COMBINING ENVIRONMENTAL CHEMISTRY, SOMATIC BIOMARKERS, AND POPULATION GENETICS: AN INNOVATIVE APPROACH IN WILDLIFE ECOTOXICOLOGY

A Dissertation

by

COLE WESLEY MATSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Wildlife and Fisheries Sciences

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Approved as to style and content by:

John W. Bickham (Chair of Committee)	Theo Colborn (Member)
Rodney L. Honeycutt (Member)	Gilbert T. Rowe (Member)
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ABSTRACT

Combining Environmental Chemistry, Somatic Biomarkers, and Population Genetics: an Innovative Approach in Wildlife Ecotoxicology. (May 2004)

Cole Wesley Matson, B.S., Texas A&M University;

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Chair of Advisory Committee: Dr. John W. Bickham

The Caspian region and specifically the Apsheron peninsula of Azerbaijan is known to be polluted with a variety of environmental contaminants, making risk assessment difficult. The wetlands of Sumgayit contain particularly complex mixtures of contaminants. Flow cytometry and the micronucleus assay were used to assess chromosomal damage in aquatic turtles and frogs inhabiting contaminated wetlands in Azerbaijan. By evaluating biomarkers that are indicative of somatic effects, elevated chromosomal damage was documented at several sites in Azerbaijan relative to reference sites. Sediment samples were analyzed for polycyclic aromatic hydrocarbons (PAHs), organochlorines (OCs), and mercury to evaluate contaminant associations with genetic damage. Sediment samples revealed heterogeneous patterns of PAH and mercury concentrations throughout Sumgayit. Significant positive correlations were documented between both PAH and mercury sediment concentrations and chromosomal damage. Population genetic methods were employed to study the effects of long-term chronic contaminant exposure in marsh frogs from Sumgayit. The Sumgayit region has

reduced levels of genetic diversity, likely due to environmental degradation. One of the most contaminated sites in Sumgayit, WTP, appears to be a source of new mutations as a result of an increased mutation rate. Finally, the Sumgayit region seems to act as an ecological sink, with levels of gene flow into the region exceeding gene flow out of the region. This study provides not only exposure and biomarker data, but also an integrated method for assessing the cumulative population impacts of contaminant exposure by studying both population genetic and evolutionary effects. The results presented here will be used in conjunction with those of ongoing research involving both wildlife and humans to develop comprehensive ecological and human risk assessments.

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NOMENCLATURE

π	Nucleotide Diversity
μ	Mean
μ	Mutation Rate
AA	Alti-Agach
ALI	Ali Bairamly
AUP	Animal Use Protocol
BaP	Benzo(a)Pyrene
bp	Base Pair
CAP	Chlor-Alkali Plant
CV	Coefficient of Variation
DDE	2,2-Bis-(*p*-chlorophenyl)-1,1-dichloroethylene
DDT	2,2-Bis-(p-chlorophenyl)-1,1,1-trichloroethane
FCM	Flow Cytometric Method
FPCV	Full Peak Coefficient of Variation
h	Haplotype Diversity
Hg	Mercury
HMW	High Molecular Weight
HPCV	Half Peak Coefficient of Variation
LMW	Low Molecular Weight
ML	Maximum Likelihood
MN	Micronucleus
mtDNA	Mitochondrial DNA
n	Sample Size
N_e	Effective Population Size
NEF	Neftchala
Nm	Effective Migrants
OC	Organochlorines
PAH	Polycyclic Aromatic Hydrocarbons
PCB	Polychlorinated Biphenyl
PI	Propidium Iodide
ppb	Parts Per Billion
ppm	Parts Per Million
RBC	Red Blood Cell
TAMU	Texas A&M University
WTP	Wastewater Treatment Plant

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CHAPTER I

INTRODUCTION

The purpose of this dissertation is to integrate several normally separate areas of research to provide a more in depth understanding of the impacts of chronic contaminant exposure on populations. In particular, the application of population genetic methods to ecotoxicology is a relatively new and rapidly developing area of toxicology. This study was conducted in Azerbaijan, a former Soviet republic located on the Caspian Sea. Azerbaijan is an interesting area for ecotoxicological research for several reasons. First, Azerbaijan contains complex mixtures of a wide variety of environmental contaminants. Second, there are populations of both wildlife and humans living within the most contaminated areas of Azerbaijan. Third, the sources of contamination are relatively well known. And finally, knowledge gained through ecotoxicological studies can be utilized to reduce human risk via education, remediation, and prevention.

This dissertation consists of three separate but interwoven projects. Chapters II-IV represent the findings of these three projects. Each of the projects builds off of the results and conclusions of the previous chapter. The first project involved the use of

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This dissertation follows the style and format of *Ecotoxicology*.

genetic biomarkers to estimate genetic damage in turtle populations from both experimental and reference sites in Azerbaijan. The results of this study are presented in Chapter II.

The second project involved the use of genetic biomarkers to estimate genetic damage in frog populations from multiple experimental populations with Sumgayit and two reference populations from other areas of Azerbaijan. The results of this study are presented in Chapter III.

The third project consisted of a population genetics approach to ecotoxicology. By studying the genetic patterns found in marsh frogs throughout Azerbaijan I wanted to investigate possible reductions in genetic diversity and/or increased mutation rates at experimental sites in Sumgayit. I also examined gene flow for evidence that Sumgayit might be acting as an ecological sink. The results of the population genetics study are presented in Chapter IV.

CHAPTER II

CHROMOSOMAL DAMAGE IN TWO SPECIES OF AQUATIC TURTLES (*EMYS ORBICULARIS* AND *MAUREMYS CASPICA*) INHABITING CONTAMINATED SITES IN AZERBAIJAN

Introduction

The Azerbaijan Republic is a former Soviet republic located on the western shore of the Caspian Sea. The Caspian region is well known for its major oil reserves and its unique fauna. In recent years the severe environmental problems of the Caspian have become more widely known (Dumont, 1995; Cullen, 1999; Watanabe et al., 1999; Kajiwara et al., 2002; Kajiwara et al., 2003; Tanabe et al., 2003). In particular, Azerbaijan is currently in the midst of an environmental crisis, primarily resulting from the poor environmental practices of the Soviet era. In addition to large scale oil production, the Apsheron Peninsula also housed a major concentration of the former Soviet Union's chemical and petroleum processing plants. Much of this industry was based in the city of Sumgayit on the northern shore of the peninsula. This intense industrial development, coupled with decades of inadequate environmental protection resulted in the environmental devastation of Sumgayit (Bickham et al., 2003; Shelton, 2003). Environmental and economic conditions became so dire that the government of Azerbaijan asked the United Nations Development Programme (UNDP) and World

Health Organization (WHO) to assist in the rehabilitation of Sumgayit. As a result, researchers have been investigating various human cancer rates and morbidity in Sumgayit. Cancer rates have now been shown to be significantly higher in Sumgayit than other parts of Azerbaijan (Andruchow, 2003). There is also concern regarding possible increased frequencies of neural tube and other birth defects in Sumgayit. The full extent of the environmental contamination in Sumgayit is just beginning to be understood. This research and a number of other studies are being used to develop better human and ecological risk assessments for Sumgayit.

Three sites in Azerbaijan were selected to investigate chromosomal damage in aquatic turtles (Fig. 2-1), including a wetland adjacent to the industrial wastewater treatment plant within the industrial zone of Sumgayit. The other two sites (Neftchala and Ali Bairamly) were selected as reference sites, as neither site has significant industrial activity. Ali Bairamly and Neftchala are both located on the Kura River in eastern Azerbaijan. Neftchala is downstream from Ali Bairamly and nearly at the mouth of the Kura River. While no previous contaminant analyses have been done on Neftchala, sediment and turtle tissue contaminant levels presented in Swartz et al. (2003) suggest that Ali Bairamly is a relatively good reference site and that Sumgayit is highly contaminated. A number of organochlorines, metals, and polycyclic aromatic hydrocarbons (PAHs) were elevated in sediments and turtle tissues from Sumgayit. Although the environmental problems of the Caspian Sea and its wildlife have been well documented (Dumont, 1995; Bickham et al., 1998b; Cullen, 1999; Watanabe et al.,

1999; Kajiwara et al., 2002; Kajiwara et al., 2003; Tanabe et al., 2003), few articles have addressed the extensive environmental damage done to Sumgayit. However, Bickham et al. (2003) discuss the environmental challenges faced by this highly contaminated city in detail, while Swartz et al. (2003) provide the first wildlife ecotoxicological studies of Sumgayit.

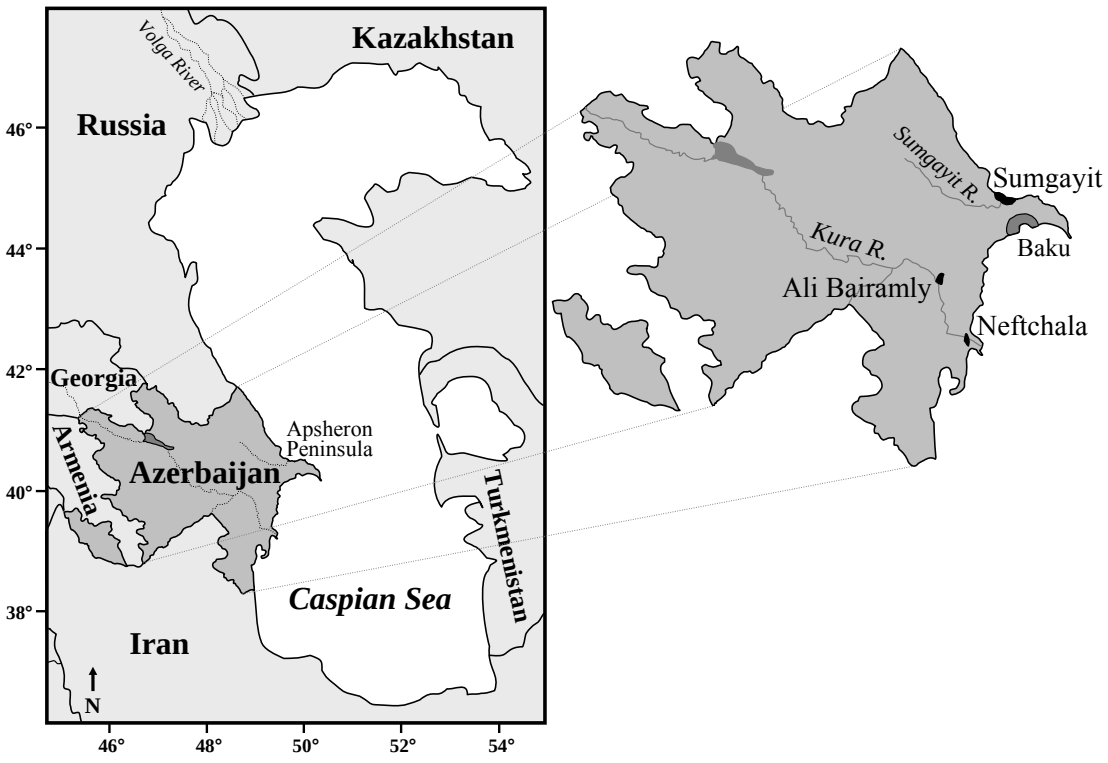


Figure 2-1. Map of Azerbaijan and turtle collecting localities.

Due to the variety of chemical plants and factories in Sumgayit, there are numerous potentially toxic chemical contaminants throughout this region. Of particular interest are the PAHs. There are several PAHs that are known to be highly clastogenic, mutagenic,

and genotoxic. Perhaps the most notorious of these is benzo(a)pyrene (BaP). However, BaP often accounts for only a small proportion of mutagenicity (Chuang et al., 1992; Matsumoto et al., 1998). There is a large number of possible and known clastogens within the PAH family, which makes it difficult to determine which individual chemical or group of chemicals may be the primary cause of observed genetic damage. One approach has been to only consider documented clastogens, while another has been to analyze PAH levels in a combined method. The problem with only looking at known clastogens is that many PAHs have not been fully studied and characterized, and more importantly the possible additive or synergistic effects of the complex mixtures one finds in nature are almost impossible to accurately predict (Reeves et al., 2001). In this study, we calculated sediment concentrations for forty eight individual PAHs. In order to simplify the analyses, we grouped the individual chemicals by the number of rings in the molecule. The potential effects of PAHs with three or fewer rings were analyzed separately from those with four or more and these groups are referred to as low and high molecular weight PAHs, respectively.

Mercury is a well known contaminant that is clastogenic and a spindle inhibitor. Mercury is of particular interest in Sumgayit as there has been an estimated 1,566 tons spilled in the vicinity of the chlor-alkali plant. World Bank is currently funding a cleanup at this site; however, there is significant mercury contamination well outside of the proposed cleanup area. In fact, elevated concentrations of mercury have been documented in both sediments and turtle tissues from the wetland adjacent to the

wastewater treatment plant in Sumgayit (Swartz et al., 2003). Mercury was detected in sediments from all three of our experimental sites.

The complex mixtures of contaminants found in Sumgayit makes theoretical risk assessments difficult. To better understand the damage caused by inhabiting these polluted environments, we performed two genetic biomarker tests on aquatic turtles. We used the flow cytometric method (FCM) and the micronucleus (MN) assay. FCM was used to estimate DNA content variation within red blood cells (RBC) of both European pond turtles (*Emys orbicularis*) and Caspian turtles (*Mauremys caspica*). This method has been shown to be an effective biomarker of chromosomal damage, caused by a variety of contaminants including PAHs, radionuclides, and some pesticides, in many wildlife studies (Bickham et al., 1988; McBee and Bickham, 1988; Dallas and Evans, 1990; George et al., 1991; Lamb et al., 1991; Bickham et al., 1992; Bickham et al., 1994; Custer et al., 1994; Lamb et al., 1995; Lingenfelser et al., 1997a; Lingenfelser et al., 1997b; Lowcock et al., 1997; Theodorakis et al., 2001; Matson et al., submitted). Of more importance to this study is the fact that flow cytometry data tend to correlate well with petroleum products and PAHs in particular (Bickham et al., 1998a; Custer et al., 2000). The MN assay was also used as a biomarker of chromosomal damage in RBCs. This test has an extensive record of successfully documenting elevated levels of chromosomal damage resulting from clastogens. More importantly, micronucleus frequency has been used in many wildlife ecotoxicological studies where animals are

exposed to a variety of contaminants (Lyne et al., 1992; Alsabti and Metcalfe, 1995; Bickham et al., 1998b; Bresler et al., 1999; Bombail et al., 2001; Izquierdo et al., 2003).

There are only two species of aquatic turtle in Azerbaijan. The European pond turtle (*Emys orbicularis*) and the Caspian turtle (*Mauremys caspica*) are both found throughout the large rivers, wetlands, and ponds of Azerbaijan. Pond turtles were collected from Sumgayit, Neftchala, and Ali Bairamly to be tested for chromosomal damage using the FCM and the MN assay. We were unable to catch a sufficient number of Caspian turtles from Ali Bairamly, which limited the analysis for that species to a comparison between Neftchala and Sumgayit. Pond turtles and Caspian turtles are both carnivorous, feeding on small invertebrates, aquatic insects, amphibians, and small fish (Ernst and Barbour, 1989). This places these species at the upper end of the aquatic food web. The propensity of many contaminants to bioaccumulate and biomagnify is well documented and of real concern regarding predators in a contaminated ecosystem. In fact, increased levels of diverse contaminants were documented previously by Swartz et al. (2003) in both pond turtles and Caspian turtles inhabiting our study site in Sumgayit as compared to reference values and levels found at Ali Bairamly. Swartz et al. (2003) also compared micronucleus frequencies between *Emys* from Sumgayit and Ali Bairamly. Micronucleus frequencies were higher in turtles from Sumgayit, although not statistically significant, due to a small sample size. The objectives of this study were to evaluate whether environmental contaminants have caused genetic damage in aquatic

turtles inhabiting three different locations in Azerbaijan, and to attempt to identify potential causal agents of any documented genetic damage.

