatment facility at EFK 13.4 together constitute 39% of EFPC mean annual flow at EFK 5.3. The section of EFPC located off the DOE reservation also receives agricultural runoff. Since 1992, however, the water flow in EFPC has been reduced due to decreased waste stream flow at the plant. Water and sediment in EFPC are contaminated with metals, organic chemicals, and radionuclides released from the Y-12 plant. Pollutants include ten priority pollutant organics (including PAHs, PCBs, and total phenol), metals (As, Cd, Pb, Hg, Ni, Ag, Zr), copper, ammonia, oil and grease, perchloroethylene and residual chlorine (Hinzman et al., 1993; Jimenez et al., 1990).

According to ORNL's First and Third BMAP reports (Hinzman et al., 1993; Hinzman et al., in press) mercury and PCB concentrations in fish collected from EFPC between May 1986 and May 1991 were elevated above levels in fish collected from a reference stream (Hinds Creek). EFPC has a distinct downstream gradient in contaminant loading and a similar decreasing concentrations of mercury

and PCB in sunfish populations progressively further downstream from Lake Reality (Adams et al., 1992). Although mercury in all EFPC sites is predominately particle-associated inorganic mercury and inorganic mercury accumulates in small proportions in redbreast sunfish, the predominant form of mercury found in fish from EFPC is methylmercury (Southworth et al., 1995). For the sampling period of May 1986 to May 1988, 30% of the fish in EFPC exceeded the USFDA (United States Food and Drug Administration) tolerance limit for mercury of 1 mg/g Hg averaging 0.92 mg/g total mercury, an 11-fold increase above the 0.08 mg/g observed in fish from reference streams. Although abnormally high concentrations of mercury were still found for the sampling period of December 1988 to May 1991, the average concentration of total mercury in redbreast sunfish was slightly smaller at 0.68 mg/g. Mercury concentrations followed a downstream decrease from EFK 23.4 until May 1990 whereafter the highest concentrations of mercury were found at EFK 18.3. The decrease in mercury concentrations in exposed sunfish is concurrent with the decrease in the mass of mercury discharged from the Y-12 plant via EFPC over the last few years. While below the FDA limit of 2 mg/g for all sites except a small portion of EFK 23.4, PCB concentrations in EFPC redbreast sunfish were elevated over that observed in fish from reference sites. EFPC sunfish ranged from 0.82 mg/g PCB at EFK 23.4 to 0.18 mg/g at EFK 2.1 (Southworth, 1990) and averaged 0.46 mg/g PCB, while fish from Hinds Creek averaged 0.04 mg/g. Data from the first and third BMAP reports (Hinzman et al., 1993; Hinzman et al., in press) suggests that a continuing though episodic source of PCB input exists upstream

from EFK 23.4 (Southworth, 1990).

In addition to these chronic low level exposures, fish in EFPC are subject to episodic contaminant loading from point sources in the upper sections of the stream and from nonpoint sources when contaminants are mobilized from the floodplain sediments during heavy rainfall (Adams et al., 1992). This contaminant exposure is believed to be detrimental to fish health. Fish closest to the headwaters of EFPC display the poorest health which is attributed to toxicological exposure evidenced by high levels of detoxification enzymes, metallothionens, and DNA damage (Hinzman et al., 1993; Hinzman et al., in press).

Total residual chlorine (TRC) concentrations were very high near the headwaters of EFPC before December 1992 generating a level of toxicity which permitted only small, transitory populations of fish in these waters (Jimenez et al, 1990). However, in November 1992 a system was installed to reduce concentrations of TRC to below toxic levels which resulted in rapid colonization of these waters with fish (Southworth et., 1995). A reduction of species diversity in benthic invertebrates and of species richness in fish attributed to toxicological exposure decreases downstream from Lake Reality (Jimenez et al, 1990).

Some of the changes in EFPC following remediation efforts may be reflected in the results of a study conducted by Etnier et al. (1994) summarized here. From 1988 to 1993, EFK 23.4 and the reference site Brushy Fork were sampled for measures of species richness (number of species of fish present), fish density (number of fish per square meter of surface), and total fish biomass (total weight of

fish per square meter of surface). Following opening Lake Reality in 1988, the upper reaches of EFPC above EFK 23.4 were colonized with fish from downstream until about 1991 at which point reproduction rather than colonization became the mechanism of population growth. Over the four year study, Brushy Fork consistently showed a higher species richness than EFK 23.4. Some fish are unable to live near the headwaters of EFPC due to sensitivity to the numerous pollutants found there. The small number of species at EFK 23.4 suggests higher pollutant stresses which would make this area uninhabitable for species of low tolerance (Etnier et al., 1994). EROD (ethoxyresorufin-O-deethylase) values, indicative of exposure to chemicals, were high at EFK 23.4 (S.M. Adams, Oak Ridge National Laboratory, personal communication). After November 1992 EFPC experienced a significant decrease in water flow which resulted in a decrease in food availability and a significant decrease in sunfish per unit area. Although the density of sunfish decreased following November 1992, the overall fish density and total fish biomass at EFK 23.4 was found to be higher than that for Brushy Fork. During the March-April 1993 sampling period, approximately 93% of fish at EFK 23.4 were found to be striped shiners (*Luxilus chrysocephalus*) and stonerollers (*Campostoma anomalum*) (Etnier et al., 1994).

EFPC Pollutants

Of the major pollutants in the EFPC watershed, mercury appears to be of the most concern relative to its potential toxicity. Mercury compounds (elemental,

inorganic, and organic) represent environmental pollutants which produce highly toxic effects in exposed organisms. From 1950 to 1963 elemental mercury was used extensively at the Y-12 plant for weapons production and subsequently 89 000 kg mercury entered the EFPC watershed (Talmage and Walton, 1993) and represents the largest pollutant hazard for this site. Mercury is present in EFPC in various forms including inorganic mercury and low concentrations of methylmercury (Southworth et al., 1995). Elemental and organic mercury are lipophilic and accumulate in fat stores of organisms and also effects the central nervous system where they act as potent neurotoxins (Miura et al., 1995; Clarkson, 1993). Specifically, methylmercury is highly lipophilic and is accumulated in exposed organisms with the highest concentrations being found in long lived top predatory fish, as seen in redbreast sunfish (Southworth et al., 1995). Inorganic mercury, however, is less lipophilic and more readily excretable. Inorganic mercury and methylmercury may accumulate in the kidney where they cause nephrotoxicity (Miura et al., 1995; Clarkson, 1993).

Mercury's mechanism of cellular toxicity is not well understood but may be due to depletion of reduced glutathione, generation of oxygen radicals, and lipid peroxidation (Miura et al., 1995; Cantoni et al., 1984; Cantoni and Costa, 1983). Superoxide anions have been shown to react with hydrogen peroxide forming hydroxyl radicals which potentially induce single strand breaks in DNA. These radicals have been implicated in the production of DNA breakage in mercury induced DNA cleavage (Costa et al., 1991; Cantoni et al., 1984). Both inorganic and

organic mercury have been shown to react with purified DNA (Ariza et al., 1994), and mercury (II) compounds have been demonstrated to bind to DNA in intact cells resulting in single strand breaks and low levels of chromosomal aberrations (Ariza et al., 1994; Burkart and Ogorek, 1986). Mercury binds very strongly to DNA (Costa et al., 1991) particularly to A:T rich DNA where it binds to the ring nitrogen (N3) in thymidine. Here it chelates between base pairs forming strong bonds to the nitrogen atoms in a linear configuration (Ariza et al., 1994; Cantoni et al., 1984).

Although Cantoni and Costa (1983) suggest that single strand breaks in DNA may be indirectly mediated by the generation of oxygen radicals, Costa et al. (1991) reports that the quantity of oxygen radicals generated by mercury does not appear sufficient to account for the high degree of strand breaks. The mechanism of cytotoxicity of mercury may lie in the ferocity with which it binds to DNA. Costa et al. (1991) suggests that because mercury binds so strongly to DNA and the bound mercury prevents repair of DNA, mercury induced DNA damage is irreparable and ultimately lethal to the cell. In view of this mechanism of DNA damage and repair inhibition, the mutagenicity and carcinogenicity of mercury is unlikely (Costa et al., 1991).

Research Objectives

Redbreast sunfish (*Lepomis auritus*) in EFPC have been exposed to toxic chemicals and, therefore, provide an opportunity to investigate possible alteration of population genetic diversity. RAPD analysis will be applied to determine whether

genetic structural differences exist between exposed and reference populations. Numerous oligonucleotide primers will be applied to scan the genome for polymorphisms. Polymorphisms which indicate significant differences between impacted and reference populations will be identified for potential future use as biomarkers of exposure to similar pollutants in other redbreast populations.

The objectives of this study are two-fold. The first is to determine whether the RAPD technique is an effective tool in determining changes in population genetic structure. The second is to determine using the RAPD technique whether chemically exposed redbreast sunfish in EFPC have altered genetic diversities from those collected in reference streams. The primary hypothesis (H_1) is that genetic diversity will decrease in exposed populations. A secondary hypothesis (H_2) is that certain genotypes will have a selective advantage in contaminated environments and the frequency of these genotypes will be greater in contaminated populations.

II. Materials and Methods

Specimen Collection

Six populations of redbreast sunfish (*Lepomis auritus*) from six sample sites were examined for possible changes in genetic diversity: four populations from East Fork Poplar Creek (EFPC) and two from reference streams (Hinds Creek and Brushy Fork). Redbreast sunfish were selected as the study organism because they are the only species which are present in sufficient numbers at all sites and are also collected for use in concurrent biomarker studies in this laboratory. (Adams, personal communication).

During the spring sampling periods of 1994 and 1995, fish were collected by electrofishing at four sites along EFPC : at EFK 23.4 (Below Lake Reality), EFK 18.3 (Rocky Top), EFK 13.8 (Jackson Farm), and EFK 5.3 (Gauging Station). See Figure 2 for location of sampling sites within EFPC watershed and Figure 3 for location of all watersheds sampled. For this study, a total of 410 fish were sampled; approximately 70 fish were sampled at each site except EFK 23.4 where due to low density only 49 fish were sampled.

DNA Extraction

Upon capture, fish were bled via caudal vein puncture and blood was collected using EDTA-coated Vacutainer tubes to prevent clotting of blood samples. Samples were stored on ice until they could be transported to the lab where blood samples were centrifuged at 5000 rpm for 5 min and plasma samples were

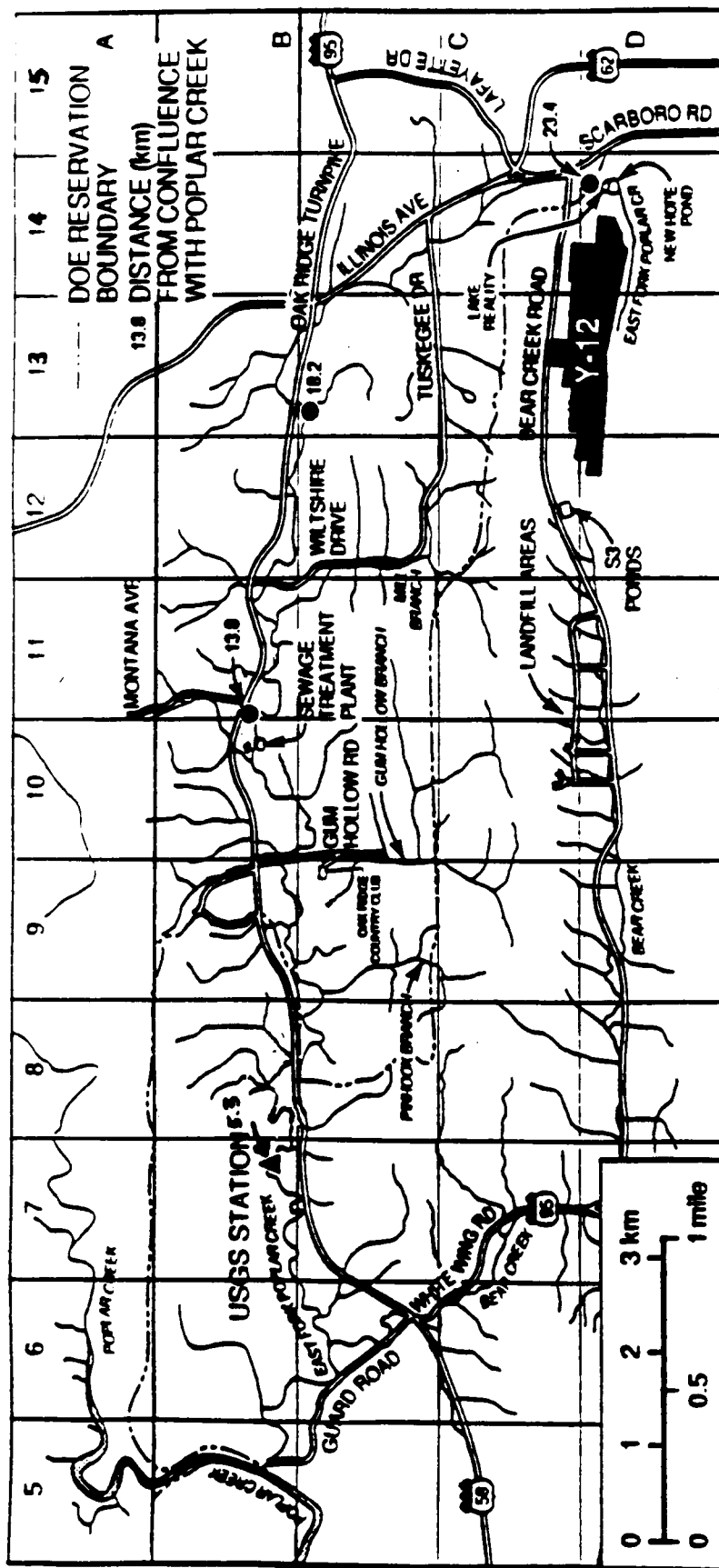


Figure 2. Map of East Fork Poplar Creek sampling sites. EFK 23.4 (Below Lake Reality), EFK 18.3 (Rocky Top), EFK 13.8 (Jackson Farm), and EFK 5.3 (Gauging Station) (Hinzman et al., 1993).

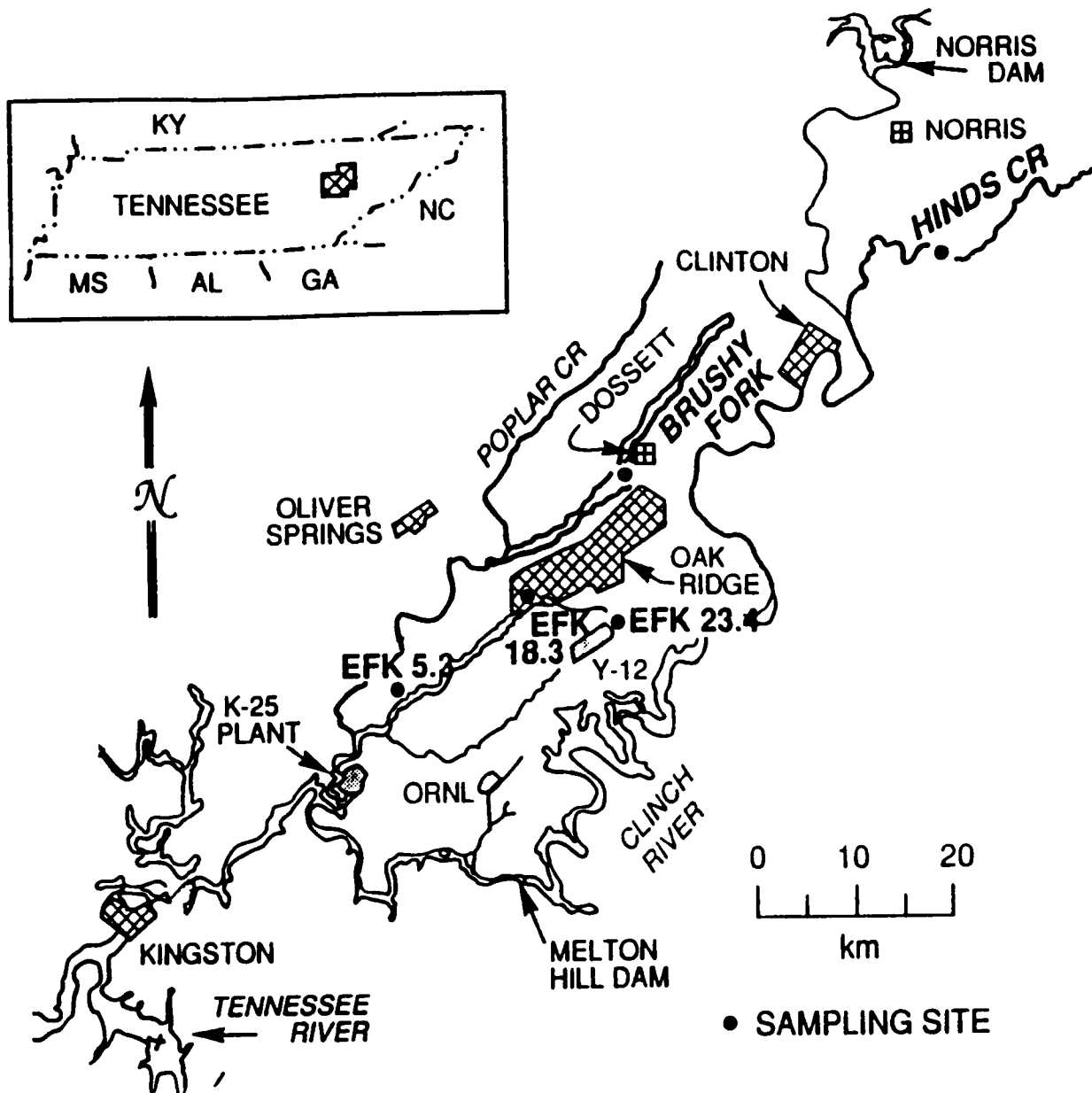


Figure 3. Map of Oak Ridge area showing locations of watersheds sampled. Reference watersheds are Brushy Fork and Hinds Creek and the impacted watershed is East Fork Poplar Creek (Hinzman et al., 1993).

removed. Fish physiological saline (FPS: 6.44g NaCl, 11 mg KCl, 22 mg CaCl₂, 12 mg MgSO₄, 7 mg KH₂PO₄, and 10 mg NaHCO₃ per liter in distilled water) was used to dilute the remaining blood samples. Blood samples were washed twice and suspended in FPS. Red blood cells were lysed using 10% sodium dodecyl sulfate (SDS) followed by two phenol:chloroform:isoamyl alcohol (25:24:1) extractions to remove cell membrane material and protein fractions. DNA was precipitated using 0.25 M NaCl and ethanol, washed in 80% ethanol, and redissolved in TE (10 mM Tris-HCl, .01mM EDTA, 0.15 M NaCl, pH 8.0). Digestion with proteinase K (200µg/ml) was carried out for one hour in a 50°C water bath followed by a phenol:chloroform:isoamyl alcohol (25:24:1) extraction. An additional chloroform extraction was performed to enhance DNA purity and remove extraneous phenol. DNA was again recovered by ethanol precipitation and dissolved in TE as before. DNA concentrations were measured using the TKO mini-fluorometer (Hoeffer Scientific Instruments, San Francisco) against known DNA concentration standards and DNA stock solutions were diluted to 3.6 µg/ml in sterile water and stored at -70° C until analysis.

RAPD Analysis

For RAPD analysis, amplification reactions were carried out in a DNA thermal cycler GeneAmp PCR System 9600 (Perkin-Elmer Corp. , Norwalk, CT). Reactions were carried out in a volume of 15 µl. The reaction mixture consisted of 2.5 mM

MgCl₂, 100 mM of each nucleotide triphosphate, 3 pmoles primer, 27 ng DNA, 0.6 unit Taq polymerase (Promega Corporation (Madison, WI), 10mM Tris-HCl, pH 8.3, 50 mM KCl, and 0.1 mg of bovine serum albumin (BSA). Primers were obtained from Operon Technologies (Alameda, CA). The reaction mixture was overlaid with mineral oil to prevent evaporation. For biomonitoring a single protocol was used so as not to introduce variation. Following a series of optimization experiments in which concentrations of template DNA, primer, dNTPs and amplification parameters were varied, a protocol was developed which optimized reaction conditions and amplification program parameters and produced reliable and distinct banding patterns for all primers used.