Materials and methods

Sample Collection

Two species of turtles were collected from three sites in Azerbaijan (Fig. 2-1). European pond turtles (*Emys orbicularis*) were collected from Neftchala (1999, *n*=11), Ali Bairamly (2002, *n*=14), and Sumgayit (2002, *n*=31). Caspian turtles (*Mauremys caspica*) were collected from Neftchala (1999, *n*=10) and Sumgayit (2002, *n*=9). Coordinates for collecting sites are: Neftchala, N 39º24', E 49º15'; Ali Bairamly, N 39º56.777', E 48º55.060'; Sumgayit, N 40º36.857', E 49º37.280'. Blood samples were collected by venipuncture of the jugular vein, and subsequently placed in heparinized cryovials to prevent clotting (Jenkins, 1996). Samples were immediately placed on dry ice until they could be permanently archived at -80ºC. Collection methods were conducted as specified in Texas A&M AUP # 2001-132, "Molecular analyses of the genetic structure of wildlife populations exposed to environmental contamination."

Flow Cytometry

Heparinized blood samples were used to determine cell to cell variation in DNA content following the methods of Vindelov and Christiansen (1994). Briefly, samples were randomized prior to processing to avoid experimental bias. The samples were quickly thawed and 50 µl added to a trypsin/detergent solution for digestion. After 10 minutes,

trypsin inhibitor and RNAse were added to stop the reaction and to degrade RNA, which will also take up the dye. The solution was filtered through 30 μm nylon mesh and propidium iodide (PI) was added. After 15 minutes on ice, the samples were analyzed on a Coulter Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA). Cells were illuminated with a Coherent laser at 514 nm at 500 mW of power to excite the PI. Fluorescent emission was then measured. Cells were gated on side scatter, forward scatter and the ratio of peak to integrated fluorescence. Ten thousand nuclei, which satisfied all gating parameters, were measured from each sample and the intercellular variation in DNA content reported as the full peak coefficient of variation (FPCV). Samples that did not have 10,000 nuclei counted by the end of the four minute run were excluded from subsequent statistical analyses. *Emys* population comparisons were performed for all three localities, while *Mauremys* were only compared for Sumgayit and Neftchala.

Micronucleus Assay

Blood samples were used to make duplicate air-dried smears on clean glass slides for each turtle. Smears were stained with the Bayer Hema-Tek® modified Wright-Giemsa stain pack (Bayer Diagnostics, Elkhart, IN) following the manufacturer's protocol. Five thousand RBCs, in the monolayer portion of the smear, were examined under 1000X magnification with oil immersion. The number of micronucleated RBCs was determined, and recorded as micronuclei per one thousand RBC. All MN examinations were performed by G.P. without any knowledge of where samples came from or what

species they belonged to. MN comparisons were only performed on *Emys* from Sumgayit and Ali Bairamly, as smears were not available from *Emys* or *Mauremys* from Neftchala. MN data collected for this study were added to the *Emys* MN data presented in Swartz et al. (2003), which were also performed by G.P.

Biomarker Data Analysis

All biomarker population comparisons were made using SPSS ver. 11.0.1 (SPSS Inc., Chicago, IL). Parametric ANOVAs were performed where assumptions of normality were met, while those that failed were analyzed using nonparametric methods. In addition to univariate biomarker comparisons, a multivariate analysis was performed with both biomarkers for *Emys* comparing Sumgayit and Ali Bairamly. This was the only comparison for which we had both flow cytometric and micronucleus data.

Sediment Contaminant Analysis

Sediment samples were collected from each of the collecting sites. One sample was collected for Neftchala (1999) and Ali Bairamly (2002), and three sediment samples were collected for Sumgayit (2002). Each sample was tested for mercury and individual PAH concentrations. Mercury was only tested for one of the Sumgayit sediment samples. Total mercury was calculated using EPA method 7473 (U.S. Environmental Protection Agency, 2000) on a Milestone DMA-80 mercury analyzer (Milestone Inc., Monroe, CT). Sediment samples were extracted following the methods of Schantz et al. (1997). PAH concentrations were determined following the methods of Cizmas et al.

(2003). All three Sumgayit samples were tested for forty eight individual PAHs. Concentrations presented for Sumgayit represent means of the three samples. PAHs were separated into high molecular weight (HMW) and low molecular weight (LMW) classes. Individual PAHs were considered HMW if the molecule consisted of four or more rings, while those with three or fewer were considered LMW. A Pearson correlation analysis was performed to look for significant associations between population mean FPCV and our three contaminant groups (Hg, HMW PAHs, LMW PAHs). No correlations were attempted with the MN data, as we only have this data for two populations.

Results

Emys orbicularis *Flow Cytometry*

The pond turtle locality FPCV data was not significant for the Shapiro-Wilk's test of normality. The ANOVA test resulted in highly significant population differences ($P<.001$; Fig. 2-2A). Levene's test of homogeneity of variances was moderately significant ($P=.026$). Fisher's LSD with a Bonferroni correction for multiple comparisons was then employed to test population pairwise comparisons. Neftchala FPCVs ($\mu=5.10$) were significantly higher than both Ali Bairamly ($\mu=4.58$; $P<.001$) and Sumgayit ($\mu=4.76$; $P=.002$). While the mean and median values at Sumgayit were higher than Ali Bairamly, the population difference was not significant ($P=.114$; Fig. 2-2A). A weighted least squares procedure was then conducted on the FPCV data following the recommendations of Misra and Easton (1999). The results of the weighted

ANOVA were again highly significant ($P<.001$), however, in the weighted analysis the LSD with Bonferroni correction yielded significant differences for all population pairwise comparisons. Neftchala was again higher than both Ali Bairamly ($P<.001$) and Sumgayit ($P=.004$), while Sumgayit was now significantly higher than Ali Bairamly ($P=.038$).

Emys orbicularis *Micronucleus Assay*

Pond turtles from Ali Bairamly ($n=23$) were compared to the population of turtles inhabiting the ponds adjacent to the wastewater treatment plant ($n=36$). On average, turtles from Sumgayit ($\mu=9.71$) had more micronuclei than those from Ali Bairamly ($\mu=5.96$; Fig. 2-2B). The data presented here represents the data presented previously by Swartz et al. (2003) in addition to samples collected in 2002 (Ali Bairamly $n=14$, WTP $n=31$). The combined MN data were not normally distributed ($P<.001$; Shapiro-Wilk's Test), therefore, a nonparametric method was used to compare the two populations. The two sample Kolmogorov-Smirnov test resulted in a significant difference between the Sumgayit and Ali Bairamly populations ($P=.024$).

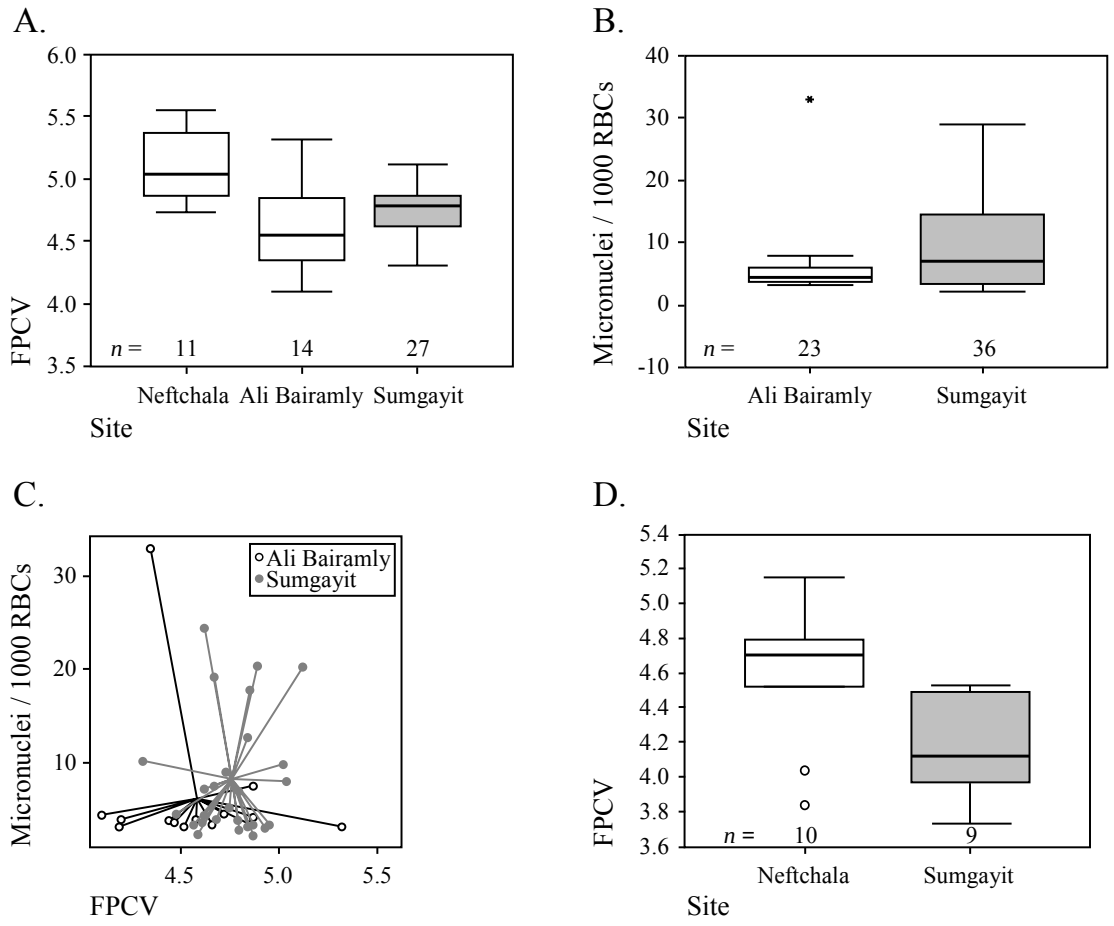


Figure 2-2. Turtle biomarker data. A. Box plots of FPCVs from European pond turtles; B. Box plots of Micronucleus Data for European pond turtles; C. Biomarker response in European pond turtles; D. Box plots of FPCV data from Caspian turtles. Box plots consist of boxes representing interquartile range transected by medians, vertical lines represent range, while ○ and * represent outliers and extreme outliers, respectively.

Emys orbicularis *Biomarker Response*

Biomarker response was significantly higher in *Emys* from Sumgayit when compared to those from Ali Bairamly (Fig. 2-2C). A multivariate analysis was performed using both FCM and MN to compare combined biomarker response in pond turtles from Sumgayit

and Ali Bairamly. Box's test of equality of covariance matrices was not significant (P=.147). Wilks' Lambda was significant for the unweighted multivariate analysis (P=.032).

Mauremys caspica *Flow Cytometry*

Caspian turtles inhabiting the fish hatchery in Neftchala (n=10) were compared to turtles from the ponds adjacent to the wastewater treatment plant in Sumgayit (n=9). Population FPCVs for Neftchala and Sumgayit were μ=4.59 and μ=4.19, respectively. The population FPCV data were normally distributed according to the Shapiro-Wilk's Test (Neftchala, P=.168; Sumgayit, P=.155). Equal variances were not assumed in the t-test as Levene's test for equality of variances was significant (P=.032). The t-test for equality of means was significant (P=.024). FPCVs were significantly higher in the turtles from Neftchala (Fig. 2-2D). In addition to documenting differences in DNA content variability among groups, we also discovered a triploid individual while doing genome size comparisons. The only triploid turtle observed within this study was from Sumgayit (TAMU specimen AK17352).

Table 2-1. Sediment individual PAH (ppb) and mercury (ppm) concentrations*.

	Sites		
LMW PAH Analytes	Neftchala	Ali Bairamly	Sumgayit
Naphthalene	12.2	0.3	2.0
C1-Naphthalenes	18.0	0.7	6.2
C2-Naphthalenes	33.3	1.0	8.0
C3-Naphthalenes	38.2	1.0	9.7
C4-Naphthalenes	29.1	0.6	6.4
Biphenyl	9.6	0.5	3.7
Acenaphthylene	0.9	< D.L.	4.3
Acenaphthene	1.5	0.9	1.2
Dibenzofuran	4.5	0.1	1.2
Fluorene	4.9	0.2	5.7
C1-Fluorenes	14.3	< D.L.	7.6
C2-Fluorenes	23.4	< D.L.	3.1
C3-Fluorenes	23.9	< D.L.	4.2
Carbazole	2.6	0.2	2.1
Dibenzothiophene	3.8	0.3	1.0
C1-Dibenzothiophene	6.8	0.7	1.3
C2-Dibenzothiophene	11.7	1.1	1.9
C3-Dibenzothiophene	2.7	< D.L.	1.1
C4-Dibenzothiophene	< D.L.	< D.L.	0.6
Anthracene	2.8	0.1	3.9
Phenanthrene	26.1	1.3	24.0
C1-Phenanthrene/Anthracene	75.1	2.6	21.5
C2-Phenanthrene/Anthracene	54.4	1.2	13.6
C3-Phenanthrene/Anthracene	33.2	0.3	6.9
C4-Phenanthrene/Anthracene	12.9	< D.L.	5.6
2-ring PAHs	140.4	4.1	35.9
3-ring PAHs	305.5	8.9	110.7
Total LMW PAHs	446	13	147

Table 2-1. Continued.

	Sites		
HMW PAH Analytes	Neftchala	Ali Bairamly	Sumgayit
Naphthobenzothiophene	5.9	< D.L.	11.2
C1-Naphthobenzothiophene	7.8	< D.L.	4.2
C2-Naphthobenzothiophene	8.2	< D.L.	1.9
C3-Naphthobenzothiophene	4.2	< D.L.	0.8
Fluoranthene	6.3	0.6	40.7
Pyrene	7.0	0.4	30.2
C1-Fluoranthenes/Pyrenes	14.6	0.3	25.0
C2-Fluoranthenes/Pyrenes	21.1	< D.L.	19.5
C3-Fluoranthenes/Pyrenes	17.8	< D.L.	5.9
Benz(a)anthracene	3.8	1.0	32.9
Chrysene	17.7	0.4	46.1
C1-Chrysenes	26.2	< D.L.	14.1
C2-Chrysenes	20.7	< D.L.	9.6
C3-Chrysenes	8.2	< D.L.	2.0
C4-Chrysenes	2.2	< D.L.	5.0
Benzo(b)fluoranthene	11.0	0.3	57.7
Benzo(k)fluoranthene	1.6	0.2	30.0
Benzo(e)pyrene	13.2	0.2	40.7
Benzo(a)pyrene	2.5	0.1	36.6
Dibenzo(a,h)anthracene	2.6	< D.L.	11.0
Perylene	20.9	1.1	8.7
Indeno(1,2,3-c,d)pyrene	3.4	< D.L.	47.4
Benzo(g,h,i)perylene	10.3	0.1	37.6
4-ring PAHs	171.7	2.7	249.1
5-ring PAHs	51.8	1.8	184.7
6-ring PAHs	13.7	0.1	85.1
Total HMW PAHs	237	5	519
Total PAHs	683	18	665
Mercury	0.0283	0.0193	1.49

*< D.L. represents samples below method detection limit.

Sediment Contaminants

Individual PAH and mercury levels were calculated for each of the collecting localities (Table 2-1). Total PAH levels were much higher at Neftchala and Sumgayit (683 and 665 ppb, respectively) than at Ali Bairamly (18 ppb). While similar in total PAH levels, Neftchala and Sumgayit differed in their PAH profiles with Neftchala having more LMW PAHs and Sumgayit heavily weighted towards HMW PAHs (Fig. 2-3). Mercury levels were low at both Neftchala and Ali Bairamly compared to the high level found in Sumgayit. Mercury concentrations found at Ali Bairamly and Neftchala are within accepted background ranges for freshwater sediments, while the concentration found in Sumgayit is more than double the upper effects threshold (Buchman, 1999). Pearson correlations of sediment contaminant levels and *Emys orbicularis* flow cytometric data resulted in only one significant comparison. LMW PAHs were significantly correlated with mean FPCVs (P=.027). Upon finding a significant correlation between our biomarker and LMW PAHs we further separated our LMW PAHs into two and three ring molecules. While two ring PAHs show no association with chromosomal damage (FPCV), three-ring PAHs show a significant association (P=.003) with R^2=1.00 (Fig. 2-4). This suggests that the correlation between LMW PAHs and chromosomal damage is being driven by the three ring molecules.

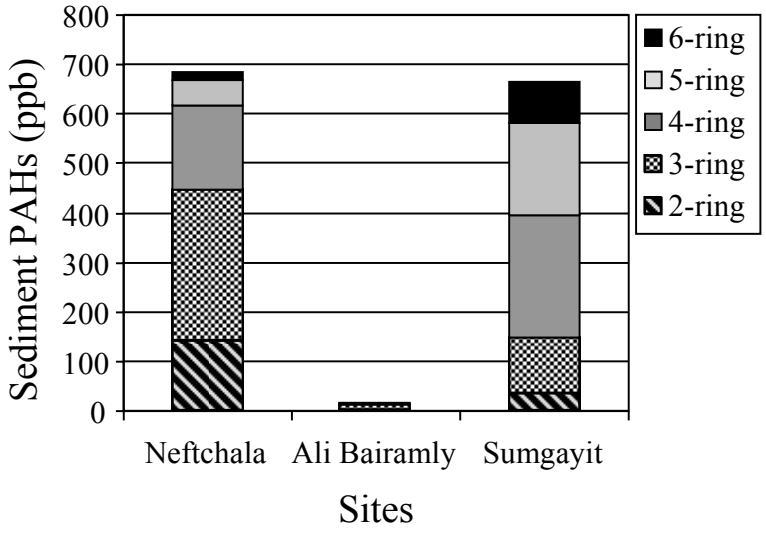


Figure 2-3. Chart of sediment PAH concentrations broken down by ring number.

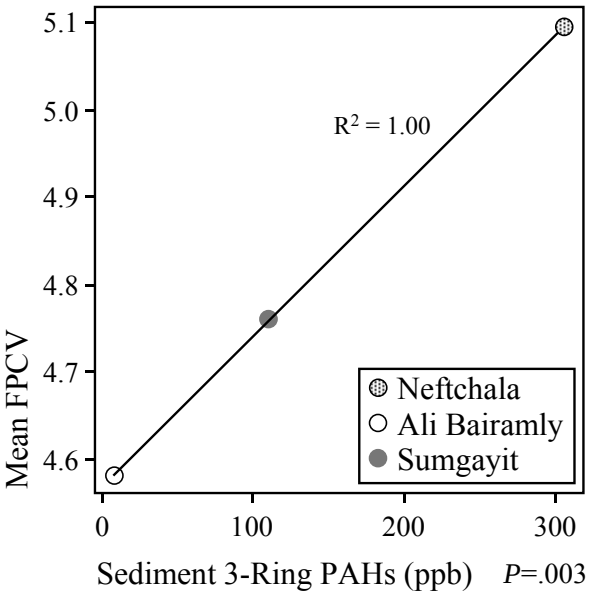


Figure 2-4. Linear regression of sediment three-ring PAH concentrations and *Emys* mean FPCV.

Discussion

The highest estimates of chromosomal damage in both *E. orbicularis* and *M. caspica* were from the Neftchala populations. These are unexpected results as there is no significant industrial complex near Neftchala. However, the sediment PAH data are consistent with the FCM data. Neftchala is not an acceptable reference site and should be considered an additional experimental site. Results from the Ali Bairamly site are consistent with those of Swartz et al. (2003) in suggesting that Ali Bairamly is an appropriate reference site in regards to both PAHs and mercury. Both Neftchala and Sumgayit show significantly elevated levels of genetic damage as estimated by FCM and MN.

Trying to determine which contaminants or sets of contaminants are responsible for this increase in damage is complicated by the numerous chemicals and possible routes of exposure. Just considering PAHs does not simplify this problem, as it has been shown that the predicted genotoxicity of complex mixtures is not a good estimate of actual effects (Chuang et al., 1992; Matsumoto et al., 1998; Reeves et al., 2001). It is difficult to investigate possible associations between biomarkers and contaminants data when there are only three data points for each comparison. Considering the large number of individual PAHs, determining which individual contaminant is likely responsible for any observed genotoxicity is futile. It is likely that the observed differences are due to the cumulative effects of many individual contaminants, some of which will remain unknown. By grouping PAHs based on ring number we can look for associations with

more confidence. It is generally thought that most two-ring PAHs lack significant genotoxicity, although there is some debate about a few of the molecules. For example, naphthalene has been shown to be clastogenic in a few human and mouse cell lines (Schreiner, 2003). Chuang et al. (1992) found that alkylated three-ring and four-ring PAHs accounted for the majority of mutagenicity in some complex mixtures. It has also been shown that some of the five-ring PAHs are highly genotoxic (Reeves et al., 2001). Our results seem to agree with Chuang et al. (1992), in that our chromosomal damage correlates best with the three-ring PAHs. One must except that there are many other PAHs that could have played a role in our results. The PAHs found at Neftchala are weighted towards molecules with fewer rings, while Sumgayit is weighted towards molecules with more rings. Neftchala has a higher concentration of 84% of the LMW PAHs; whereas, Sumgayit has a higher concentration for 61% of the HMW PAHs. The high molecular weight PAHs at Sumgayit include some genotoxic molecules (i.e. benzo(a)pyrene). Ali Bairamly has the lowest concentrations of every PAH found at any one of the three sites.

Mercury has been shown to be clastogenic and to disrupt spindle fiber formation (Betti et al., 1992; De Flora et al., 1994; Ogura et al., 1996; Queiroz et al., 1999; Amorim et al., 2000; Ochi, 2002). Mercury concentration was substantially higher in Sumgayit than in either Neftchala or Ali Bairamly. This suggests that mercury is not a significant factor in the elevated genotoxicity (as estimated by FCM) at Neftchala. Although our FCM data suggests that mercury is not a significant factor in the observed genotoxicity,

the presence of a triploid *Mauremys* at Sumgayit could be evidence that mercury (or some other contaminant) is acting as a spindle inhibitor. Triploidy can result from inhibition of spindle formation; however, no conclusions can be made regarding the triploid *M. caspica* as it represents only a single observation. Mercury has been shown to increase MN frequency (Nepomuceno et al., 1997; Sanchez-Galan et al., 1999; Ayllon and Garcia-Vazquez, 2000; Sanchez-Galan et al., 2001). Swartz et al. (2003) found a significant correlation between mercury concentrations in liver and MN frequency in *E. orbicularis* from Sumgayit. However, the observed increase in MN frequency could also be caused by PAHs. We cannot discriminate between these two factors as possible causal agents in the MN frequency increase at Sumgayit. In fact, it is likely that both mercury and PAHs play some role in the observed differences in MN between Sumgayit and Ali Bairamly.

Sediment PAH data revealed that contaminant levels in Sumgayit declined over the period of this study. Swartz et al. (2003) documented extremely high levels of PAHs in sediment samples collected in 1996. Total sediment PAH concentrations at the WTP in Sumgayit decreased from approximately 95 ppm to 665 ppb. This is the result of three factors. The first and most important is the substantial reduction in chemical plant output over the last ten to fifteen years. Chemical processing is down to about ten or fifteen percent of production capacity, with several plants no longer in operation. This factor explains the reduced inflow of new contaminants. Biotransformation and physical movement of contaminants are the other two major factors likely to have led to this

substantial decrease in PAH concentrations. Biotransformation and photolysis of PAHs is well documented (Mill et al., 1981; Heitkamp and Cerniglia, 1987; Chen et al., 2001; Mills et al., 2003) and has likely played an important role in Sumgayit. In addition, the geology of Sumgayit lends itself to rapid physical transfer of contaminants. The Apsheron peninsula is porous allowing movement of contaminants down into the substrate and out to the Caspian Sea. The close proximity of our study site in Sumgayit to the Caspian Sea also permits runoff from rainfall to sweep contaminants directly into the sea. In the case of mercury, our levels (1.49 ppm) are not substantially lower than those documented by Swartz et al. (2003) from 1996 (2.7 ppm). Although the magnitude of change for mercury is much smaller, the trend is still in the right direction. Photographs taken at this site in May of 1996 and 2001 document a significant improvement in this Sumgayit wetland habitat which corresponds with the substantial reductions of contaminant concentrations (Fig. 2-5).

Figure 2-5. Photographs of the wetlands adjacent to the industrial wastewater treatment plant in Sumgayit. Photographs were taken in May of 1996 (left) and 2001 (right).

There are many other contaminants, besides PAHs and mercury, which could have caused or contributed to the chromosomal damage that we documented in Azerbaijan. The increased MN frequencies at Sumgayit could be the result of dozens of contaminants that have been produced or used within the industrialized zone of Sumgayit. Many of these contaminants are known clastogens and aneugens. There are large amounts of asbestos contamination within the industrial sector of the city due to the deterioration of pipe insulation which consisted of asbestos wrapped with spun glass. Asbestos is known to cause chromosomal damage and to disrupt mitosis, resulting in both aneuploidy and polyploidy (Dopp et al., 1995; Jaurand, 1997; Dopp and Schiffmann, 1998). There have also been a large number of other chemicals produced in Sumgayit and used throughout Azerbaijan that could be involved in this chromosomal damage. One group of chemicals that has also been shown to be genotoxic is the polychlorinated aromatic hydrocarbons. One of the most toxic of these is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Some organochlorines and organophosphates

have also been shown to clastogenic (Thies et al., 1996; Cicchetti et al., 1999; Gauthier et al., 1999; Kovalkovicova et al., 2000). As a number of organochlorines and organophosphates have been produced in Sumgayit and used throughout Azerbaijan, we cannot rule out these classes of contaminants as having been involved in our observed chromosomal damage.

The FCM data presented in this paper support the conclusion that Neftchala exhibits significantly higher levels of genetic damage than Sumgayit or Ali Bairamly. This demonstration of the genotoxicity of the Kura River in Neftchala is problematic for a number of reasons. Discovery of sites with this level of environmental contamination are never a positive event, however, in this situation it is far worse. Neftchala is the site of one of the largest sturgeon hatcheries in Azerbaijan and it receives its water directly from the Kura River. In fact, the turtles used in this study were collected from this hatchery. Additionally, the World Bank is funding the construction of an even larger hatchery in this same area. Several studies have shown that sturgeon fry exhibit increased mortality when exposed to contaminated sediment (Kocan et al., 1996; Bickham et al., 1998b; Swartz et al., 2003).

Emys orbicularis from Sumgayit show evidence of increased genetic damage compared to a population at Ali Bairamly based on both FCM and MN data. This multiple biomarker response in *E. orbicularis* demonstrates that the environmental degradation of Sumgayit is causing chromosomal damage in at least one species, and suggests that it is

likely to be causing similar damage in other wildlife and possibly humans. The data presented in this paper extend and confirm the observations of Swartz et al. (2003) that high levels of environmental contamination occur in the wetland adjacent to the wastewater treatment plant in Sumgayit. We further confirm a significant increase in genetic damage in *E. orbicularis* from Sumgayit compared to a reference population at Ali Bairamly. The MN data presented for these populations by Swartz et al. (2003) was based on a much smaller sample size and was non-significant. Despite the fact that some contaminant levels in Sumgayit sediment have declined appreciably since 1996, biomarker responses in wildlife indicate continued health impacts.

CHAPTER III

PATTERNS OF GENOTOXICITY AND CONTAMINANT EXPOSURE: EVIDENCE OF GENOMIC INSTABILITY IN THE MARSH FROGS (*RANA RIDIBUNDA*) OF SUMGAYIT, AZERBAIJAN

Introduction

Azerbaijan, a recently independent republic of the former Soviet Union, is well known for its vast oil reserves. The majority of Azerbaijan's petrochemical industry is housed on the Apsheron Peninsula, and many of the physical facilities from the Soviet era are still in use. Baku, the capital and largest city in Azerbaijan, is the location of one of the largest oil fields, while Baku and Sumgayit are the major processing sites. Sumgayit is located on the northern coast of the Apsheron Peninsula adjacent to the Caspian Sea while Baku is located on the southern coast (Fig. 3-1). In addition to being a center of the Soviet petrochemical industry, Sumgayit also contained a major concentration of the former Soviet Union's chemical and manufacturing industries. There were 23 factories operating in Sumgayit during the height of production in the 1980's. Although the chemical industry in Sumgayit collapsed following the breakup of the Soviet Union, the environmental damage remains. Environmental protection was never a priority in Sumgayit, and as such, tremendous amounts of contaminants were released into this area. These contaminants were intentionally released into the air and water as well as by

dumping solid wastes onto open ground. Accidental releases also occurred because of a lack of maintenance on plants which led to leaky pipes and other problems. However, this environmental degradation was not contained around Sumgayit. Contamination originating in Sumgayit has certainly played a role in the environmental problems now facing the Apsheron Peninsula and the entire Caspian Sea. The international community is becoming increasingly aware of the pollution problems facing this region (Dumont, 1995; Cullen, 1999; Watanabe et al., 1999; Kajiwara et al., 2002; Kajiwara et al., 2003; Moore et al., 2003; Tanabe et al., 2003). The environmental problems of Azerbaijan, specifically, have also come to the attention of the world (Bickham et al., 2003; Shelton, 2003; Swartz et al., 2003). The World Bank is currently funding several projects in Azerbaijan. The most important in Sumgayit is the cleanup of mercury-contaminated soils from the chlor-alkali plant. There has been an estimated 1,566 tons of mercury spilled at this site (Bickham et al., 2003). The World Bank has also started construction of the first industrial landfill. This landfill is located near Sumgayit and will be used to safely contain contaminants from a variety of past and present sources.