This protocol includes the addition of BSA to the reaction mixture prior to amplification. The desired outcome would be two fold: first, BSA would prevent the polymerase enzyme from binding to the reaction tube (Wittwer et al., 1994); second, BSA would stabilize the polymerase enzyme enabling it to continue functioning through more cycles increasing the amount of amplified product and therefore the intensity of the resulting banding pattern. With the addition of BSA to the reaction mixture, an amplification protocol with 55 cycles produced a more clear and pronounced banding pattern.

The amplification protocol consisted of 1 min. at 95°C followed by 55 cycles of 20 sec at 94°C, 30 sec at 37°C, and 2 min at 72°C. Each cycle was shortened compared to Williams' (1990) protocol to decrease assay time while maintaining specificity and reproducibility. Not only did this decrease amplification time but also

improved the performance of the polymerase enzyme allowing it to remain biologically active through more cycles and produce better banding patterns. The denaturation time was reduced while maintaining intensity and reproducibility of amplification products. The annealing time is important to the specificity of the reaction as longer annealing times may lead to increased misincorporations. A decreased annealing temperature is used due to the short primers but also may decrease specificity of annealing. Therefore, it was found a shorter annealing time of 30 s produced a more clear and intense banding pattern. The size and intensity of amplification products was maximized at a 2 min extension time. Decreased extension times produced streaking.

Following amplification, samples were either stored at 4°C for short periods of 1 to 2 days or -20°C for longer periods until gel electrophoresis could be performed. The samples were electrophoresed in a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide and run at 5 V/cm. Molecular length standards (bacteriophage ϕ X174 DNA digested with *Hae* III) were run with samples for relative determination of molecular length. Gels were run to produce maximum separation of bands which required various lengths of time depending on the banding patterns produced. Consistently sharp and reproducible bands were selected for scoring while bands which were faint or not reproducible over several runs were not considered for scoring. Banding patterns were confirmed by repeating assays for each primer. Band patterns were visualized using ethidium bromide staining and UV illumination. The gels were documented by photography.

For this analysis, the presence or absence of bands was scored visually.

Analytical Procedures

Genetic diversity was determined by using methods conventionally employed for other DNA fingerprinting methods, specifically RFLPs but adapted for use in RAPD analysis. Two methods were used to calculate genetic diversity using programs created by Theodorakis (1994) from the formulas of Lynch (1990) and Nei (1987). The first method involves calculating the fraction of bands which are shared between individuals in a population to approximate the average identity-in-state for random pairs of individuals. The band sharing index (BSI) (Lynch, 1990) uses the formula:

$$BSI_{ij} = 2S_{ij} / (N_i + N_j)$$

where S_{ij} is the number of bands shared by individuals i and j , and N_i and N_j are the number of bands displayed by individuals i and j , respectively. The BSI was calculated for all i, j pairs and averaged within the population. The value of the index may range between 0 and 1 indicating that all individuals are completely different or completely alike with reference to their banding patterns, respectively. Using the mean BSI and its associated variance for each population, a linear regression was performed for each primer to investigate any distance effect between the four impacted populations from EFPC similar to the gradient contaminant loading mentioned above. A z test was performed to determine any significant difference between reference populations with respect to BSI. The

statistical significance of the difference in BSI between impacted sites and reference sites was determined by a one way weighted ANOVA.

The second method used to calculate genetic diversity was also developed for use with RFLPs: the nucleon diversity index (h) (Nei, 1987). This is calculated according to the formula:

$$h = [(n / (n - 1))] (1 - \sum_{i=1}^m x_i^2)$$

where n is the sample size, x_i is the frequency of the i^{th} genotype (banding pattern), and m is the number of genotypes in the population. A variance of this index was calculated for each primer and used to perform a linear regression to investigate any distance effect between impacted populations within EFPC. A z test was performed to determine any significant difference between reference populations with respect to nucleon diversity. The statistical significance of the difference in nucleon diversity between impacted sites and each reference site was determined by one way weighted ANOVA.

Genetic distances between populations were also calculated using a method developed for allozyme assays but adapted here for our purposes. The formula for Roger's genetic distance (Nei, 1987) was used for this calculation:

$$D_{ij} = (1 / n) \sum_{k=1}^n (x_{ik} - x_{jk})^2$$

where n is the number of bands scored, x_{ik} is the frequency of the k^{th} band in population I and x_{jk} is the frequency of the k^{th} band in population J. D_{ij} takes on

values between 0 and 1 when the populations are completely similar and completely different, respectively. Using a program containing this equation created by Theodorakis (1994), distances were calculated for each pair of populations. Distances were calculated using genotypes generated from all primers and the differences between different pairwise comparisons were examined.

RAPDPLOT (Kambhampati et al, 1992), a computer program which calculates a band sharing index, was used to create an input file for the NEIGHBOR program of PHYLIP 3.5c (Felsenstein, 1993). The formula for band sharing used here is

$$M = NAB/NT$$

where M is the fraction of shared bands, NAB is the total number of matches in individuals A and B , and NT is the total number of loci scored. Unlike BSI (Lynch 1990) used earlier, the denominator here is fixed and the absence of a band (representing the recessive phenotype at a locus) is scored. M can range from 0 to 1 indicating completely different and completely identical banding patterns, respectively. NEIGHBOR utilizes the neighbor joining method of Saitou and Nei (1987) in which genetic distance computed for all pairs of individuals is used to generate an unrooted phenogram. According to the DRAWGRAM program documentation, the term phenogram is used to designate a pictorial style of representing the relationship between individuals. It makes no implications to considering these diagrams as genealogies or phenetic clustering diagrams. Phenograms were assessed to determine the proportions of individuals from various

sites constituting clusterings of similar genotypes. Phenograms were plotted for all populations sampled using the DRAWGRAM program of PHYLIP (Felsenstein, 1993).

As it is hypothesized that pollutant sensitive genotypes will decrease in frequency and pollutant resistant genotypes will increase in frequency in contaminated environments (Chagnon and Guttman, 1989; Gillespie and Guttman, 1989), certain banding patterns which displayed significantly different frequencies across impacted sites were used for further analysis using the RAPDPLOT neighbor joining method. A group of bands which displayed increased frequency in impacted sites further downstream from EFK 23.7 were used to generate a phenogram. These bands are OPD12₁₁₆₀, OPA1₁₅₂₅, OPA9₁₀₅₀, OPA18₁₀₇₀, OPA18₅₈₀. This phenogram was analyzed for clusterings of individuals with similar genotypes which increased in frequency in impacted sites as compared to reference sites. Additionally, a phenogram was generated using primer OPA15 because of the highly significant difference in band sharing index between reference sites and impacted sites for this primer.

III. Results

RAPD Analysis

In this study a total of 410 fish were examined. Sample sizes for each population are 49 fish at EFK 23.4, 71 fish at EFK 18.3, 74 fish at EFK 13.8, 75 fish at EFK 5.3, 73 fish from Brushy Fork, and 68 fish from Hinds Creek.

Forty different primers, which included the OPD and OPA sets, were initially screened for polymorphic banding patterns. Of these, thirteen were selected for use based on production of polymorphic banding patterns in at least one population and the production of distinct, reproducible banding patterns. Primers which produced faint bands or inconsistently produced bands in repeated assays were not selected for further use in this analysis. The results using the remaining thirteen primers are reported here.

The nomenclature for each primer is based in part on an abbreviation for the name of the company which manufactured the oligonucleotide followed by a number assigned by the company to designate each primer. Operon technologies produces sets of 20 separate 10 base pair oligonucleotide primers and each set is given a letter and a number designation. For example, set D was used in this experiment with primers named OPD1, OPD2,... OPD20. Each primer has a unique nucleotide sequence (Table 1) and produced different banding patterns. Bands are designated by which primer produced it and by its molecular length relative to the ϕ X174/Hae III molecular length standard. For example, band OPB3₅₈₀ would be produced from primer OPB3 and would represent a 580 nucleotide fragment.

Table 1. Decamer oligonucleotides used as random primers, their sequences and variability.

Primer	Sequence 5' to 3'	Polymorphic Bands	Monomorphic Bands	Total Bands
OPD3	GTCGCCGTCA	4	3	7
OPD5	TGAGCGGACA	6	8	14
OPD8	GTGTGCCCCA	3	4	7
OPD11	AGCGCCATTG	5	2	7
OPD12	CACCGTATCC	2	1	3
OPD15	CATCCGTGCT	2	0	2
OPD16	AGGGCGTAAG	2	3	5
OPA1	CAGGCCCTTC	3	4	7
OPA2	TGCCGAGCTG	2	4	6
OPA9	GGGTAACGCC	4	1	5
OPA11	CAATCGCCGT	2	1	3
OPA15	TTCCGAACCC	3	3	6
OPA18	AGGTGACCGT	6	0	6

Total: 44 34 78

The total number of bands produced in impacted sites was not different from the total number produced in reference sites for each primer. However, some bands which were produced in impacted sites were not seen in one of the reference sites. For example, OPD5_{1400,1380} were present in impacted sites and Hinds Creek samples but were absent for Brushy Fork samples. OPA11₁₅₂₅ and OPA18₅ were produced in all sites except Hinds Creek.

Band Frequency

There were a total of 78 bands produced by the 13 primers for fish from all sites. Forty-four of the bands produced were polymorphic and thirty-four were monomorphic in all populations. The approximate molecular lengths and frequency of each of the bands is indicated in Table 2. Two of these bands significantly decreased in frequency in impacted sites which were progressively more distant downstream from Lake Reality while 6 increased in frequency. Of these, one band (OPA15₁₅₅₀), which decreased in frequency in impacted sites downstream from Lake Reality, was present in increased frequency in reference sites as compared to impacted sites. A second band (OPA1₁₅₂₅) which increased in frequency in impacted sites progressively further downstream from Lake Reality was present in decreased frequency in reference sites. For bands with similar frequencies in the two reference sites, 3 bands decreased in frequency from impacted to reference sites while 5 increased in frequency. For 24 bands, the frequency between the two reference sites was statistically different and of these 15 also showed a difference

Table 2. Band frequencies of polymorphic bands. Thirteen oligonucleotide primers were used in RAPD assay for 6 populations of redbreast sunfish.

Band	EFK 23.4	EFK18.3	EFK13.8	EFK3.5	BF	HC
OPD3 ₁₄₅₀	0.449	0.3239	0.4865	0.3333	0.2603	0.2059
OPD3 ₇₂₅	0.6327	0.6761	0.7838	0.6133	0.5205	0.9412
OPD3 ₆₇₅	0.3469	0.3944	0.4324	0.3867	0.5342	0.0588
OPD3 ₆₂₅	0.3061	0.2535	0.3784	0.44	0.1507	0.07353
OPD5 ₁₄₀₀	0.2245	0.169	0.05405	0.04	0	0.1324
OPD5 ₁₃₈₀	0.2245	0.169	0.05405	0.04	0	0.1324
OPD5 ₉₅₀	0.1429	0.2394	0.2838	0.2133	0.3836	0
OPD5 ₇₃₀	0.6531	0.6056	0.7027	0.76	0.6438	0.4559
OPD5 ₇₂₀	0.2857	0.3803	0.2297	0.2133	0.2877	0.4118
OPD5 ₇₀₀	0.3061	0.3944	0.2703	0.24	0.3425	0.5441
OPD8 ₉₀₀	0.449	0.5211	0.3784	0.36	0.0274	0.4265
OPD8 ₈₂₀	0.5306	0.4225	0.5811	0.6133	0.9726	0.5735
OPD8 ₇₇₀	0.898	0.7606	0.8108	0.76	0.9041	0.7206
OPD11 ₁₅₂₅	0.1429	0.1408	0.06757	0.08	0.05479	0.4412
OPD11 ₁₅₀₀	0.1429	0.1408	0.06757	0.08	0.05479	0.4412
OPD11 ₁₂₄₀	0.6327	0.6056	0.5405	0.6933	0.3699	0.5588
OPD11 ₁₁₈₀	0.4694	0.493	0.3649	0.5333	0.4521	0.5588
OPD11 ₁₁₄₀	0.7959	0.7606	0.7973	0.84	0.8767	0.9118
OPD12 ₁₁₆₀	0.551	0.5915	0.7703	0.68	0.8082	0.5
OPD12 ₁₀₃₀	0.4286	0.3239	0.2838	0.3067	0.2877	0.1471

Table 2. (Cont'd)

OPD15 ₁₀₄₀	0.7347	0.7746	0.7838	0.7733	0.7397	0.8088
OPD15 ₉₉₀	0.2653	0.2676	0.2162	0.24	0.3425	0.3529
OPD16 ₂₀₀₀	0.3265	0.4789	0.4324	0.5467	0.6027	0.3235
OPD16 ₆₀₀	0.1837	0.0845	0.1216	0.1867	0.2466	0.3382
OPA1 ₁₅₂₅	0.0408	0.1127	0.1486	0.12	0.0548	0.02941
OPA1 ₁₂₅₀	0.6327	0.6479	0.7297	0.6133	0.6986	0.8035
OPA1 ₉₈₀	0.2449	0.3662	0.2432	0.2533	0.6438	0.0735
OPA2 ₁₂₁₀	0.0225	0.3099	0.2838	0.16	0.4795	0.3971
OPA2 ₁₂₀₀	0.2245	0.1549	0.1829	0.2	0.0959	0.4706
OPA9 ₁₀₅₀	0.0408	0.0845	0.054505	0.16	0.1507	0.05882
OPA9 ₁₀₀₀	0.0408	0.0845	0.054505	0.16	0.1507	0.05882
OPA9 ₆₅₀	0.8367	0.7746	0.8649	0.88	0.7945	0.6324
OPA9 ₆₂₅	0.1633	0.2254	0.1351	0.12	0.2055	0.3676
OPA11 ₂₀₀₀	0.2041	0.1408	0.2162	0.2933	0.3669	0
OPA11 ₈₅₀	0.7551	0.6901	0.7027	0.7733	0.6986	0.5294
OPA15 ₁₈₀₀	0.0612	0.0282	0.1081	0.02667	0.05479	0.0294
OPA15 ₁₇₀₀	0.0816	0.169	0.08108	0.2533	0.3425	0.7794
OPA15 ₁₅₅₀	0.0612	0.0986	0	0.02667	0.1781	0.2794
OPA18 ₁₈₀₀	0.5714	0.4085	0.3784	0.52	0.06849	0.6618
OPA18 ₁₁₀₀	0.9796	1	0.9324	0.96	0.9863	1
OPA18 ₁₀₇₀	0.898	0.7606	0.8108	0.92	0.4932	0.8529
OPA18 ₉₀₀	0.9388	0.9296	0.9595	0.8667	0.9726	0.8676
OPA18 ₆₀₀	0.102	0.1549	0.0946	0.1067	0.3288	0
OPA18 ₅₈₀	0.2857	0.1831	0.2162	0.4	0.3562	0.2647

Band Sharing Index

The genetic diversity as measured by the band sharing index (Lynch, 1990) calculated for each primer revealed no differences as a function of distance downstream from the point source contaminant and no significant difference in diversity between impacted sites within EFPC for all primers examined. No significant difference was found between the two reference sites using this index. Because no statistical difference was observed between impacted sites within EFPC and between the two reference sites, impacted sites were grouped and compared with the grouped reference sites using a one way weighted ANOVA for each primer. See Table 3 for a summary of these analyses. For the three primers OPD3 ($p=0.0770$), OPD8 ($p=0.0704$), and OPA1 ($p=0.0773$), there is a slight indication of a difference between impacted and reference sites. Impacted sites were shown to be more diverse than the reference sites for these primers. Primers OPD16 ($p=0.0419$), OPA9 ($p=0.0436$), and OPA15 ($p=0.0099$) indicate a significant difference between the grouped impacted and grouped reference sites. Reference sites were shown to be more diverse for these primers. The seven remaining primers indicated no difference between impacted and reference populations.

If an α value of 0.10 rather than the traditional 0.05 is used for analysis of this

Table 3. Results of the one way weighted ANOVA by primer for Band Sharing Index. Populations were grouped into two groups: impacted populations from EFPC(i) and reference populations (r).

Primer	Weighted Mean	p Value
OPD3 i r	0.8092 0.8888	0.077
OPD5 i r	0.8826 0.8914	0.5327
OPD8 i r	0.8869 0.9570	0.0704
OPD11 i r	0.7938 0.7944	0.9781
OPD12 i r	0.7724 0.8034	0.314
OPD15 i r	0.6148 0.6170	0.9188
OPD16 i r	0.9016 0.8854	0.0419
OPA1 i r	0.8976 0.9330	0.0773
OPA2 i r	0.9266 0.9140	0.2626
OPA9 i r	0.8219 0.7477	0.0436
OPA11 i r	0.8041 0.8012	0.8818
OPA15 i r	0.0997 0.0768	0.0099
OPA18 i r	0.78 0.7795	0.9835

data, the interpretation of the results are somewhat different. For the one way weighted ANOVA for Band Sharing Index, the primers which indicated a slight difference between impacted and reference populations, OPD3 ($p = 0.0770$), OPD8 ($p = 0.0704$), and OPA1 ($p = 0.0773$), indicate a significant difference at $\alpha = 0.10$ in addition to the three primers which indicated a significant difference at $\alpha = 0.05$. No additional primers indicate a significant difference between impacted and reference sites at $\alpha = 0.20$.

Nucleon Diversity Index

Using the nucleon diversity index (Nei, 1987) calculated for each primer, no significant difference within EFPC and no difference correlating to distance from the point source contaminant sites was indicated for all primers examined. However, this index indicated differences between the two reference sites for seven of the thirteen primers. Therefore, for further analysis using this index reference sites were considered independently and each tested for differences in genetic diversity from the impacted sites taken together using a one way weighted ANOVA. See Table 4 for a summary of these analyses.

The four primers OPD3 ($p = 0.0031$), OPA1 ($p = 0.0432$), OPA9 ($p = 0.0538$), and OPA15 ($p = 0.0212$) showed a significant difference between impacted sites and Hinds Creek though the former two and the latter two primers showed decreased and increased levels of diversity in Hinds Creek, respectively. Primers OPD8

Table 4. Results of the one way weighted ANOVA for nucleon diversity index.

ANOVA was calculated for three groups: impacted sites on EFPC (i), Brushy Fork (bf), and Hinds Creek (hc).

Primer		Weighted Mean	p Value
OPD3	i	0.8837	-----
	bf	0.8550	0.2025
	hc	0.4846	0.0031
OPD5	i	0.7351	-----
	bf	0.7321	0.9666
	hc	0.7120	0.7402
OPD8	i	0.6849	-----
	bf	0.1796	0.0053
	hc	0.6984	0.6874
OPD11	i	0.7445	-----
	bf	0.7461	0.9801
	hc	0.8288	0.1665
OPD12	i	0.5918	-----
	bf	0.5133	0.4011
	hc	0.6207	0.5849
OPD15	i	0.4986	-----
	bf	0.5225	0.8208
	hc	0.5632	0.5673
OPD16	i	0.6166	-----
	bf	0.6842	0.1620
	hc	0.7011	0.1124
OPA1	i	0.7377	-----
	bf	0.7327	0.7842
	hc	0.4350	0.0432
OPA2	i	0.5968	-----
	bf	0.5887	0.7773
	hc	0.6124	0.6008
OPA9	i	0.4288	-----
	bf	0.5278	0.1173
	hc	0.5351	0.0538

Table 4. (Cont'd).

OPA11 i	0.5700	-----
bf	0.6975	0.1996
hc	0.5057	0.2900
OPA15 i	0.4074	-----
bf	0.6792	0.0087
hc	0.6453	0.0212
OPA18 i	0.8514	-----
bf	0.8774	0.0866
hc	0.8117	0.1964

($p=0.0053$) and OPA15 ($p=0.0087$) showed a difference and OPA18 ($p=0.0866$) a slight difference between impacted sites and Brushy Fork with a similar division of primers showing differing levels of diversity for Brushy Fork as compared to the impacted sites of EFPC. One primer, OPA15, showed a significant difference between impacted sites and both reference sites. Seven primers showed no significant difference between impacted sites and either reference site.

If alternate α values are applied to the one way weighted ANOVA for Nucleon Diversity Index, no more primers indicate a difference between impacted sites and either reference site at $\alpha = 0.10$ than at $\alpha = 0.05$. However, when a value of $\alpha = 0.20$ is applied to this data, primers OPD3 ($p= 0.2025$), OPD16 ($p= 0.1620$), OPA9 ($p= 0.1173$), and OPA11 ($p= 0.1996$) indicate a significant difference between impacted sites and Brushy Fork. Primers OPD11 ($p= 0.1665$), OPD16 ($p= 0.1124$), and OPA18 ($p=0.1964$) indicate a significant difference between impacted sites and

Hinds Creek at $\alpha = 0.20$. Using this α value, five primers indicate a significant difference between impacted sites and both of the reference sites and four additional primers indicate a significant difference between impacted and at least one of the reference sites.