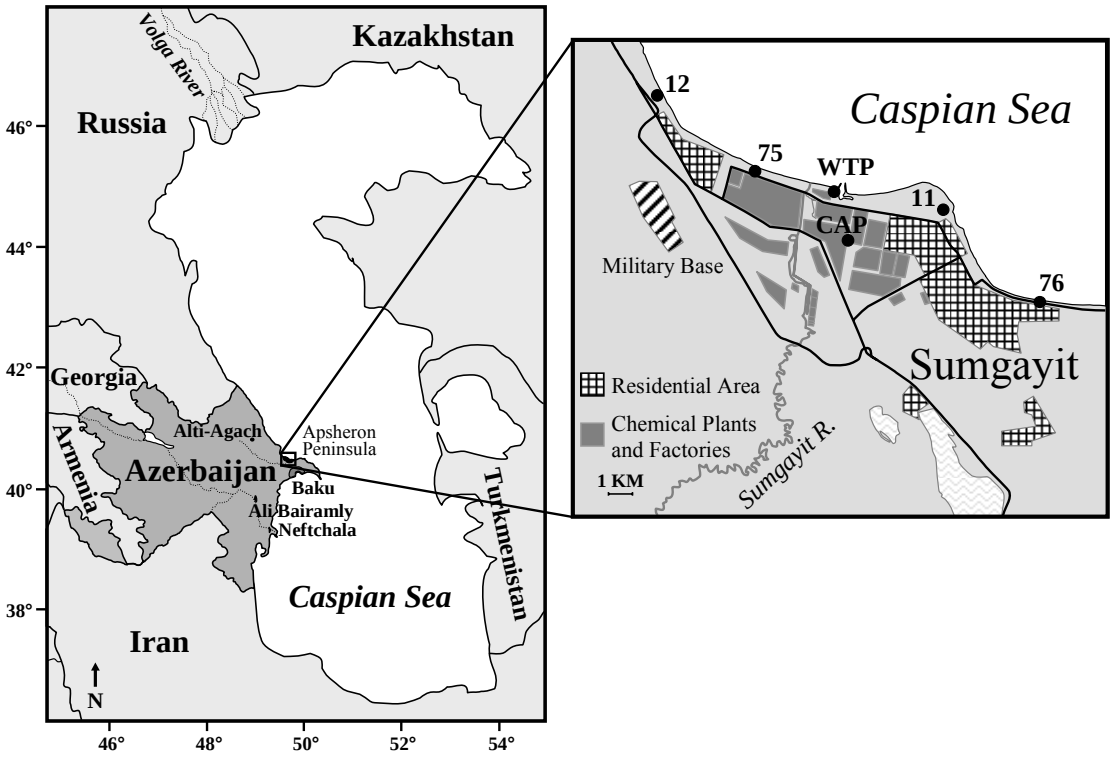


Figure 3-1. Map of marsh frog collecting localities.

There has been a significant reduction in pollution levels since the collapse of the Soviet Union. This reduction is not due to new environmental legislation or through the use of cleaner technology; rather, most of the plants have either closed altogether or are running well below capacity. Although there has been a substantial reduction, the legacy of decades of unrestricted air, water, and soil pollution from the Soviet era will last well into the future. A recently completed study of cancer rates in Azerbaijan found elevated rates in a few types of cancer in Sumgayit (Andruchow, 2003). There is currently research investigating the purported elevated rates of neural tube defects in Sumgayit. This human epidemiological research will be combined with wildlife ecotoxicological

research and basic environmental chemistry to provide us with a better understanding of the risks resulting from exposure to contaminants in Sumgayit. A complicating factor is the fact that there are many Azeri refugees living within the Sumgayit industrial zone (Bickham et al., 2003). These individuals almost certainly have higher contaminant exposures than average Sumgayit residents.

Previous studies have shown that Sumgayit and more specifically the wetlands adjacent to the industrial wastewater treatment plant (WTP) are highly contaminated and capable of causing chromosomal damage in turtles (Swartz et al., 2003). We wanted to build on these studies by investigating the marsh frog (*Rana ridibunda*) which is broadly distributed within Sumgayit, and by adding several additional sites within Sumgayit to look for small-scale patterns in contaminant distributions and biomarker responses. We established eight sampling sites for this study. Six of the sites are within Sumgayit while the remaining two serve as independent reference sites. By adding additional sites and using larger sample sizes than in the previous studies we hoped to increase our ability to discriminate biomarker patterns and identify significant correlations with contaminant levels. We used the marsh frog for a number of reasons. Ranid frogs were used successfully in a number of toxicology studies (Gillan et al., 1998; Monson et al., 1999; Glennemeier and Denver, 2001; Papadimitriou and Loumbourdis, 2002; Gilbertson et al., 2003) and they have proven to be effective indicators of genotoxicity (Clements et al., 1997; Lowcock et al., 1997; Ralph and Petras, 1997; Ralph and Petras, 1998; Rajaguru et al., 2001; Campana et al., 2003). Frogs are highly susceptible to aquatic

contaminants. Not only are they exposed to contaminants via dietary routes, but many are absorbed directly across their permeable skin. *Rana ridibunda* is the only species of frog abundant and widespread throughout Azerbaijan. In fact, this frog can be found in almost all freshwater habitats. Ranids are known to disperse distances as large as two to three kilometers under some circumstances (Peter, 2001; Pilliod et al., 2002; Bulger et al., 2003), but in urban environments movements are far less likely even without absolute barriers (Hitchings and Beebee, 1997). This site specificity is an obvious advantage in ecotoxicological studies.

Previous ecotoxicology studies in Azerbaijan have provided detailed contaminant profiles from both sediment and wildlife tissues (Swartz et al., 2003). The complex mixtures that have been documented in Sumgayit limit our ability to accurately predict the risks associated with contaminant exposure. For this reason, previous studies have relied on biomarker data to provide an estimate of genetic damage in resident wildlife populations. From these data we hope to improve the ability to accurately assess risk to humans and the environment. Previous studies have shown the flow cytometric method (FCM) and the micronucleus assay (MN) to be sensitive biomarkers of chromosomal damage. This manuscript provides evidence of chromosomal damage in turtles inhabiting contaminated areas in Azerbaijan using both the FCM and MN. Flow cytometry has been used effectively as a biomarker of genetic damage resulting from polycyclic aromatic hydrocarbons (PAHs), radionuclides, and even some pesticides (Bickham et al., 1988; McBee and Bickham, 1988; Dallas and Evans, 1990; George et

al., 1991; Lamb et al., 1991; Bickham et al., 1992; Bickham et al., 1994; Custer et al., 1994; Lamb et al., 1995; Lingenfelser et al., 1997a; Lingenfelser et al., 1997b; Lowcock et al., 1997; Bickham et al., 1998a; Custer et al., 2000; Theodorakis et al., 2001; Matson et al., submitted). This manuscript has documented a significant correlation between three-ring PAHs and chromosomal damage as estimated using the FCM. The micronucleus assay is also a widely used biomarker of genetic damage in red blood cells (RBCs). As with the FCM, MN have been used extensively to document genetic damage in wildlife (Lyne et al., 1992; Alsabti and Metcalfe, 1995; Bickham et al., 1998b; Bresler et al., 1999; Bombail et al., 2001; Izquierdo et al., 2003).

The complex mixtures of contaminants found in Sumgayit make it difficult to determine which individual or classes of contaminants are likely to be the cause of any observable biomarker effects. Of particular concern are the genotoxic PAHs (Chuang et al., 1992; Matsumoto et al., 1998; Custer et al., 2000). Sumgayit contains elevated levels of many PAHs as a result of the extensive oil industry on the Apsheron Peninsula (Swartz et al., 2003). Although most PAH congeners have been tested to quantify genotoxicity, there is still debate over how to estimate potential risk with complex mixtures and degradation products (White, 2002). It has been shown that predicted genotoxicity is not a good estimator of risk or effects (Chuang et al., 1992; Matsumoto et al., 1998; Reeves et al., 2001). However, another approach is to group PAHs based on the number of rings in the individual molecules. By either grouping low and high molecular weight PAHs, or grouping based on individual ring number, one can test for associations with a little more

accuracy. High molecular weight (HMW) PAHs are generally more mutagenic than low molecular weight (LMW) PAHs (Chuang et al., 1992; Reeves et al., 2001).

Mercury also has been found in environmentally significant concentrations in sediment and turtle tissue in Sumgayit (Swartz et al., 2003). Mercury is known to disrupt spindle fiber formation in addition to being clastogenic (Betti et al., 1992; De Flora et al., 1994; Ogura et al., 1996; Queiroz et al., 1999; Amorim et al., 2000; Ochi, 2002). We measured sediment mercury concentrations at all sites and frog tissue levels at selected contaminated sites.

Several organochlorines (OCs) have been shown to be clastogenic via the micronucleus assay (Cicchetti et al., 1999; Gauthier et al., 1999; Kovalkovicova et al., 2000). This class of contaminants is also known to have a variety of negative impacts on frogs (Glennemeier and Denver, 2001; Glennemeier and Begnoche, 2002). Swartz et al. (2003) documented elevated levels of several OCs in Sumgayit, so they are clearly contaminants of concern. Additionally, organochlorine contamination of the adjacent Caspian Sea is well documented (Watanabe et al., 1999; Kajiwara et al., 2002; Kajiwara et al., 2003; Tanabe et al., 2003).

Our objectives in this study were to attempt to confirm previous results regarding the genotoxicity of Sumgayit, while expanding on those earlier studies by utilizing *Rana ridibunda* as our model organism. Additionally, by using frogs we were able to assess

the Sumgayit environment at a finer scale. This allowed us to test not only Sumgayit as a region, but also to look for patterns within the Sumgayit area.

Materials and methods

Sample Collection

Marsh frogs were collected from a total of eight sites during 2001 and 2002. Collecting areas consisted of marshes, ponds, and in one case a drainage ditch. A map of collecting locations is presented in Figure 3-1. Site coordinates and sample size information is provided in Table 3-1. Six of the sites are located within the city of Sumgayit, while the remaining two are from reference areas. Five of the sites follow a transect along the Caspian Sea starting at the northwest edge of town well outside of the industrial zone (Site 12), continuing to a pond in front of the Organic Synthesis plant (Site 75), with the middle site representing the ponds along the industrial wastewater treatment plant (WTP), followed by a marsh well within the residential area, with the last site located in a drainage ditch near the eastern edge of the city separated from the industrial zone by the majority of Sumgayit's residential area (Site 76). The final site within Sumgayit was located in the center of the industrial zone in a marsh adjacent to the Chlor-alkali plant (CAP). The reference sites were a small pond in the Caucus Mountains to the northwest of Sumgayit near Alti-Agach (AA) and a fish hatchery in the city of Ali Bairamly on the Kura River (ALI). The transect sites along the Caspian were sampled in May of 2001. Additional samples were collected from three of the transect sites in May/June 2002

(WTP, 12, and 75). During the 2002 collecting trip, we also established the last experimental site (CAP) and the two reference sites (AA and ALI).

Frogs were anaesthetized with MS222 (CAS # 886-86-2; Sigma-Aldrich Corp., St. Louis, MO). Blood samples were then obtained via abdominal venipuncture and placed in heparinized microcentrifuge tubes. Samples were immediately frozen on dry ice until they could be archived at -80ºC. Animals were then sacrificed via pithing. Carcasses were then frozen for contaminant screening. Tissue samples were also collected and subsequently archived at Texas A&M University. Collection and processing methods were conducted as specified in Texas A&M AUP # 2001-132, "Molecular analyses of the genetic structure of wildlife populations exposed to environmental contamination."

Table 3-1. Sampling data and biomarker statistics.

Site	Location	FCM *n*=	HPCV Mean	HPCV St.Dev	MN *n*=	MN Mean	MN St.Dev
12	N40º38.671', E49º32.381'	-	-	-	15	3.03	1.27
75	N40º37.402', E49º34.696'	-	-	-	19	3.60	4.37
WTP	N40º36.857', E49º37.280'	20	3.85	1.58	35	9.20	6.49
CAP	N40º35.788', E49º37.700'	17	5.46	2.57	18	6.64	6.07
11	N40º36.380', E49º40.477'	-	-	-	16	4.44	1.86
76	N40º34.468', E49º43.129'	-	-	-	12	6.48	2.78
AA	N40º53.012', E48º59.800'	-	-	-	24	2.45	1.33
ALI	N39º56.777', E48º55.060'	15	3.61	0.68	23	1.59	1.28

Flow Cytometry

Blood samples were used to determine cell to cell variation in DNA content following the methods of Vindelov and Christiansen (1994). Briefly, samples were randomized prior to processing to avoid experimental bias. The samples were quickly thawed and 50 µl added to a trypsin/detergent solution for digestion. After 10 minutes, trypsin inhibitor and RNAse were added to stop the reaction and to degrade RNA, which will also take up the dye. The solution was filtered through 30 µm nylon mesh and propidium iodide (PI) was added. After 15 minutes on ice, the samples were analyzed on a Coulter Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA). Fluorescent emission was measured from cells illuminated with a Coherent laser at 514 nm at 500 mW of power to excite the PI. Cells were gated on side scatter, forward scatter, and the ratio of peak to integrated fluorescence. Ten thousand nuclei, which satisfied all gating parameters, were measured from each sample and the intercellular variation in DNA content reported as the half peak coefficient of variation (HPCV). Samples that did not have 10,000 nuclei counted by the end of the four minute run were excluded from subsequent statistical analyses. Population comparisons were only performed for WTP, CAP, and Ali Bairamly. All samples included in this experiment were from 2002 collections. Genome size was also estimated for each individual.

Micronucleus Assay

Fresh blood samples were used to make duplicate air-dried smears on clean glass slides for each frog. Smears were stained with the Bayer Hema-Tek® modified Wright-Giemsa stain pack (Bayer Diagnostics, Elkhart, IN) following the manufacturer's protocol.

Three thousand RBCs, in the monolayer portion of the smear, were examined at 1000X magnification with oil immersion for all 2001 samples. Five thousand RBCs were counted in samples collected in 2002. The number of micronucleated RBCs was determined, and recorded as micronuclei per one thousand RBC. All MN examinations were performed by G.P. without knowledge of the population or species to which samples belonged.

Biomarker Data Analysis

All data analyses were performed using SPSS ver. 11.0.1 (SPSS Inc., Chicago, IL). Tests of normality were performed using the Kolmogorov-Smirnov test. Levene's test for equality of variances was used to test for significant differences in population variances. Statistical significance was accepted as $P<0.05$ for all tests.

Contaminant Analyses

Sediment samples were collected from sites 12, 75, 11, and 76 during 2001. Three samples from WTP, in addition to one sample from CAP, AA, ALI, and a replicate for site 12 were collected in 2002. One sample for each year was tested for each site, where

available, except for PAH analyses, where all samples were tested. Each sample was tested for mercury, organochlorines, and individual PAH concentrations. Concentrations presented here represent the site mean when more than one sample was analyzed (sites 12 and WTP). Frog carcasses collected in 2001 were tested for organochlorines and PAHs. A select number of frog livers from 2002 were tested for mercury concentrations. Total mercury was calculated using EPA method 7473 (U.S. Environmental Protection Agency, 2000) on a Milestone DMA-80 mercury analyzer (Milestone Inc., Monroe, CT). Sediment samples were extracted following the methods of Schantz et al. (1997). Sediment PAH and OC concentrations were determined following the methods of Cizmas et al. (2003) and Frank et al. (2001), respectively. PAHs were separated by number of rings and into HMW and LMW classes. Individual PAHs were considered HMW if the molecule consisted of four or more rings, while those with two or three were considered LMW. Frog carcass organochlorine screening was performed following a slightly modified version of the methods provided in Wu et al. (2000).

Pearson correlation analyses were performed, using SPSS ver. 11.0.1 (SPSS Inc., Chicago, IL), to look for significant associations between population HPCV means, MN frequencies (means and standard deviations), and contaminants.