Roger's Genetic Distance

Genetic distance was calculated from genotypes generated using all primers. The genetic distance between EFK 13.8 (JF) and EFK 5.3 (GS) was the smallest (0.0382) while the genetic distance between EFK 23.4 (BLR) and EFK 18.3 (RT) was likewise small (0.0398), indicating the small genetic distance between all EFPC populations. Comparisons of the genetic distance between all EFPC populations and each of the reference populations indicated that the genetic distance between EFPC and Brushy Fork populations was smaller than that between EFPC and Hinds Creek populations. See Table 5 for a summary of these analyses.

RAPDPLOT Neighbor Joining Analysis

The results of the neighbor joining analysis using RAPDPLOT (Kambhampati et al., 1992) confirmed and demonstrated that individuals within all population sites are highly variable. Figure 4 is a phenogram generated using all primers and including all individuals examined in this assay. This phenogram is partitioned into 5 major clusters and the number of individuals from each population within each

Table 5. Roger's genetic distance for RAPD genotypes. Pairwise comparisons between all populations examined were calculated by genotypes generated using all primers. EFPC populations (impacted sites) include: BLR (EFK 23.4), RT (EFK 18.3), JF (EFK13.8), and GS (EFK 5.3). Reference sites are Brushy Fork (BF) and Hinds Creek (HC).

	BLR	RT	JF	GS	BF	HC
BLR	----	.0398	.0429	.0431	.0875	.0882
RT	----	----	.0427	.0482	.0749	.0833
JF	----	----	----	.0382	.0741	.0985
GS	----	----	----	----	.0733	.0999
BF	----	----	----	----	----	.1209
HC	----	----	----	----	----	----

cluster is given in Table 6. Populations did not form discrete clusters as might have been expected for genetically similar and distinct groups. Clusters were observed but incorporated fish from various sites within EFPC and reference sites. There was no observed relationship between physical distance from the more contaminated upper reaches of EFPC or among sites within EFPC and the estimates of similarity as indicated by the neighbor joining method.

The phenogram generated using five bands which displayed increased frequency in impacted sites progressively further downstream from EFK 23.7 showed a similar pattern of clusterings of individuals from all sites (Figure 5).

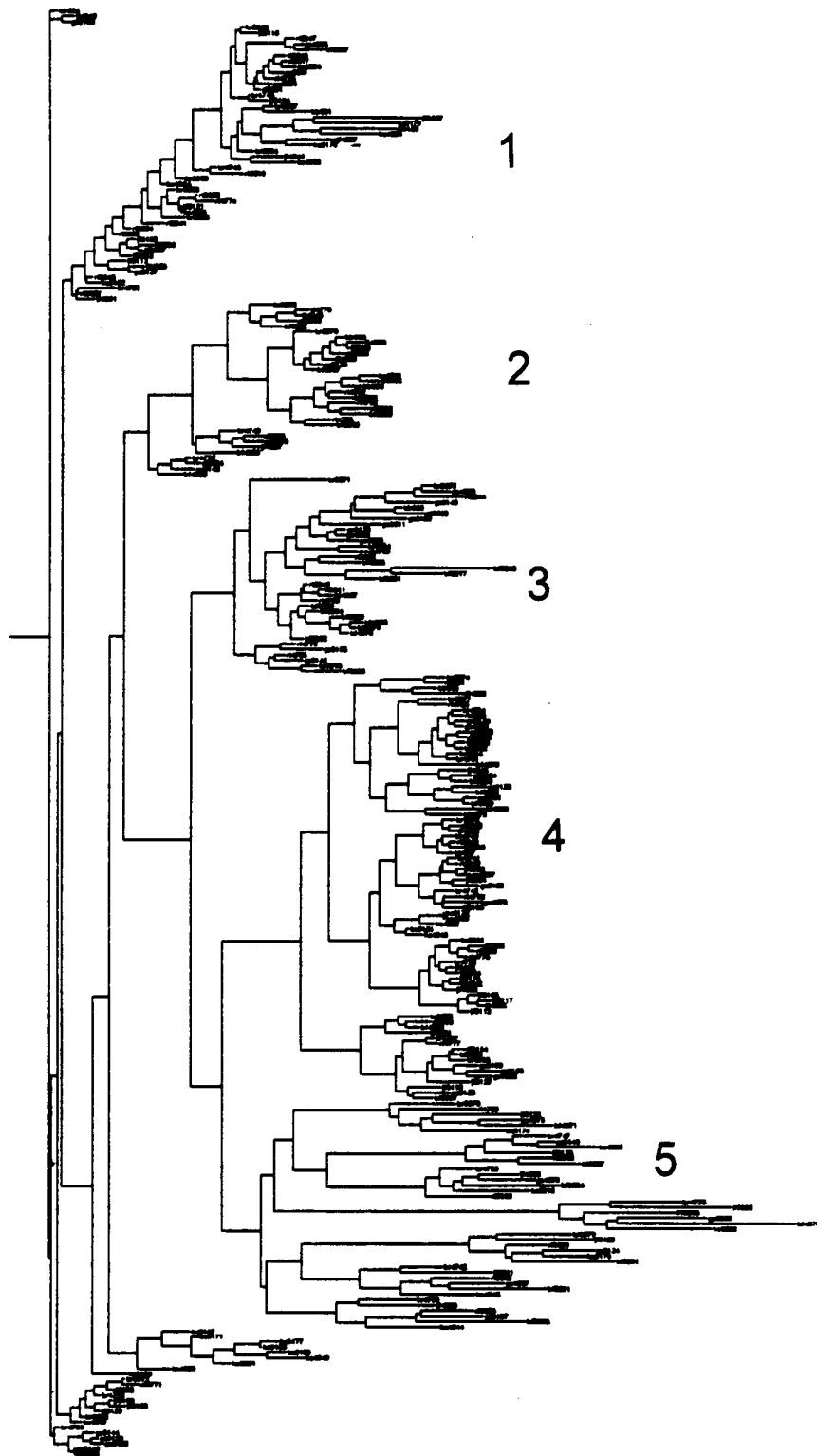


Figure 4. Phenogram generated using neighbor joining method (Saitou and Nei, 1987). Phenogram includes the four impacted sites on East Fork Poplar Creek and the two reference sites Brushy Fork and Hinds Creek. Duplicate genotypes have been removed. Composition of clusters is given in Table 6.

Table 6. Number of individuals from each site comprising clusters in phenogram. Phenogram is shown in Figure 4 and is comprised of 5 major clusters of individuals from all sites.

Site	Cluster				
	1	2	3	4	5
EFK 23.4	10	7	4	13	10
EFK 18.3	13	7	9	10	10
EFK 13.8	14	6	0	17	11
EFK 5.3	5	5	9	10	11
Brushy Fork	1	7	14	8	11
Hinds Creek	7	0	0	5	7

However, one cluster of individuals was produced in which individuals from more highly impacted sites were present in higher frequency than individuals from sites less impacted or from reference sites. The individuals within this cluster share a common genotype. This genotype is found in 30.6% of the EFK 23.4 population, in 18.3% of the EFK 18.3, 10.8% of the EFK 13.8, 12% of the EFK 5.3, 4% of Brushy Fork and 7.4% of Hinds Creek populations. Additionally, a phenogram generated using data from primer OPA15 produced a clustering of individuals with decreased frequency in highly impacted sites as compared to reference sites (Figure 6). The individuals within this cluster share a common genotype. This genotype is found in 8.16% of the EFK 23.4 population, 16.9% of the EFK 18.3, 8.10% of the EFK 13.8, 24% of the EFK 5.3, 32.9% of Brushy Fork and 50% of Hinds Creek populations.