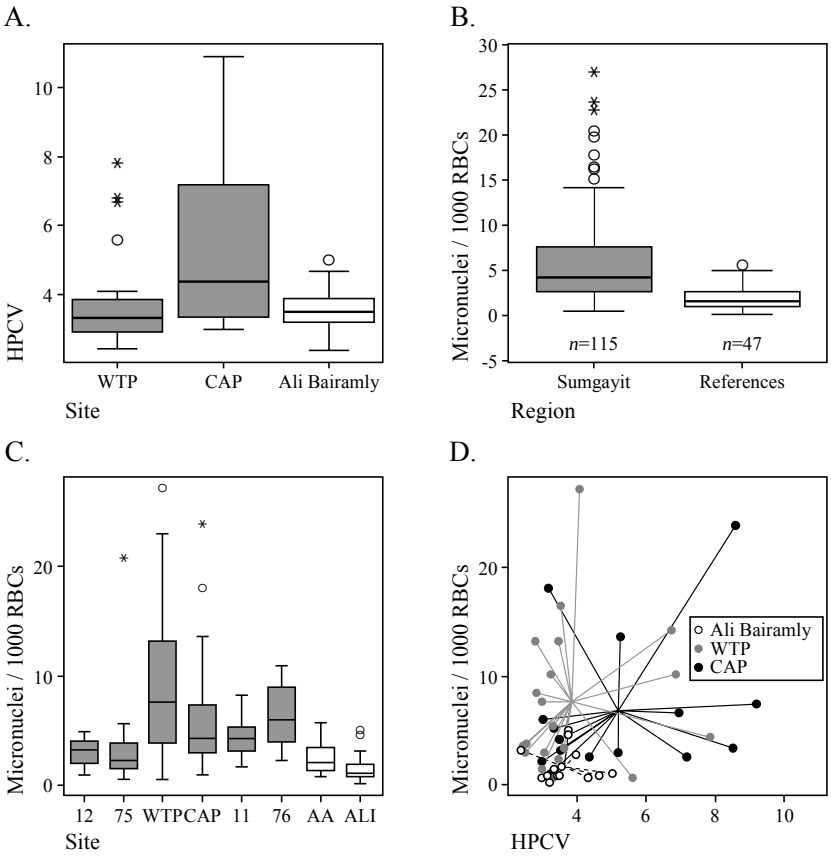


Figure 3-2. Marsh frog biomarker data. A. Box plots of HPCV data from two experimental sites in Sumgayit (shaded) and one reference. B. Box plots of MN data for Sumgayit (shaded) and references. C. Box plots of MN data from six experimental sites in Sumgayit (shaded) and two references. D. Scatter plot of total biomarker response for frogs from two experimental sites (WTP, CAP) and one reference site. Box plots consist of boxes representing interquartile range transected by medians, vertical lines represent range, while ○ and * represent outliers and extreme outliers, respectively.

Results

Flow Cytometry

A population comparison was performed for Ali Bairamly, WTP, and CAP (Fig. 3-2A). Population HPCV data was normally distributed for Ali Bairamly ($P>0.200$) and CAP ($P=0.079$), however, data from WTP was not normally distributed ($P<0.001$). Because WTP data was not normally distributed, we performed a Kruskal-Wallis test. HPCVs were significantly different among populations ($P=0.036$). To test for significant pairwise differences with WTP, we performed Mann-Whitney tests for the WTP-CAP and WTP-Ali Bairamly comparisons. CAP HPCVs were significantly larger than those from WTP ($P=0.015$), while Ali Bairamly and WTP HPCVs were not significantly different from one another ($P=0.463$). Since HPCVs from both Ali Bairamly and CAP were normally distributed, we performed an independent samples *t*-test for their comparison. Given that Levene's test for equality of variance was significant for the CAP-Ali Bairamly comparison ($P<0.001$), equal variances were not assumed in the *t*-test. CAP HPCVs were significantly greater than those from Ali Bairamly ($P=0.010$). CAP HPCVs were significantly greater than those from both Ali Bairamly ($P=0.03$) and WTP ($P=0.045$) after a Bonferroni correction to account for multiple comparisons and avoid an increased Type I error. A comparison of population means and standard deviations are presented in Table 3-1. While the population HPCV means are not significantly different for WTP and Ali Bairamly, the population HPCV variance is significantly greater for the WTP population ($P=0.023$). During our FCM experiment,

we discovered a single triploid individual in the CAP population. This was the only triploid identified within the study.

Micronucleus Assay

A comparison was made between samples from the experimental sites in Sumgayit and samples from the two reference sites (AA and ALI; Fig. 3-2B). MN data were not normally distributed for either the experimental ($P<0.001$) or reference ($P=0.013$) group. A Kruskal-Wallis test was performed to compare groups. Frogs from Sumgayit had significantly more micronucleated RBCs than the reference sites ($P<0.001$). We also wanted to check for significant differences among all of the individual localities (Fig. 3-2C). Population MN summary data are presented in Table 3-1. To test for temporal variation within sites, we performed independent samples *t*-tests for sites where MN data were normally distributed (WTP and 12) and Mann-Whitney tests for sites that did not have normal MN distributions (75). Temporal sample comparisons were not significant ($P>0.05$), therefore, 2001 and 2002 MN data were combined for each site. MN frequency data were not normally distributed for site 75, CAP, AA, or ALI. The Kruskal-Wallis test resulted in significant differences among localities ($P<0.001$). Simple Mann-Whitney comparisons of each combination of localities would have resulted in an unacceptable Type I error probability, while the use a Bonferroni correction of this magnitude would have given an unacceptable probability of a Type II error. Because of the large number of localities in this test, we decided to perform an ANOVA on ranked values with a Bonferroni multiple comparison. The departure from

normality in several of our samples prevented us from being able to use standard multiple comparison tests. To avoid any problems associated with breaking the assumption of normality in parametric tests, we have used a modified ANOVA based on ranks as a nonparametric alternative that avoids any major violations of assumptions. All but two Sumgayit sites are significantly larger than the two reference sites for HPCVs (Table 3-2). Sumgayit sites 12 and 75 are not significantly different from the reference sites. The WTP and site 76 are significantly larger than the references and sites 12 and 75.

Table 3-2. Fisher's LSD with a Bonferroni correction. *P*-values for site MN data. Significant values are in bold.

Site	12	75	WTP	CAP	11	76	AA	ALI
12	-							
75	1	-						
WTP	**0.001**	**<0.001**	-					
CAP	0.561	0.135	1	-				
11	1	0.436	1	1	-			
76	**0.037**	**0.007**	1	1	1	-		
AA	1	1	**<0.001**	**0.004**	**0.021**	**<0.001**	-	
ALI	0.085	0.142	**<0.001**	**<0.001**	**<0.001**	**<0.001**	1	-

Sediment Contaminant Analyses

Sediment PAH and mercury data are provided in Table 3-3. PAHs were elevated at WTP, CAP, and site 11. However, PAH profiles differed significantly between sites (Fig. 3-3). While, site 11 had LMW PAH concentrations much higher than any other site, it also had elevated levels of HMW PAHs. The proportion of low to high molecular weight PAHs was very high at site 11. WTP and CAP both were weighted toward HWM PAHs, although they also had elevated LMW PAH concentrations.

We were unable to detect OCs in any of our sediment samples. This may simply be a function of the heterogeneity of sediments and soils.

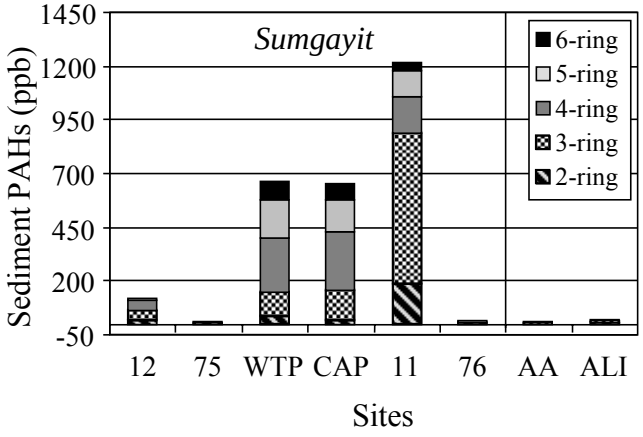


Figure 3-3. Sediment PAH concentrations.

Table 3-3. Sediment PAH (ppb) and total mercury (ppm) concentrations*.

	Sites							
LMW Analytes	12	75	WTP	CAP	11	76	AA	ALI
Naphthalene	0.35	1.28	1.99	5.48	21.13	2.66	0.74	0.3
C1-Naphthalenes	1.51	0.26	6.17	2.33	50.35	1.29	0.19	0.71
C2-Naphthalenes	3.10	0.19	7.98	2.19	28.50	1.01	0	1.04
C3-Naphthalenes	2.32	0.20	9.70	1.82	29.06	0.71	0	1.02
C4-Naphthalenes	4.47	0.17	6.38	1.2	34.32	0.43	0	0.55
Biphenyl	0.97	0.15	3.68	1.2	17.14	0.28	0.19	0.45
Acenaphthylene	10.20	0.12	4.33	6.95	77.35	0.13	0.05	0.04
Acenaphthene	0.46	0.02	1.19	1.23	48.76	0.09	0.89	0.94
Dibenzofuran	0.36	0.05	1.18	1.09	15.16	0.06	0.11	0.14
Fluorene	1.00	0.05	5.65	1.26	27.17	0.12	0.17	0.15
C1-Fluorenes	3.97	< D.L.	7.59	4.25	48.98	< D.L.	0.7	< D.L.
C2-Fluorenes	7.50	< D.L.	3.14	5.9	240.70	< D.L.	< D.L.	< D.L.
C3-Fluorenes	4.58	< D.L.	4.16	10.14	185.18	< D.L.	< D.L.	< D.L.
Carbazole	< D.L.	-	2.06	2.13	-	-	1.01	0.15
Dibenzothiophene	0.16	0.03	0.96	5.3	1.82	0.09	0.03	0.27
C1-Dibenzothiophene	0.31	0.09	1.31	1.01	3.60	0.26	< D.L.	0.73
C2-Dibenzothiophene	0.87	0.16	1.87	2.02	3.77	0.31	< D.L.	1.1
C3-Dibenzothiophene	0.68	0.11	1.09	1.97	1.52	0.18	< D.L.	< D.L.
C4-Dibenzothiophene	< D.L.	< D.L.	0.60	0.74	< D.L.	< D.L.	< D.L.	< D.L.
Anthracene	2.65	0.06	3.93	4.15	17.71	0.13	0.03	0.06
Phenanthrene	4.65	0.33	24.02	36.55	2.51	0.87	0.27	1.27
C1-Phenanthrene/Anthracene	6.99	0.36	21.47	28.09	12.52	0.83	0.1	2.55
C2-Phenanthrene/Anthracene	5.10	0.28	13.64	14.52	9.10	0.56	< D.L.	1.19
C3-Phenanthrene/Anthracene	2.96	0.13	6.91	6.93	5.24	0.31	< D.L.	0.32
C4-Phenanthrene/Anthracene	1.69	< D.L.	5.58	3.42	3.88	0.09	< D.L.	< D.L.
Total 2-ring PAHs	12.7	2.2	35.9	14.2	180.5	6.4	1.1	4.1
Total 3-ring PAHs	54.1	1.8	110.7	137.7	705.0	4.0	3.4	8.9
Total LMW PAHs	66.8	4.0	146.6	151.9	885.5	10.4	4.5	13.0

Table 3-3. Continued.

	Sites							
HMW Analytes	12	75	WTP	CAP	11	76	AA	ALI
Naphthobenzothiophene	< D.L.	-	11.17	8.7	-	-	< D.L.	< D.L.
C1-Naphthobenzothiophene	< D.L.	-	4.16	5.86	-	-	< D.L.	< D.L.
C2-Naphthobenzothiophene	< D.L.	-	1.88	5.61	-	-	< D.L.	< D.L.
C3-Naphthobenzothiophene	< D.L.	-	0.82	4.89	-	-	< D.L.	< D.L.
Fluoranthene	4.68	0.17	40.73	42.16	32.25	0.69	0.17	0.56
Pyrene	4.67	0.18	30.19	37.48	23.50	0.56	0.14	0.4
C1-Fluoranthenes/Pyrenes	7.94	0.23	24.95	24.75	11.14	0.34	< D.L.	0.3
C2-Fluoranthenes/Pyrenes	7.06	0.81	19.49	28.04	11.75	0.27	< D.L.	< D.L.
C3-Fluoranthenes/Pyrenes	2.28	0.10	5.95	12.37	5.05	0.15	< D.L.	< D.L.
Benz(a)anthracene	2.76	0.22	32.92	24.99	22.12	0.32	< D.L.	1.03
Chrysene	4.79	0.18	46.09	52.53	37.08	0.64	< D.L.	0.42
C1-Chrysenes	3.62	0.17	14.14	9.87	12.46	0.30	< D.L.	< D.L.
C2-Chrysenes	1.55	0.09	9.65	12.95	6.68	< D.L.	< D.L.	< D.L.
C3-Chrysenes	1.22	< D.L.	2.01	4.43	4.05	< D.L.	< D.L.	< D.L.
C4-Chrysenes	0.93	< D.L.	4.96	3.92	8.62	< D.L.	< D.L.	< D.L.
Benzo(b)fluoranthene	3.88	0.21	57.72	66.74	44.25	0.39	0.19	0.3
Benzo(k)fluoranthene	1.18	0.05	29.99	16.57	14.83	0.18	0.07	0.16
Benzo(e)pyrene	2.30	0.19	40.68	36.2	29.37	0.24	0.24	0.2
Benzo(a)pyrene	1.34	0.09	36.63	21.25	16.68	0.15	0.03	0.1
Dibenzo(a,h)anthracene	0.43	0.02	11.04	6.57	5.47	0.03	< D.L.	< D.L.
Perylene	1.04	0.04	8.68	3.04	3.72	0.03	1.28	1.07
Indeno(1,2,3-c,d)pyrene	1.55	0.11	47.43	43.94	27.77	1.11	< D.L.	< D.L.
Benzo(g,h,i)perylene	0.80	0.09	37.63	28.23	17.01	0.12	0.26	0.14
Total 4-ring PAHs	41.5	2.2	249.1	278.6	174.7	3.3	0.3	2.7
Total 5-ring PAHs	10.2	0.6	184.7	150.4	114.3	1.0	1.8	1.8
Total 6-ring PAHs	2.3	0.2	85.1	72.2	44.8	1.2	0.3	0.1
Total HMW PAHs	54.0	3.0	518.9	501.1	333.8	5.5	2.4	4.7
Total PAHs	120.8	7.0	665.5	653.0	1219.3	15.9	6.9	17.7
Hg (ppm)	2.277	0.03	1.49	19.4	0.207	0.019	0.0373	0.0193

*< D.L. represents samples below method detection limit.

Frog Contaminant Analyses

Frog carcass organochlorine concentration data is presented in Table 3-4. Aldrin values are not included as no frogs contained detectable levels at any site tested. We tested mercury concentrations in frog liver samples from WTP (*n*=8) and CAP (*n*=8). Median mercury concentration from WTP was 1.83 ppm with a range of 0.96-2.77 ppm. CAP had a median concentration of 7.17 ppm with a range of 4.65-12.60 ppm. Frog carcass PAH concentrations did not reach method detection limits.

Correlations

We found no significant associations between frog carcass OC or liver mercury concentrations with either of our biomarkers. We did observe two significant associations between sediment contaminant levels and population biomarker responses. Population mean HPCV was significantly correlated with sediment mercury concentrations (Fig. 3-4A). Population mean MN frequency showed a significant association with sediment total HMW PAH concentrations (Fig. 3-4B).

Table 3-4. Number of frog carcasses with detectable organochlorines and population median and maximum concentrations (ppb).

	Site				
	12	75	WTP	11	76
n^t=	10	14	21	23	15
Lindane					
n^+=	1	5	7	8	10
median	7	42	22	16.5	7
max.	7	616	52	113	25
Heptachlor					
n^+=	3	1	0	0	6
median	5	28	-	-	7.5
max.	131	28	-	-	16
Dieldrin					
n^+=	0	0	0	0	1
median	-	-	-	-	7
max.	-	-	-	-	7
DDE					
n^+=	2	7	12	8	5
median	9	57	48.5	19	10
max.	10	319	156	52	27
DDT					
n^+=	3	8	11	10	2
median	50	81.5	22	20	11.5
max.	96	317	140	67	13
Endrin					
n^+=	1	8	1	0	14
median	5	41.5	8	-	28
max.	5	91	8	-	82
Methoxychlor					
n^+=	0	0	1	0	1
median	-	-	20	-	10
max.	-	-	20	-	10

n^t is the total number of animals tested for each site. N^+ is the number of individuals at each site that contained detectable levels of given contaminant.

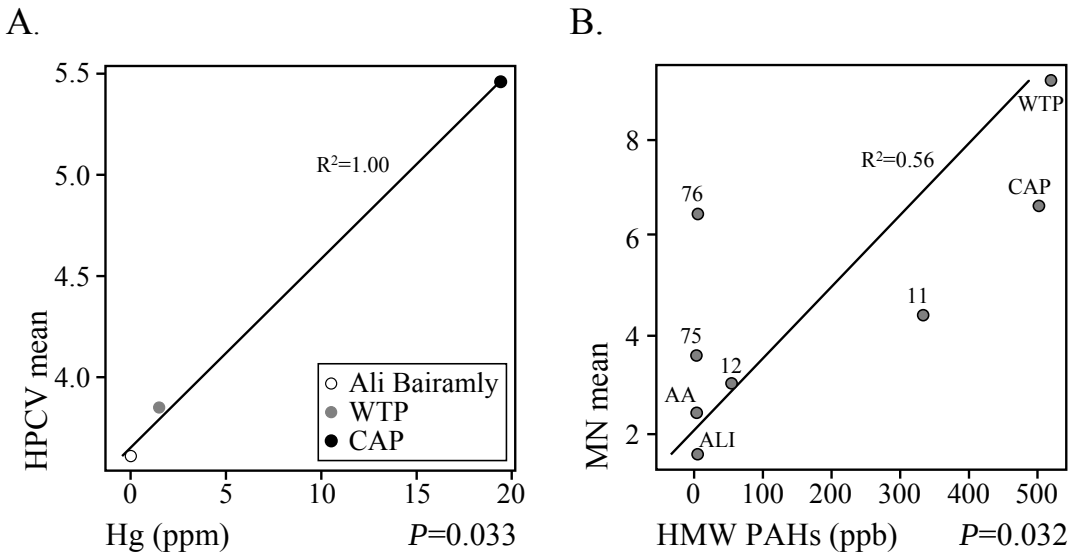


Figure 3-4. Sediment correlations with biomarkers. A. Linear regression of sediment mercury concentrations and population HPCV means. B. Linear regression of sediment HMW PAHs and population MN means.

Discussion

These experiments expand on previous studies by utilizing a different model organism and providing a more detailed evaluation of Sumgayit by including multiple localities within the area. The results of this study agree with those of Swartz et al. (2003) in that the contamination in Sumgayit is sufficient to cause chromosomal damage. Both the MN and FCM data support this conclusion by showing significantly higher estimates of genetic damage in at least some Sumgayit populations compared to one or more reference populations. HPCV data from CAP were significantly greater than those from ALI. While HPCV data from WTP and ALI were not significantly different based on the Mann-Whitney test, it has been suggested that measures of central tendency may not always be the most sensitive for population comparisons in ecotoxicology. Orlando and

Guillette (2001) have shown that biomarker variances can be more sensitive indicators of effect than the more traditional measures of central tendency. The standard deviation in HPCVs at WTP is more than twice as large as that of Ali Bairamly (P=0.023). Therefore, we conclude that there is an effect at both Sumgayit sites relative to the reference site. We also found significant differences in estimates of genetic damage between WTP and CAP, both sites within Sumgayit. Our FCM data suggest that the *R. ridibunda* population at CAP is exhibiting significantly greater amounts of genetic damage than the nearby WTP population. Although we have FCM data only for three sites, we were able to document a significant association between sediment mercury concentrations and population mean HPCV (P=.033).

In addition to showing elevated estimates of genetic damage based on flow cytometry, frogs inhabiting the various wetlands of Sumgayit have significantly greater frequencies of micronucleated RBCs (P<.001) than reference animals. These MN data are in agreement with both our FCM data and the results of turtle studies presented by Swartz et al. (2003) and those of this manuscript. However, we also wanted to look at patterns of genetic damage within the Sumgayit region. Thus, we investigated possible biomarker patterns and correlations with contaminant levels among six experimental sites within Sumgayit. We found that localities east of the Sumgayit River have significantly increased MN frequencies, while sites west of the river are not elevated over references (Table 3-2). These biomarker data are also significantly correlated with sediment concentrations of HMW PAHs (P=0.032). The portion of the Sumgayit

industrial zone east of the Sumgayit River consistently shows the highest contaminant levels and the highest estimates of genetic damage. The Sumgayit River appears to be acting as a barrier to the movement of both PAHs and mercury. The source of the majority of Sumgayit's mercury contamination, the Chlor-Alkali plant, is east of the Sumgayit River. The sites with the worst mercury contamination are east of the river. The one exception is site 12 which has elevated mercury concentrations and is the site farthest to the northwest. The levels at this site might be explained by the presence of large amounts of fill material brought in to build the highway that runs along the wetland collecting site. We have no information regarding the origin of this material, however, it is clear that contaminated materials from all around the industrial zone have been transported to other areas. Historically, there has been no designated industrial land fill to dispose of Sumgayit's contaminated materials.

Overall biomarker response was evaluated for WTP, CAP, and ALI, the only sites to have both FCM and MN data. A clear pattern is evident from the biomarker plot (Fig. 3-2D). Variation is elevated in both experimental sites for both biomarkers, suggesting elevated levels of genetic damage.

Additionally, we found a triploid frog at CAP. This discovery is not entirely unexpected as our FCM and MN data show evidence of genetic damage and possibly genomic instability. Perhaps more interestingly, we previously documented the presence of a triploid turtle in Sumgayit, although that animal came from the WTP. Finding two triploid animals of different species within a few kilometers of one another seems to represent more than mere coincidence. Triploidy can be the result of improper spindle fiber formation. Various contaminants are known to disrupt spindle fiber formation. Mercury is a known clastogen that has been documented to disrupt spindle fiber formation (Betti et al., 1992; De Flora et al., 1994; Ogura et al., 1996; Queiroz et al., 1999; Amorim et al., 2000; Ochi, 2002). To put these two observations in perspective, we have conducted FCM studies on wildlife populations for more than 20 years, examining thousands of fish, amphibians, reptiles, birds and mammals. These are the only two observations we have made of triploids from natural populations.

A variety of organochlorine pesticides were detected in the frogs including DDT and it's persistent metabolite, DDE. The significant presence of DDT relative to DDE also suggests a more recent exposure. With a few exceptions, median and maximum OC concentrations were consistently higher in frogs from site 75 and WTP. The lack of pesticide detections in sediments suggests that the OCs may not be biologically available to frogs directly; frog food items may serve as a more direct source of OC exposure.

The biomarker data presented here for frogs and the turtle data presented by Swartz et al. (2003) and previously in this manuscript combine to suggest that humans living in or utilizing resources from these areas are likely to be negatively impacted by exposure to the complex mixtures of contaminants present. The fact that multiple taxa are showing similar responses to these contaminant mixtures suggests that this is likely a generalized response common to most animals inhabiting these environments or that have similar levels of exposure. At a minimum, it raises significant concern for the refugees living in and around these highly contaminated areas within the Sumgayit industrial area. Refugees have been observed grazing livestock, gardening, and fishing within the most contaminated sites of the industrial zone and we can only assume that significant opportunity for exposures must exist. The need for research regarding human exposure and risk assessment is certainly apparent for refugees in the industrial zone as well as individuals in the residential areas of the city. As our micronucleus data show, frog populations inhabiting residential areas of Sumgayit east of the industrial zone also have elevated levels of genetic damage.

We were able to document significant correlations with both mercury and HMW PAHs. However, it is important to understand that while these contaminants are likely to be important factors, they are but a part of the total contaminant load that animals in Sumgayit face. The mixtures of contaminants in this area are quite complex and most likely dozens of individual chemicals combine and interact to cause the damage that we have documented. These complex mixtures of petroleum products, pesticides, insecticides, heavy metals, and many other industrial chemicals found in Sumgayit make exact determinations and linkages of cause and effect essentially impossible. By using correlation analyses to explore contaminant effects on biomarker responses, we can identify contaminants that are likely causing a majority of the observed biomarker responses. While the Sumgayit environment is quite complex, HMW PAHs and mercury appear to be important contaminants for human and wildlife risk assessment modeling.

CHAPTER IV

POPULATION GENETIC EFFECTS OF CHRONIC CONTAMINANT EXPOSURE ON MARSH FROGS (*RANA RIDIBUNDA*) IN SUMGAYIT, AZERBAIJAN

Introduction

Evolutionary toxicology is the study of the effects of contaminants on the genetics of natural populations and is rapidly becoming an essential component of ecotoxicology. The use of population genetic data to investigate effects of chronic contaminant exposure has proven effective in studies representing a diversity of organisms and ecological settings. Molecular genetics is a powerful tool to identify reductions in genetic diversity (Murdoch and Hebert, 1994), contamination induced natural selection (Foré et al., 1995a; Foré et al., 1995b; Theodorakis and Shugart, 1997; Peles et al., 2003), ecological sinks (Matson et al., 2000; Baker et al., 2001; Theodorakis et al., 2001), and increased mutation rates (Ellegren et al., 1997; Forster et al., 2002). The application of evolutionary or population genetic approaches to problems in ecotoxicology were reviewed in several articles (Hebert and Luiker, 1996; Bickham et al., 2000; Belfiore and Anderson, 2001; Van Straalen and Timmermans, 2002). Bickham et al. (2000) provide a thorough discussion of the complex pathways through which contaminants can affect wildlife populations.

Three specific effects can be readily investigated. First, environmental contaminants can lead to a reduction in genetic diversity resulting from population declines. Reductions in fecundity or viability, or increased mortality as a result of contaminant exposure can lead to a reduction of the number of breeding adults. More complex scenarios involve ecosystem degradation leading to reductions in carrying capacity thus resulting in smaller populations. Populations that have experienced a significant bottleneck are expected to suffer a reduction in population genetic diversity. The severity and duration of the bottlenecks will determine the amount of diversity loss. Genetic diversity is generally measured as either "haplotype" or "nucleotide" diversity (Nei, 1987). Where gene diversity looks only at the number and frequencies of haplotypes, nucleotide diversity also includes a measure of haplotype sequence similarity or phylogenetic relationship (Tajima, 1983; Nei, 1987). The loss of population genetic diversity has been linked in several previous studies to environmental contaminants (Kopp et al., 1992; Murdoch and Hebert, 1994; Street and Montagna, 1996; Krane et al., 1999).

Secondly, contaminant exposure can lead to an increased mutation rate. This increased mutation rate can offset some of the genetic diversity lost due to bottlenecks. The haplotype patterns found in these populations can be informative in determining whether populations have gone through bottlenecks and/or have elevated mutation rates. Elevated levels of heteroplasmy, the presence of more than one mtDNA variant within an individual, in contaminated populations suggest the presence of an elevated mutation

rate. However, it is important to understand that different types and patterns of heteroplasmy suggest different sources. There have been several instances where the differences between the heteroplasmic haplotypes have been too large to suggest a simple gametic or somatic mutational event. This pattern suggests that paternal leakage is the cause and not gametic or somatic mutation. Where paternal leakage is indicated, heteroplasmic haplotypes are likely to be common haplotypes (Magoulas and Zouros, 1993) or else they might be differentiated by more than a single mutational event (Magoulas and Zouros, 1993; Kvist et al., 2003). In perhaps the most comprehensive examination of population heteroplasmy, paternal leakage was ruled out by using people with known pedigrees (Forster et al., 2002). The heteroplasmy documented was proven to be the result of gametic mutations as the heteroplasmy was subsequently passed down the maternal lineage. This heteroplasmy was also shown to be linked to natural radiation exposure, as populations from radioactive areas had an elevated frequency of heteroplasmy (Forster et al., 2002). These data combine to show that heteroplasmy can be an effective means for investigating mutation rates. It appears that mutational heteroplasmy is generally more common than complete mutations and thus a more sensitive means of detecting increased mutation rates in populations.

The third effect to be investigated is whether or not contaminant exposure has resulted in an ecological sink. Although this is in fact an ecological effect, it can be investigated using population genetics. Theodorakis et al. (2001) used population genetics analyses to investigate the genetic patterns of kangaroo rats (*Dipodomys merriami*) inhabiting the

radionuclide contaminated Nevada Test Site. Based on both genetic diversity and gene flow estimates, they concluded that the atomic blast sites are acting as ecological sinks. Additionally, they concluded that immigration into the blast sites was masking the genotoxic effects of the radiation contamination on the resident kangaroo rat populations. Population genetic studies of bank voles (*Clethrionomys glareolus*) living near Chornobyl, led researchers to conclude that the most contaminated areas were likely acting as ecological sinks (Matson et al., 2000; Baker et al., 2001). While the authors were unable to eliminate an increased mutation rate as being the cause of observed genetic patterns, subsequent heteroplasmy studies suggest that mutation rates are not elevated at Chornobyl (Wickliffe et al., 2002; Wickliffe et al., 2003).

Azerbaijan is a former Soviet republic located on the western shores of the Caspian Sea. This small country is well known for its large oil reserves, which have been exploited for more than a century. Oil production, as well as the chemical and manufacturing plants of Sumgayit, has left the Apsheron Peninsula of Azerbaijan with a complex mixture of environmental contaminants. Environmental degradation and contamination in Azerbaijan and Sumgayit specifically has been explored in several recent articles (Andruchow, 2003; Bickham et al., 2003; Shelton, 2003; Swartz et al., 2003). Elevated levels of numerous potentially mutagenic contaminants, including PAHs, mercury, PCBs, and organochlorines, have been documented in wildlife, sediments, and soils from Sumgayit and the capitol city of Baku (Bickham et al., 1998b; Swartz et al., 2003). There is no doubt that the contaminants of Sumgayit affect both wildlife and humans;

however, there is still debate regarding the magnitude and types of effects. Previously in this manuscript we have shown elevated levels of chromosomal damage in wildlife, while a recent study has also shown increased cancer rates in humans (Andruchow, 2003). The complex mixtures of contaminants found in Sumgayit make it difficult to prove causal links between individual contaminants and observed effects. Therefore, associations and correlations are used to identify contaminants that are likely to be involved in a particular biomarker effect. In addition, it is difficult to fully understand what the ultimate consequences of increased genetic damage as well as other effects might be on individuals or populations.