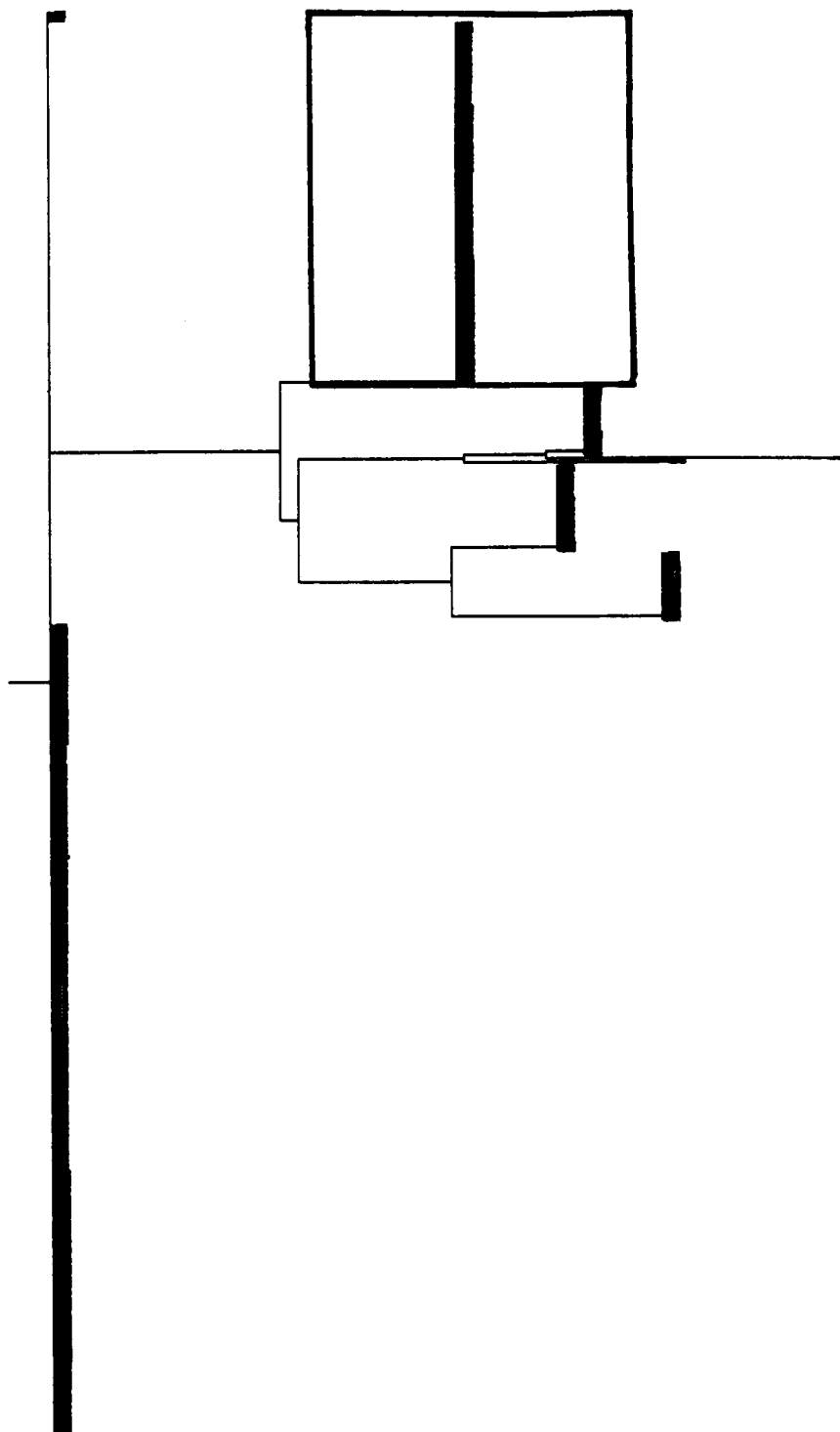


Figure 5. Phenogram generated using bands with increased frequency in impacted sites. Boxed cluster of individuals represents a genotype present in increased frequency in highly impacted sites as compared to reference sites: EFK 23.4 (30.6%), EFK 18.3 (18.3%), EFK 13.8 (10.8%), EFK 5.3 (12%), BF (4%), and HC (7.4%).



Figure 6. Phenogram generated using banding pattern information from OPA15. Boxed cluster of individuals represents a genotype present in decreased frequency in highly impacted sites as compared to reference sites: EFK 23.4 (8.16%), EFK 18.3 (16.9%), EFK 13.8 (8.10%), EFK 5.3 (24%), BF (32.9%), and HC (50%).

IV. Discussion

The RAPD Technique

The relative simplicity of the RAPD assay and the requirement for only small amounts of DNA, even degraded DNA, make it an appealing technique for biomonitoring processes. Because no prior knowledge of the genome is required the assay can be applied to the selected study organism without additional work to determine sequences of interest to be amplified. A large number of commercially available primers can be used though primers with at least 60% G:C content are recommended due to the high temperatures used in cycling (Chong et al, 1994). The RAPD technique scans the entire genome for randomly located priming sites producing amplified fragments from both coding and noncoding regions. Utilizing the entire genome increases the chances of finding polymorphisms which are used as biomarkers (Bachman, 1994; Lynch and Milligan, 1994; Liu and Funier, 1993).

Polymorphisms detected by band presence/absence may be caused by (1) failure to prime at a site in certain individuals because of nucleotide sequence differences possibly due to deletions of the DNA fragment carrying one of the two annealing sites or nucleotide substitutions within the annealing site, or (2) insertions or deletions in the targeted fragment between the priming sites such that the target is too large to be amplified or the size of the fragment produced is changed causing it to migrate to a different position during gel electrophoresis and to be scored as a different band (Weising et al., 1995; Beckman, 1994; Clark and Lanigan, 1993; Williams et al., 1990).

Although there are several advantages to RAPDs, care must be taken when applying this technique in the field for biomonitoring purposes. The genetic function of the amplified fragments produced by RAPDs and the physiological and ecological significance of the alteration of RAPD banding patterns produced are not yet known, making interpretation of results from this assay difficult. Results of RAPD assays are also primer specific. Because primers target different sections of the genome the resulting banding pattern is different for each primer used. While one primer or even one band produced by a primer may indicate a small genetic diversity within a population, a different primer or band may indicate a large diversity. An example of this is the primer OPA2 which indicates a large BSI, or a large diversity for impacted sites, and primer OPD15 which indicates a small BSI, or a small diversity for impacted sites.

Despite the dilemma of not knowing the biological function of RAPD markers, the RAPD technique is useful in biomonitoring studies. As discussed above, exposure to stressors within an organism's environment can lead to rapid changes at the molecular level (Adams et al., 1992). Biomarkers, such as RAPDs, may reflect changes due to exposure to particular stressors and are therefore beneficial in biomonitoring studies. Identifying RAPD markers which are indicative of exposure to particular pollutants permits further application in biomonitoring studies to assess exposure for the same species in other aquatic systems. Thus, the RAPD technique permits a quick scanning of the genome in order to detect differences between populations.

Genetic Diversity in EFPC Populations

Due to its toxicity and potential to inhibit DNA repair and cause cell death, mercury exposure may represent a powerful selection force in exposed populations, potentially narrowing the genetic diversity and overall fitness of exposed populations (Diamond et al, 1989). A downstream gradient in mercury contamination in EFPC water and fish populations has been demonstrated and may represent a selection force on these populations. Although mercury compounds, primarily methylmercury (Southworth et al., 1995), have been shown to accumulate in redbreast sunfish with decreasing levels of accumulation in populations progressively further downstream from Lake Reality, the genetic diversity of these populations does not seem to significantly differ. The results of this study provide evidence that the genetic structure of populations within EFPC are not significantly different reflecting no symptomatic alteration to genetic structure due to the graded levels of exposure to the various contaminants found in EFPC, particularly mercury. The RAPD technique showed only small differences between populations within EFPC. However, it did reveal differences between EFPC populations and reference populations which were free of the potential selection pressures of mercury exposure.

The results of this study provide little evidence of differences among sunfish populations within EFPC despite a difference in levels of exposure to and concentrations of mercury in redbreast sunfish among sites. Results are highly primer-specific and no generalities among primers are evident. Several primers

indicated decreased diversity while others indicate increased diversity in EFPC populations compared to reference populations. Primer specificity may depend on the sites being amplified, although at this point the function of amplified fragments is not known. While some bands increased in frequency in impacted sites progressively further downstream from EFK 23.4 others decreased in frequency in these same populations. Calculating genetic diversity with each band individually may reflect differences in genetic diversity among sites but when taken together bands with increased and decreased frequency may cancel this effect. It is not known what these individual bands mean, but if they represent genotypes with either resistance to or sensitivity to EFPC pollutants this may mask a difference in these populations. If such genotypes are representative of pollutant exposure, they could be applied to assess exposure in other similarly polluted environments.

Other studies of contaminant exposed populations have shown changes in the levels of genetic diversity. A study using RAPDs in sunfish populations exposed to paper mill effluents showed an apparent increase in diversity (Kai-Lin Lee, Oak Ridge National Laboratory, personal communication). In a study using the RAPD technique to evaluate genetic diversity in fish populations from radionuclide contaminant ponds, exposed fish were found to have higher levels of genetic diversity as compared to fish from uncontaminated ponds (Theodorakis, 1994).

The results of the calculations of genetic distance seem to indicate that impacted populations are at least to some degree genetically distant from reference populations. All populations in EFPC seem to be more dissimilar to reference

populations than to one another. All EFPC populations display small genetic distances between each other and larger distances to the reference populations. The EFPC populations are less genetically distant to the Brushy Fork population than to the Hinds Creek population. Potential reasons for this difference are discussed later. Differences in genetic distance between populations may reflect not only a population difference between EFPC and reference populations but an additional difference within EFPC populations.

Results of the RAPDPLOT neighbor joining method using all primers in all populations examined in this study indicated no significant difference between populations. However, when a phenogram was generated using bands which significantly increased in frequency in impacted sites progressively further downstream from EFK 23.7, a cluster of individuals with the same genotype was produced which from the distribution of individuals from all sites may indicate a pollutant resistant genotype. Individuals from more highly impacted sites were present in higher frequency than from less impacted EFPC sites and from reference sites. This may reflect selection pressures in EFPC which favor this genotype. The phenogram generated using primer OPA15 produced a cluster of individuals with the same genotype in which the proportion of individuals from more highly impacted sites was lower than for less impacted sites and for reference sites. This may reflect a pollutant sensitive genotype which has been selected against in EFPC.

The primary hypothesis of this study, that genetic diversity is decreased in EFPC redbreast sunfish populations, was not supported by the results of this study.

The data here show slight differences in populations of exposed sunfish as compared to reference populations. Differences in levels of genetic diversity as measured by RAPD assay between impacted and reference sites may be due to a number of factors including (1) population differences due to geographical isolation, (2) a possibly small degree of migration between EFPC and Brushy Fork populations due to the moderate proximity and connectedness of these two watersheds, (3) recent remediation efforts within EFPC, and (4) a small degree of migration between populations within EFPC. Additionally, the fact that little difference in genetic diversity between impacted and reference sites is seen from this study may be due to the power of statistical analysis used and will be discussed later.

Comparison of genetic diversity between geographically isolated populations must consider the dynamics of population genetic structure which are independent of directional selection from pollution. Geographically isolated populations are likely to diverge due to natural selection due to environmental differences and random genetic drift. However, populations with some degree of gene flow between them are expected to be more closely related than if they were completely isolated. Because Brushy Fork and EFPC both drain into Poplar Creek (Figure 2), fish from these two watersheds may intermingle somewhat resulting in closer genetic relatedness. Although Gatz and Adams (1994) suggest that movement between redbreast sunfish populations within EFPC is minimal, it is possible for fish to migrate between these two watersheds because Poplar Creek serves as a common

receptor for these systems. Such gene flow between fish populations would lead to a closer genetic relatedness between these populations. Recruitment of fish from outside EFPC could increase the genetic diversity of these populations which would decrease the probability of detecting significant differences in any genetic diversity caused by pollution. Conversely, the complete geographical isolation of Hinds Creek from EFPC and Brushy Fork may cause it to be more genetically distant from these two watersheds.

RAPD assays indicate only a small difference between impacted sites within EFPC and reference sites, and this result may be due in part to recent remediation efforts. Although remediation efforts reduced TRC (Southworth et al., 1995; Etnier et al., 1994) and mercury (Hinzman et al., in press) in the upper reaches of EFPC, these same efforts decreased the water flow of the stream which may have introduced additional selection forces on and induced additional changes in redbreast sunfish populations. Mercury levels, though reduced, followed the same downstream gradient until May 1990 whereafter the highest mercury concentrations were found at EFK 18.3 (Hinzman et al, 1993).

Because prior to the remediation effort of November 1992 the upper reaches of EFPC possessed only small, transitory fish populations due to toxic TRC concentrations and experienced rapid colonization following remediation (Southworth et al., 1995), the EFK 23.4 population may have decreased in size. Fish from less contaminated downstream sites may have also migrated upstream and mixed with the population at EFK 23.4. Furthermore, since May 1990 the EFK

18.3 population has existed at the location of the highest concentrations of mercury exposure. This may, in part, account for potential similarity between EFK 23.4 and EFK 18.3 populations. A small number of fish comprising the original population of EFK 23.4 may have survived the toxicity of these waters and could have been exposed to higher levels of mercury over longer periods than fish which just recently colonized the area from areas of lower contamination. However, as mentioned before, EFK 18.3 has been reported to display the highest concentrations of mercury since May 1990 (Hinzman et al., in press). Decreased water flow and food availability permitted the increase of other species thus increasing the species richness within EFPC (Etnier et al., 1994) but caused a significant decrease in sunfish per unit area. Sunfish collected following November 1992 showed a lower average EROD (S.M. Adams, Oak Ridge National Laboratory, personal communication) at all sites which may indicate a reduction in exposure to toxic chemicals. While remediation efforts may have decreased stress from chemical pollutants, decreased water flow and food availability may have introduced additional selection pressures on the entire EFPC redbreast sunfish population and may have decreased the diversity of this population as compared with populations from other streams. Differences in genetic distances between EFPC and reference streams support this theory.

Negative Results in Toxicological Studies

Although the results of this study seem to indicate no genetic difference among EFPC sites leading to the failure to reject the null hypothesis ($H_0 = 0$) of no effect, this could also be a reflection of the statistical power used in analysis. It is clear that failing to reject H_0 does not mean H_0 is true, that there is no effect on the system. According to Hayes (1987) the extremely low power of statistical tests used in toxicological research increase the chances of obtaining negative results. He points out that the magnitude of difference that an investigator considers significant, the effect size (ES), may be too small. Additionally, the level of probability of making a Type I error (α), the probability of rejecting a true H_0 , is often too low in toxicological studies. The traditional use of a low α level (0.05) and little regard for β (probability of making a Type II error or not rejecting H_0 when it is false) is based on the belief that it is better to fail to detect an effect than to falsely report an effect. While this premise may be true for some areas of research, for toxicology research failing to detect statistically significant results may be more important than finding significant effects. Thus toxicologists should be more concerned with minimizing Type II errors. Because small sample sizes, small α values, and small ES contribute to this problem, Hayes (1987) suggests the use of large sample sizes and liberal for statistical tests of reasonable power to detect small but biologically significant effects.

Results of this study are somewhat different when analyzed at an α value of

0.10 rather than the traditional 0.05. Both band sharing index and nucleon diversity index indicate some additional difference between impacted sites of EFPC and reference sites when an α value of 0.10 is applied. An α value of 0.20 indicates additional differences between impacted sites of EFPC and reference sites for nucleon diversity index but not band sharing index. However, no significant difference between EFPC sites was seen at these levels. When α values are relaxed, additional primers show differences between impacted site of EFPC and reference sites indicating a potentially greater difference in genetic diversity among these populations.

Negative results are important in toxicology research. They may be useful in monitoring recovery of previously effected systems. Of course, when negative results are found, the statistical power and ES levels should be investigated for possible contribution to these results.

V. Conclusions

Although the genetic function of amplification sites from which RAPD bands are produced are not known, the RAPD assay is a useful tool for biomonitoring purposes permitting the detection of genetic differences between even closely related populations. The results of the RAPD assay are highly primer specific. In this study, all impacted sites were found to be less genetically distant from one another and more genetically distant to the two reference sites. Because the results of the RAPD assay are so highly primer specific, applying each primer to known impacted and non-impacted sites prior to use for biomonitoring purposes might allow investigators to establish banding patterns produced in each type of environment and relate these results to other potentially impacted sites.

Analyzing the results of the RAPD assay at the band level, may reveal bands which are indicative of increased or decreased genetic diversity. If two such bands are found one of which reflects increased and the other of which reflects decreased genetic diversity, analyzing the two together may indicate no change in diversity. Identifying such bands may provide population specific markers or biomarkers of pollutant exposure.

The results of this study indicate that either 1) stressors within EFPC, whether chemical or natural, do not seem to have significantly altered the population genetic structure compared to populations in reference streams or 2) some degree of gene flow into EFPC populations, possibly in combination with reduced selection pressure from pollutants following remediation efforts, may have increased genetic

diversity within EFPC populations. Although measures of genetic distance do seem to indicate a difference between impacted sites of EFPC and reference sites, it is not known whether or to what degree the cause of this difference may be attributed to population differences and/or to stress induced differences or the power of the statistical tests used here.

VI. Future Directions

The application of RAPD assay in this study illustrates its usefulness as a tool in biomonitoring studies. However, limited knowledge of the implications of the results of this assay as discussed above should be noted. Differences between RAPD patterns may be due to numerous causes including neutral variation, random drift, or environmental stressors.

While RAPD assay indicates differences between the populations examined here, assaying additional primers may give a better picture of the genetic makeup and therefore overall health status of sentinel fish populations of EFPC. Because RAPD bands are produced randomly, screening additional primers may produce bands which better reflect changes in population genetic structure in impacted sites. Of course, the number of primers which can be screened is limited by time and money. As more and more primers are screened the accumulation of bands scored will permit differentiation of individuals within populations which is beyond what is possible for this study. Additionally, comparing results of RAPD assays with other assays of fish health such as EROD assays may help in the interpretation of our results.

Possible avenues of future research include the investigation of the function of RAPD markers, particularly those with apparently different frequencies in highly impacted sites. While no alteration in genetic diversity among EFPC populations seems evident at this time by assessing the genotypes of individuals within EFPC populations, small portions of these populations may be found which have

characteristic genotypes. These individuals may demonstrate genotypes which have a selective advantage in the EFPC environment. This may indicate that a portion of individuals from impacted sites display altered genotypes due to pollutant induced selection. Identification of RAPD markers with direct correlation to exposure to particular pollutants would allow further research in which these markers could be employed as biomarkers of exposure in similarly contaminated populations.

References

References

Adams, S.M. 1990. Status and use of biological indicators for evaluating the effects of stress on fish. *Am. Fish. Soc. Symp.* 8:1-8.

Adams, S.M., W.D. Crumby, M.S. Greeley, Jr., M.G. Ryon, and E.M. Schiling. 1992. Relationships between physiological and fish population responses in a contaminated stream. *Environ. Toxicol. Chem.* 11:1549-1557.

Adams, S.M., W.D. Crumby, M.S. Greeley, Jr., L.R. Shugart, and C.F. Saylor. 1992. Response of fish populations and communities to pulp mill effluents: a holistic assessment. *Ecotoxicol. Environ. Saf.* 24:347-360.

Adams, S.M., L.R. Shugart, and G.R. Southworth. 1990. Application of bioindicators in assessing the health of fish populations experiencing contaminant stress. In McCarthy, J.F. and L.R. Shugart. *Biomarkers of environmental contamination*. Boca Raton, FL: Lewis Publishers. pp 333-353.

Arnheim, N. and H.A. Erlich. 1992. PCR strategy. *Annu. Rev. Biochem.* 61:131-156.

Arnheim, N., T. White, and W.E. Rainey. 1990. Application of PCR: Organismal and

population biology. *Bioscience*. 40:174-182.

Ariza, M.E., J. Holliday, and M.V. Williams. 1994. Mutagenic effect of mercury (II) in eukaryotic cells. *In Vivo*. 8:559-564.

Bachmann, K. 1994. Tansley Review No. 63: Molecular markers in plant ecology. *New Phytol*. 126:403-418.

Black, W.C., IV. 1993. RAPDPLOT-FORTRAN program for analysis of genetic relationships among individuals using RAPD-PCR markers. Colorado State University, Fort Collins, CO.

Burkart, W. and B. Ogorek. 1986. Genotoxic action of cadmium and mercury in cell cultures and modulation of radiation effects. *Toxicol. Environ. Chem*. 12:173-183.

Cantoni, O. and M. Costa. 1983. Correlations of DNA strand breaks and their repair with cell survival following acute exposure to mercury (II) and x-rays. *Mol. Pharm*. 24:84-89.

Cantoni, O., N.T. Christie, A. Swann, D.B. Drath, and M. Costa. 1984. Mechanism of HgCl₂ cytotoxicity in cultured mammalian cells. *Mol. Pharm*. 26:360-368.

Chagnon, N. L. and S.I. Guttman. 1989. Differential survivorship of allozyme genotypes in mosquitofish populations exposed to copper and cadmium. *Environ. Toxicol. Chem.* 8:319-326.

Chalmers, K.J., R. Waugh, J.I. Sprent, A.J. Simons, and W. Powell. 1992. Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPDs markers. *J. Hered.* 69:465-472.

Chong, D.K., R.C. Yang, and F.C. Yeh. 1994. Nucleotide divergence between populations of trembling aspen (*Populus tremuloides*) by RAPDs. *Curr. Genet.* 26:374-376.

Christie, N.T., O. Cantoni, R.M. Evans, R.E. Meyn, and M. Costa. 1984. Use of mammalian DNA repair-deficient mutants to assess the effects of toxic metal compounds on DNA. *Biochem. Pharm.* 33:1661-1670.

Clark, A.G. and M.S. Lanigan. 1993. Prospects for estimating nucleotide divergence with RAPDs. *Mol. Biol. Evol.* 10:1096-1111.

Clarkson, T.W. 1993. Mercury: Major issues in environmental health. *Environ. Health Perspect.* 100:31-38.

Costa, M., N.T. Christie, O. Cantoni, and J.T. Zelikoff. 1991. DNA damage by mercury compounds: An overview. In Suzuki, T., N. Imura, and T.W. Clarkson, eds. *Advances in Mercury Toxicology*. New York: Plenum Press. pp 255-273.

Dawson, I.K., K.J. Chalmers, R. Waugh, and W. Powell. 1993. Detection and analysis of genetic variation in *Hordeum spontanium* populations from Israel using RAPD markers. *Mol. Ecol.* 2:151-159.

Depledge, M.H. 1994. Genotypic toxicity: Implications for individuals and populations. *Environ. Health Perspect.* 102:101-104.

Diamond, S.A., M.C. Newman, M. Mulvey, P.M. Dixon, and D. Martison. 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard), during acute exposure to inorganic mercury. *Environ. Toxicol. Chem.* 8:613-622.