The Exxon Valdez oil spill provided scientists with an *in situ* laboratory to study the impacts of long-term chronic PAH exposure to a variety of molluscs, fish, birds, and mammals (Downs et al., 2002; Esler et al., 2002; Peterson et al., 2003). Although the effects of acute exposures to oil have been well documented and are currently taken into account in ecological risk assessments, the effects of long-term chronic exposure to weathered oil is not well understood or included in risk assessments. Peterson et al. (2003) recently concluded that chronic exposure to PAHs, even at ppb concentrations, could have significant population consequences. These population-level impacts are the result of sublethal doses of PAHs which compromised health, growth, and reproduction. Additionally, indirect ecosystem changes also contributed to population declines. These data provide strong support for including probable population-level impacts from chronic exposure to contaminants into both wildlife and human risk assessment models.

In this study, we test three hypotheses regarding marsh frog populations in Azerbaijan. First, we wanted to determine if Sumgayit populations exhibit reduced levels of genetic diversity that could be the result of a population bottleneck. Second, we test whether the most contaminated areas within Sumgayit are acting as sources of new mutations. Lastly, we investigate patterns of gene flow to determine if the Sumgayit region is acting as an ecological sink.

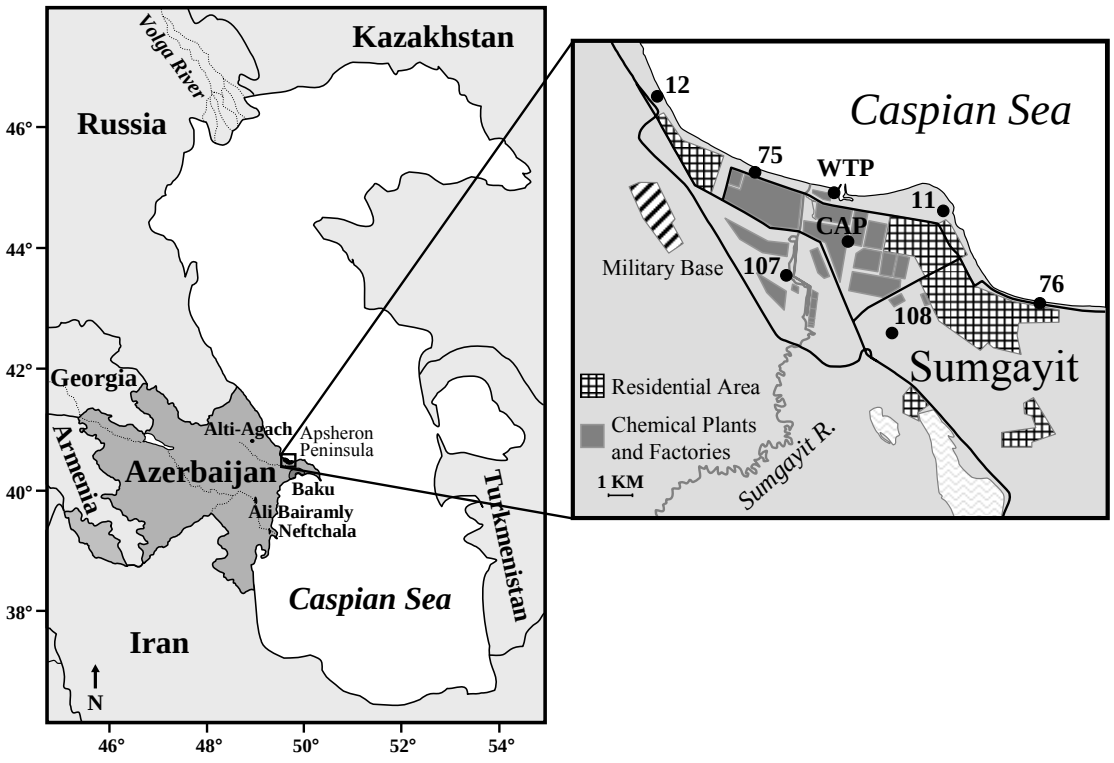


Figure 4-1. Map of Azerbaijan showing both experimental (Sumgayit blowup) and reference marsh frog collecting localities.

Materials and methods

Sample Collection

Marsh frogs were collected from a total of eleven sites between 1999 and 2002. Collecting areas consisted of marshes, ponds, and in one case a drainage ditch. A map of collecting locations is presented in Figure 4-1. Site coordinates are as follows: Neftchala, N 39º24', E 49º15'; Ali Bairamly, N 39º56.777', E 48º55.060'; Alti-Agach, N 40º53.012', E 48º59.800'; WTP, N 40º36.857', E 49º37.280'; Site 11, N 40º36.380', E 49º40.477'; Site 12, N 40º38.671', E 49º32.381'; Site 75, N 40º37.402', E 49º34.696'; Site 76, N 40º34.468', E 49º43.129'; CAP, N 40º35.788', E 49º37.700'; Site 107, N 40°35.180', E 49°36.110'; Site 108, N 40º33.917', E 49º38.778'. Eight of the sites are located within the city of Sumgayit, while the remaining three are from reference areas. Two of the reference sites are to the south of Sumgayit (Ali Bairamly and Neftchala), while the third is to the northwest and up in the Caucus Mountains (Alti-Agach). Sample sizes are presented in Table 4-1.

Frogs were anaesthetized with MS222 (CAS # 886-86-2; Sigma-Aldrich Corp., St. Louis, MO). Blood samples for DNA analyses were then obtained via abdominal venipuncture and placed in lysis buffer (Longmire et al., 1997). Samples were immediately refrigerated at 4ºC until transported back to Texas A&M University for processing. Animals were then sacrificed via pithing. Frozen tissue samples were also collected and subsequently archived at Texas A&M University. Collection and processing methods were conducted as specified in Texas A&M AUP # 2001-132,

"Molecular analyses of the genetic structure of wildlife populations exposed to environmental contamination."

Table 4-1. Population and regional haplotype counts, sample sizes, and diversity estimates.

	Haplotype																	Diversity Indices				
Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	4/15	1/5	*n*=	*h*	+/-	π	+/-
NEF	6	1	-	3	-	-	1	-	-	-	-	-	-	-	-	-	-	11	0.673	0.123	0.00245	0.00182
ALI	8	2	-	6	-	-	1	-	-	1	1	1	3	-	-	-	-	23	0.814	0.055	0.00318	0.00211
AA	1	5	1	2	-	10	-	-	2	-	-	-	-	-	1	-	-	22	0.753	0.076	0.00327	0.00216
WTP	17	3	2	3	2	15	-	1	-	-	-	-	-	2	-	2	5	52	0.803	0.036	0.00202	0.00147
Site #11	7	-	2	-	-	15	-	-	-	-	-	-	-	-	-	-	-	24	0.540	0.082	0.00112	0.00100
Site #12	12	2	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	15	0.362	0.145	0.00132	0.00114
Site #75	13	-	-	-	-	1	-	3	-	-	-	-	-	2	-	-	1	20	0.568	0.119	0.00096	0.00092
Site #76	12	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	14	0.275	0.148	0.00074	0.00079
CAP	14	1	2	1	-	2	-	-	-	-	-	-	-	-	-	-	-	20	0.511	0.128	0.00131	0.00112
Site #107	2	1	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-
Site #108	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	-
Region																						
NEF/ALI	14	3	-	9	-	-	2	-	-	1	1	1	3	-	-	-	-	34	0.761	0.053	0.00292	0.00194
Alti-Agach	1	5	1	2	-	10	-	-	2	-	-	-	-	-	1	-	-	22	0.753	0.076	0.00327	0.00216
Sumgayit	77	7	7	7	2	34	-	4	-	1	-	-	-	4	-	2	6	151	0.684	0.033	0.00164	0.00125
Total	92	15	8	18	2	44	2	4	2	2	1	1	3	4	1	2	6	207	0.744	0.025	0.00216	0.00151

h Haplotype diversity
π Nucleotide diversity

DNA Extraction and Sequencing

Total genomic DNA was extracted from blood samples using Qiagen Dneasy® Tissue kits following the manufacturer's protocol (Qiagen Inc., Valencia, CA). A 788 bp fragment of mtDNA was then directly amplified using the polymerase chain reaction

(PCR) utilizing primers designed specifically for *Rana ridibunda*: RANA CR-3F (5′-GAAGACCCCTTTATCACCATCG-3′) and RANA CR-4R (5′-GGGTACGATAGGGCTTATGAAT-3′). These primers amplify a region of mtDNA which includes a 63 bp portion of the 3′ end of the Cytochrome *b* gene and the first 725 bp of the 5′ end of the control region. This portion of the mtDNA control region is generally referred to as the hypervariable region I (HVR1). Amplifications were performed with AmpliTaq Gold® PCR Master Mix following the manufacturer's recommendations and a 50ºC annealing temperature. Fragments were then purified using QIAquick® PCR Purification Kits (Qiagen Inc., Valencia, CA).

Cycle sequencing reactions were performed using ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) following manufacturer's recommendations and the primer RANA CR-5F (5′-CCTTTATCACCATCGGTCAAATCG-3′). A subset of individuals was additionally sequenced using the primer RANA CR-2R (5′-GGCTTATGAATATTGCGTCGAG-3′). Sequencing products were subsequently cleaned with Qiagen DyeEx® 2.0 Spin Kits (Qiagen Inc., Valencia, CA) and run out on an ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Sequence chromatographs were proofed and aligned using Sequencher ver. 4.1 software (Gene Codes Corp., Ann Arbor, MI). In addition to our normal confirmation efforts, all ambiguous or heteroplasmic nucleotides, and novel haplotypes were confirmed by sequencing in the reverse direction using

RANA CR-2R. Detection limits for heteroplasmy were approximately 15 to 20 percent. Individuals with lower frequencies of secondary haplotypes were likely overlooked.

The final mtDNA fragment used for analyses was 582 bp and included the terminal 32 bp of Cytochrome *b* and the adjacent 550 bp of the control region. There are no tRNA genes separating Cytochrome *b* from the control region in many Ranid species including *R. ridibunda* (Sumida et al., 2000; Sumida et al., 2001). All haplotype sequences have been deposited in GenBank (accession numbers AY554011-AY554027).

Data Analysis

Sequence data were analyzed for both population and regional groupings. Population data were only analyzed for sites with at least ten individuals. The two cases in which there was not an adequate population sample size for analysis (Sites 107 and 108), the individuals were only utilized in regional analyses. Haplotype (h) and nucleotide diversity (π) were calculated at both scales using ARLEQUIN ver. 2.000 (Schneider et al., 2000). ARLEQUIN was also used to calculate linearized F_{ST} values and migration estimates (Slatkin, 1991; Slatkin, 1995). MIGRATE ver. 1.7.3 (Beerli, 1997-2003) was used to estimate effective population sizes (N_e) and number of effective migrants (Nm) for regional groupings utilizing a maximum likelihood methodology (Beerli and Felsenstein, 2001).

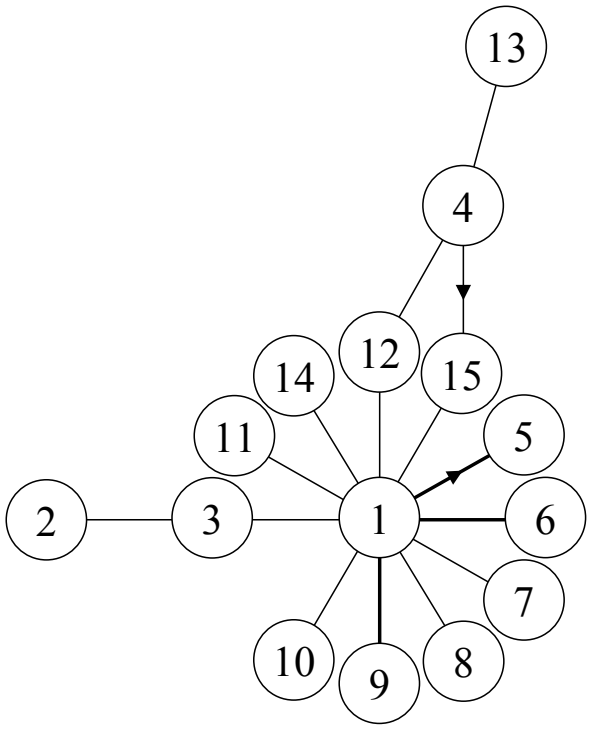


Figure 4-2. Minimum spanning network (MSN) of all *R. ridibunda* haplotypes from Azerbaijan. Each connection represents a single mutational event, with bold lines representing transversions. Triangles on connections represent heteroplasmic conditions between the two haplotypes, with the point representing inferred mutational direction.

Results

A total of 15 haplotypes were documented in *R. ridibunda* populations from Azerbaijan. Both population and regional haplotype counts are presented in Table 4-1. There were 13 variable nucleotide positions identified. Of the variable nucleotide positions, ten were transitions and three were transversions. One of the transitions was located within Cytochrome *b*, while the remaining variable positions were located in the control region. In addition to the frogs with one of the 15 haplotypes, six frogs were found to be

heteroplasmic for haplotypes 1 and 5, and two frogs were found to be heteroplasmic for haplotypes 4 and 15. Secondary heteroplasmic haplotype proportions ranged from approximately 15 to 45 percent. A minimum spanning network of all *R. ridibunda* haplotypes from Azerbaijan is presented in Figure 4-2.

Regional and population diversity estimates are presented in Table 4-1. At a regional scale, Sumgayit had reduced levels of both haplotype and nucleotide diversity relative to both reference regions (Fig. 4-3). At the population level, five out of the six sites in Sumgayit had lower haplotype diversity than our three reference populations (Fig. 4-4). The WTP site was the only Sumgayit population that appeared to have relatively normal haplotype diversity. However, all of the Sumgayit populations showed reduced levels of nucleotide diversity relative to the reference populations (Fig. 4-4).

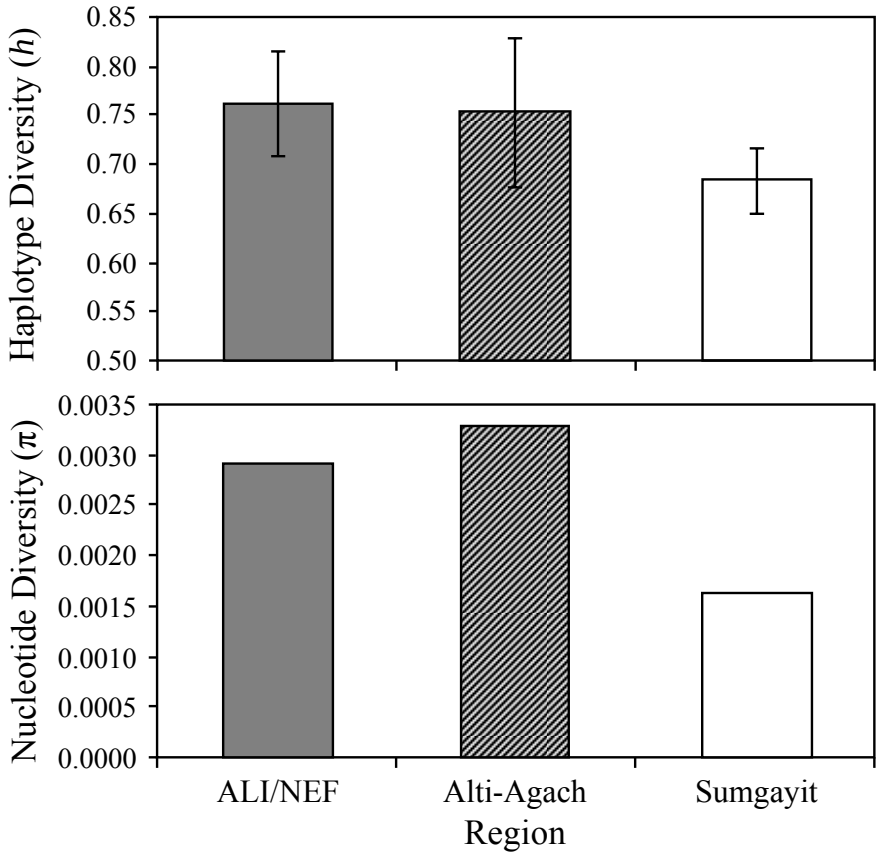


Figure 4-3. Regional diversity indices for sites from reference (shaded and striped) and experimental (white) regions. Error bars represent standard error.