Erlich, H.A., D. Gelfand, and J.J. Sninsky. 1991. Recent advances in the polymerase chain reaction. *Science*. 252:1643-1651.

Erlich, H.A. and N. Arnheim. 1992. Genetic analysis using the polymerase chain reaction. *Annu. Rev. Genet.* 26: 479-506.

Etnier, E.L., D.M. Opresko, and S.S. Talmage. 1994. Second report on the Oak Ridge Y-12 plant fish kill for upper East Fork Poplar Creek. ORNL/TM-12636 Report. Oak Ridge Y-12 Plant, Oak Ridge, Tn.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Produced and distributed by author. Department of Genetics, University of Washington, Seattle.

Ferris, S.D. and W.J. Berg. 1987. The utility of mitochondrial DNA in fish genetics and fishery management. In Ryman, N. and F. Utter, eds. Population genetics and fishery management. Seattle: University of Washington Press. pp 277-299.

Fore, S.A., S.I. Guttman, A.J. Bailer, D.J. Altfater, and B.V. Counts. 1995. Exploratory analysis of population genetic assessment as a water quality indicator. *Ecotoxicol. Environ. Saf.* 30:36-46.

Gatz, A.J. and S.M. Adams. 1994. Patterns of movement of centrarchids in two warmwater streams in eastern Tennessee. *Ecol. Freshwater Fish.* 3:35-48.

Gillespie, R.B. and S.I. Guttman. 1989. Effects of contaminants on the frequencies of allozymes in populations of the central stoneroller. *Environ. Toxicol. Chem.* 8:309-317.

Hadrys, H., M. Balick, and B. Schierwater. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* 1:55-63.

Hallerman, E.M. and J.S. Beckman. 1988. DNA-level polymorphism as a tool in fisheries science. *Can. J. Fish. Aquat. Sci.* 45:1075-1087.

Hayes, J.P. 1987. The positive approach to negative results in toxicology studies. *Ecotoxicol. Environ. Saf.* 14:73-77.

Hinzman, R.L., S.M. Adams, M.C. Black, H.L. Boston, W. Burton, A.J. Gatz, Jr., M.S. Greeley, Jr., W.R. Hill, J.M. Loar, J.F. McCarthy, M.J. Peterson, M.G. Ryon, J.G. Smith, G.R. Southworth, A.J. Stewart, and V.R. Tolbert. 1993. Second report on the Oak Ridge Y-12 Plant biological monitoring and abatement program for East Fork Poplar Creek. Y/TS-888 Report, Oak Ridge Y-12 Plant, Oak Ridge, Tn.

Hinzman, R.L., S.M. Adams, J.J. Beauchamp, S.W. Christensen, B.R. Gray, M.S. Greeley, Jr., K.D. Ham, W.R. Hill, C.G. Hull, S.R. Jardine, L.L. Kohring, J.M. Loar, J. W. Lotts, J.F. McCarthy, G.E. Napolitano, M.J. Peterson, M.G. Ryon, E.M. Schilling, S.K. Sharp, L.R. Shugart, J.G. Smith, G.R. Southworth, A.J. Stewart, C.W. Theodorakis, V.R. Tolbert. Third Report on the Oak Ridge Y-12 Plant biological monitoring and abatement program for East Fork Poplar Creek. Draft Y/TS-889 Report, Oak Ridge Y-12 Plant, Oak Ridge, Tn.

Jimenez, B.D., A. Oikari, S.M. Adams, D.E. Hinton, and J.F. McCarthy. 1990. Hepatic enzymes as biomarkers: interpreting the effects of environmental physiological and toxicological variables. In McCarthy, J.F. and L.R. Shugart. Biomarkers of environmental contamination. Boca Raton, FL: Lewis Publishers. pp 123-142.

Kambhampati, S., W.C. Black IV, and K.S. Rai. 1991. RAPD-PCR for identification and differentiation of mosquito species and populations: Techniques and statistical analysis. *J. Med. Entomol.* 29:939-945.

Kopp, R.L., T.E. Wissing, and S.I. Guttman. 1994. Genetic indicators of environmental tolerance among fish populations exposed to acid deposition. *Biochem. Syst. Ecol.* 22:459-475.

Larsson, A., C. Haux, and M. Sjobeck. 1985. Fish physiology and metal pollution: results and experiences from laboratory and field studies. *Ecotoxicol. Environ. Saf.* 9:250-281.

Liu, Z. and G.R. Funier. 1993. Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. *Theor. Appl. Genet.* 87:97-105.

Lynch, M. and B.G. Milligan. 1994. Analysis of population genetic structure with RAPD markers. *Mol. Ecol.* 3:91-99.

Lynch, M. 1990. The similarity index and DNA fingerprinting. *Mol. Biol. Evol.* 7:478-484.

McCarthy, J.F. and L.R. Shugart. 1990. Biological markers of environmental contamination. In McCarthy, J.F. and L.R. Shugart. Biomarkers of environmental contamination. Boca Raton, FL: Lewis Publishers. pp 3-14.

Miura, K., A. Naganuma, S. Himeno, and N. Imura. 1995. Mercury toxicity. In Goyer, R.A. and M.G. Cherian, eds. Toxicity of metals: Biochemical aspects. New York: Springer-Verlag. pp 163-187.

Nei, M. 1987. Molecular evolutionary genetics. New York: Columbia University Press.

Nevo, E., R. Noy, B. Lavie, A. Beiles, and S. Muchtar. 1986. Genetic diversity and resistance to marine pollution. *Biol. J. Linn. Soc.* 29:139-144.

Rossmann, T.G. 1995. Metal mutagenesis. In Goyer, R.A. and M.G. Cherian, eds. Toxicity of metals: biochemical aspects. New York: Springer-Verlag. pp 373-405.

Saiki, R.K. 1990. Amplification of genomic DNA. In Innis, M.A., D.H. Gelfand, J.J. Sninsky, and T.J. White eds. PCR protocols: A guide to methods and applications. Academic Press, Inc:New York. pp 13-20.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharff, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 239:487-491.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for constructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.

Shugart, L.R. 1990. Biological monitoring: testing for genotoxicity. In McCarthy, J.F. and L.R. Shugart. Biomarkers of environmental contamination. Boca Raton, FL: Lewis Publishers. pp 205-216.

Shugart, L.R., J. Bickman, G. Jackim, G. McMahon, W. Ridley, J. Stein, and S. Steinhart. 1992. DNA alterations. In Hugget, R.J., R.A. Kimerle, P.M. Mehrie, Jr., and H.L. Bergman, eds. 1992. Biomarkers: Biochemical, physiological, and histological markers of anthropogenic stress. Boca Raton, FL: Lewis Publishers. pp 125-153.

Sipes, I.G. and A.J. Gandolfi. 1991. Biotransformation of toxicants. In Amdur, M.O.,

J. Doull, and C.D. Klaasen, eds. *Cassaret and Doull's Toxicology: The basic science of poisons*. 4th edition. New York:Pergamon Press. pp 88-126.

Southworth, G.R., R.R. Turner, M.J. Petterson, and M.A. Bogle. 1995. Form of mercury in stream fish exposed to high concentrations of dissolved inorganic mercury. *Chemosphere*. 30:779-787.

Southworth, G.R.1990. PCB concentrations in stream sunfish (*Lepomis auritus* and *L. macrochirus*) in relation to proximity to chronic point sources. *Water Air Soil Pollut*. 51:287-296.

Strittholt, J.R., S.I. Guttman, and T.E. Wissing. 1988. Low levels of genetic variability of yellow perch (*Perca flavescens*) in Lake Erie and selected impoundments. In J.F. Downhower, ed. *The biogeography of the island region of western Lake Erie*. Ohio University Press. pp 246-257.

Talmage, S.S. and B. Walton. 1993. Food chain transfer and potential renal toxicity of mercury to small mammals at a contaminated terrestrial field site. *Ecotoxicology*. 2:243-256.

Theodorakis, C.W. 1994. Interpretation of toxicological data in an ecological setting: Physiological, molecular, and population genetic effects of genotoxicants on fish. Dissertation.

Theodorakis, C.W., S.J. D'Surney, J.W. Bickman, T.B. Lynem B.P. Bradley, W.E. Hawkins, W.L. Farkas, and J.F. McCarthy. 1992. Sequential expression of biomarkers in bluegill sunfish exposed to contaminated sediment. *Ecotoxicology*. 1:45-73.

Thorpe, J.E., J.F. Koonce, D. Borgeson, B. Henderson, A. Lamsa, P.S. Maitland, M.A. Ross, R.C. Simon, and C. Walters. 1981. Assessing and managing man's impact on fish genetic resources. *Can. J. Fish Aquat. Sci.* 38:1899-1905.

Weising, K., H. Nybom, K. Wolff, and W. Meyler. 1995. DNA fingerprinting in plants and fungi. Boca Raton, FL: CRC Press, Inc.

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nuc. Acids Res.* 18:7213-7218.

White, T.J., N. Arnheim, H.A. Erlich. 1989. The polymerase chain reaction. *Trends Genet.* 5:185-189.

Williams, J.G., A.R. Kubelik, K.J. Livak, J. A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acid Res.* 18:6531-6535.

Wittwer, C.T., G.B. Reed, and K.M. Ririe. 1994. Rapid cycle DNA amplification. In Mullis, K.B., F. Ferre, and R.A. Gibbs, eds. The polymerase chain reaction. Birkhauser, Boston. pp 174-181.

Woods, P.E., J.D. Paulauskis, L.A. Weigt, M.A. Romano, and S.I. Guttman. 1989. Genetic variation in laboratory and field populations of the midge, *Chironomus tentans* FAB: Implications for toxicology. *Environ. Toxicol.* 8:1067-1074.

VITA

Susan Gail Elizabeth Nadig was born at Edwards AFB in the Mohave Desert of California on December 18, 1970. She attended elementary schools in Alabama, California, Washington, and Tennessee, and graduated high school from University School of Jackson, Jackson, Tennessee, in May 1989. In August 1989, she entered Union University, Jackson, Tennessee, where, in May 1993, she graduated with honors and recieved a Bachelor of Science degree in Biology. In January 1994, she entered the University of Tennessee, Knoxville, and in May 1996 received a Master of Science in Life Sciences with an emphasis in Environmental Toxicology.

After graduation, Ms. Nadig will be employed by the University of Alaska, Anchorage.