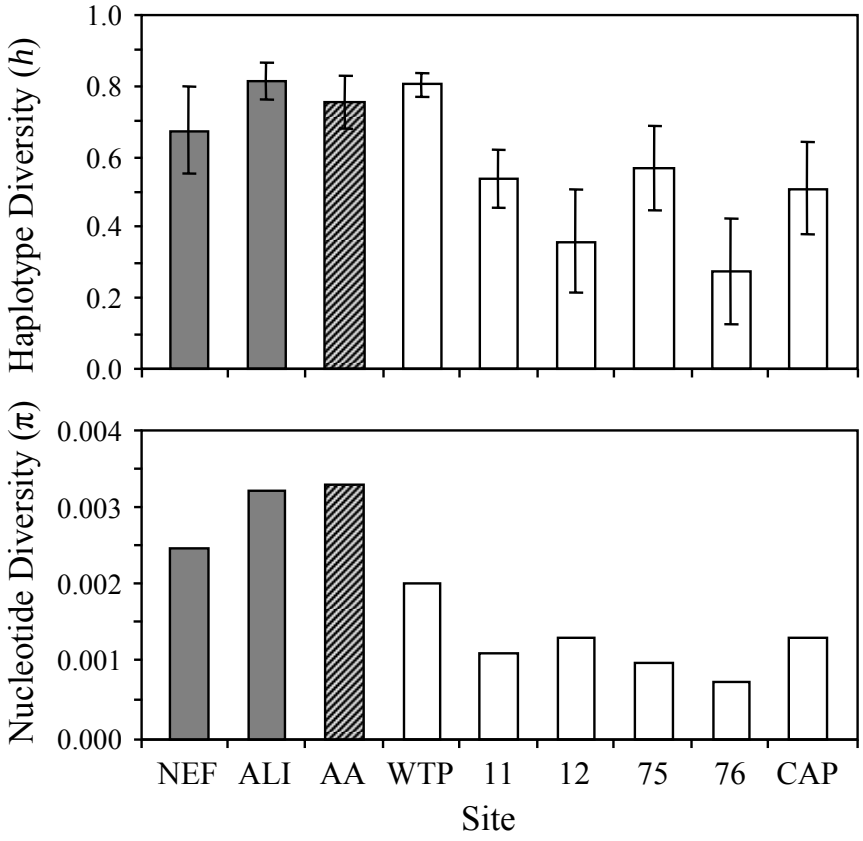


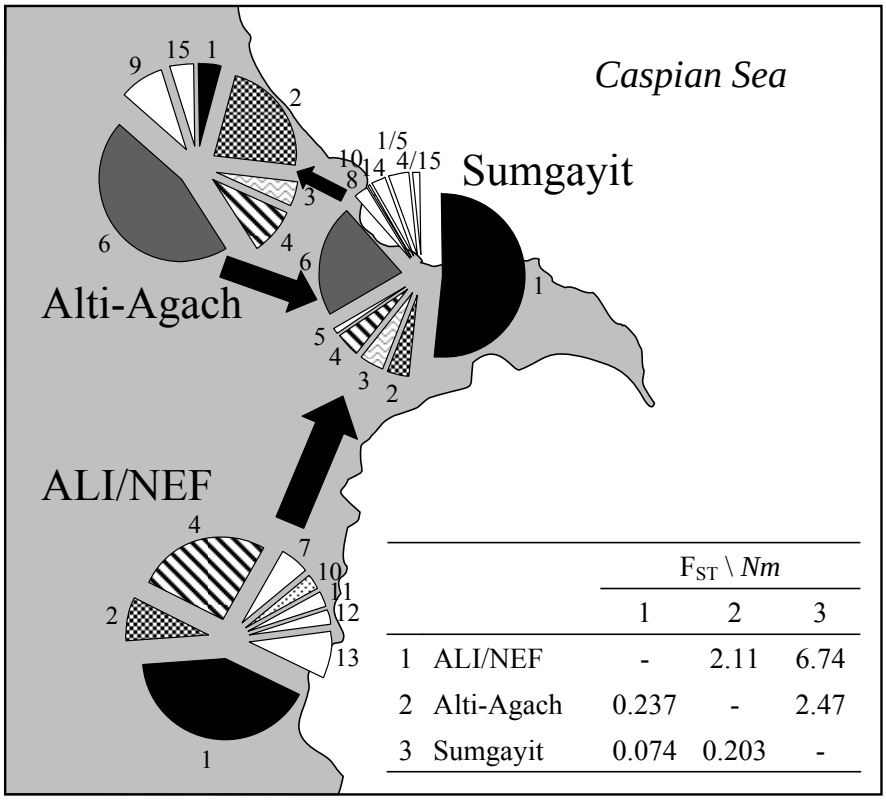
Figure 4-4. Population diversity indices for sites from reference (shaded and striped) and experimental (white) regions. Error bars represent standard error.

To investigate population substructure at both regional and local scales, F_{ST} estimates were computed and are presented in Figure 4-5 and Table 4-2, respectively. Estimates of the number of effective migrants (*Nm*) between populations is presented in Table 4-2. Regional estimates of *Nm* were calculated using two different methods, a traditional method utilizing F_{ST} values (Slatkin, 1991) and a maximum likelihood (ML) method (Beerli and Felsenstein, 2001). Traditional estimates of *Nm* only estimate total gene flow between populations (Fig. 4-5), while the ML method computes directional estimates of gene flow between populations (Table 4-3). Both methods result in the

highest estimates of gene flow occurring between Sumgayit and the ALI/NEF region and the lowest estimates between ALI/NEF and Alti-Agach. The directional nature of gene flow from traditional estimates must be deduced from population haplotype frequencies and patterns. Our interpretation of haplotype patterns suggest that gene flow among the regions is directional with the majority resulting from immigration into Sumgayit (Fig. 4-5). ML estimates suggest that gene flow between Sumgayit and ALI/NEF is almost entirely movement from ALI/NEF into Sumgayit. However, ML estimates also suggest that gene flow between Sumgayit and Alti-Agach is weighted toward emigration out of Sumgayit.

Table 4-2. Population linearized F_{ST} values (below diagonal) and estimates of *Nm* (*M*=*Nm*, above diagonal)

		1	2	3	4	5	6	7	8	9
1	Neftchala	-	∞	1.7	6.7	1.2	12.3	11.2	5.2	24
2	Ali Bairamly	0.00	-	2.5	7	1.5	3.2	4.3	2.3	4.4
3	Alti-Agach	0.30	0.20	-	7.2	6.1	0.8	1.2	0.7	1.2
4	WTP	0.07	0.07	0.07	-	7.7	2.6	5.1	2.2	5.1
5	Site #11	0.41	0.33	0.08	0.07	-	0.8	1.2	0.7	1.3
6	Site #12	0.04	0.16	0.61	0.19	0.66	-	18.4	∞	∞
7	Site #75	0.05	0.12	0.43	0.10	0.41	0.03	-	12.4	512
8	Site #76	0.10	0.21	0.76	0.23	0.75	0.00	0.04	-	∞
9	CAP	0.02	0.11	0.42	0.10	0.38	0.00	0.00	0.00	-



		F_{ST} \ *Nm*		
		1	2	3
1	ALI/NEF	-	2.11	6.74
2	Alti-Agach	0.237	-	2.47
3	Sumgayit	0.074	0.203	-

Figure 4-5. Regional haplotype patterns and estimates of genetic differentiation (F_{ST}) and gene flow (*Nm*). Shared haplotypes are shaded or patterned while unique haplotypes are white.

Effective population size (N_e) was estimated for each region using a ML approach. Theta (θ), which in the case of mtDNA, is equal to N_e multiplied by the mutation rate (μ). Mutation rates are generally assumed to be equal among populations. Therefore, we used estimates of θ to compare effective population size (Table 4-3). These estimates suggest that the ALI/NEF region has the largest population size followed by Alti-Agach and Sumgayit, respectively.

Table 4-3. Maximum likelihood estimates of theta (θ) and migrants (*Nm*).

	Population [receiving]	Theta (θ) [$Ne\mu$]	*Nm* 1	2	3
1	ALI/NEF	0.00125	-	0.16	0.00
2	Alti-Agach	0.00107	0.00	-	2.88
3	Sumgayit	0.00071	5.05	0.46	-

Discussion

Patterns of regional diversity support the hypothesis that marsh frog populations from Sumgayit have reduced genetic diversity, likely through population reductions and/or bottlenecks. The Sumgayit region has reduced levels of both haplotype and nucleotide diversity relative to both ALI/NEF and Alti-Agach (Fig. 4-3). This reduction in diversity is likely the result of genetic drift exacerbated by declines in effective population size. Comparisons of genetic diversity from individual localities also support the hypothesis that Sumgayit has reduced levels of genetic diversity. All of the experimental sites within Sumgayit exhibit reduced levels of nucleotide diversity relative to reference sites. WTP is the only Sumgayit population to not also show reduced haplotype diversity. Both regional and population genetic diversity assessments support the hypothesis that *R. ridibunda* populations in Sumgayit have reduced levels of genetic diversity. We conclude that the observed loss of diversity is likely the result of population declines and that environmental degradation is the most likely cause of the regional reductions of genetic diversity.

Patterns of diversity and gene flow can be used to evaluate whether an area is acting as an ecological sink. Reduced genetic diversity is not necessary to identify an area as an ecological sink. In fact, a sink could result in increased diversity if immigration is from multiple populations. Population history, including whether the population is recovering or declining, and genetic patterns are important factors when interpreting population diversity levels. A recovering population that has lost diversity during previous bottlenecks would be expected to continue showing reduced genetic diversity unless new diversity was being introduced to the population through gene flow or new mutations. If a population was extirpated and subsequently recolonized from a single adjacent population one would expect to see reduced levels of genetic diversity. This process would be expected to yield very high estimates of gene flow and low genetic differentiation among populations. If the extirpated population was recolonized from multiple adjacent populations, one might expect to have normal or elevated levels of genetic diversity relative to reference populations. Recent population genetic investigations of possible ecological sinks have concluded that observed genetic patterns suggested population extirpation and a subsequent recolonization from adjacent populations (Matson et al., 2000; Baker et al., 2001; Theodorakis et al., 2001). The Sumgayit population genetic patterns do not support the hypothesis that frogs were extirpated from the area and subsequently recolonized from one or more surrounding populations. This scenario would be expected to result in a current population with relatively normal or elevated levels of both haplotype and nucleotide diversity.

Additionally, under the extirpation hypothesis one would not expect to find multiple unique haplotypes in Sumgayit.

Patterns of haplotype distributions, gene flow, and diversity indices are consistent with the Sumgayit region being an ecological sink. Population genetic patterns suggest that the majority of gene flow occurring between Sumgayit and ALI/NEF is actually immigration into Sumgayit (Table 4-3, Fig. 4-5). This regional comparison is probably the most significant in that these regions are ecologically similar lowland marsh habitats that have the highest levels of gene flow among regions. Gene flow estimates between Sumgayit and Alti-Agach are contradictory. Although both of our methods for estimating gene flow suggest that the total amount is less than that between Sumgayit and ALI/NEF, directional gene flow estimates seem to point in opposite directions. Maximum likelihood estimates suggest that more frogs are emigrating than immigrating (Table 4-3), while haplotype patterns seem to suggest that gene flow is directional and mainly due to immigration into Sumgayit. Haplotypes that are common in the north are also common in Sumgayit, but do not penetrate to the south. Haplotypes that are common in the south are also found in Sumgayit but do not penetrate to the north. Therefore, it appears that gene flow (migration) is into Sumgayit from both directions, but not out of Sumgayit in either direction, or at least at a much lower rate (Fig. 4-5). Regional genetic diversity estimates support our hypothesis that diversity was lost in Sumgayit due to reductions in effective population size and that the population was almost certainly not extirpated. We have shown that many environmental contaminant

concentrations have been reduced significantly in the last decade, and Bickham et al. (2003) discuss the history of Sumgayit including the economic decline and subsequent closings and reduced production at most plants and factories following the collapse of the Soviet Union. This reduction in new pollution and the transport and weathering of old contaminants has allowed for improvements in environmental quality in Sumgayit. This improvement in environmental health has likely allowed marsh frog populations to begin to recover from decades of terrible environmental conditions. Although conditions are improving, biomarkers of genetic damage still suggest that frogs in Sumgayit are impacted by contaminant exposure. Even with the environmental improvements in Sumgayit, population genetic patterns support the hypothesis that Sumgayit is acting as an ecological sink.

Regional data suggest that frog populations in Sumgayit are being impacted by long-term contaminant exposure. We wanted to investigate these effects at individual sites throughout the region. As discussed previously, genetic diversity estimates revealed reduced levels of both haplotype and nucleotide diversity in every site within the Sumgayit region except WTP. WTP was shown to have reduced levels of nucleotide diversity but normal levels of haplotype diversity. In a population that had reduced haplotype and nucleotide diversity, an elevated mutation rate would be expected to generate an increase in haplotypic diversity more rapidly than nucleotide diversity. We propose that WTP is actually a source of new mutations in the Sumgayit region. Based on patterns of haplotype and nucleotide diversity this argument would not be convincing.

However, we were able to discover significant evidence to support this hypothesis. Eight cases of mtDNA heteroplasmy were documented within this study. All of the heteroplasmic individuals were from the Sumgayit region. In fact, seven of the eight individuals were found at WTP.

Heteroplasmy can be caused by a few different mechanisms. The two most probable mechanisms are paternal leakage and new mutations. It is not always possible to determine which mechanism led to any observed heteroplasmy. Paternal leakage was concluded to be the most likely cause of observed heteroplasmy in two studies. Magoulas and Zouros (1993) justified their conclusion that paternal leakage was the cause of observed heteroplasmy in anchovy (*Engraulis encrasicolus*) based on the fact that the haplotypes involved were all common haplotypes in the population and that the haplotypes involved were phylogenetically separated by several mutational events. Kvist et al. (2003) concluded that heteroplasmy observed in populations of great tit (*Parus major*) was the result of paternal leakage based on the phylogenetic relationship of the heteroplasmic haplotypes. In this case, the haplotypes were representative of two different subspecies and the heteroplasmic individuals were concluded to be hybrids. In our heteroplasmic marsh frogs the haplotypes vary by only a single nucleotide in both the 1/5 and 4/15 forms. Additionally, the 4/15 heteroplasmy is only found at WTP, and haplotype 15 has not been documented within the Sumgayit region. The 1/5 heteroplasmy is slightly more complicated in that both haplotypes are present at WTP, although haplotype 5 is only present at a low frequency. The most convincing evidence

that the observed heteroplasmy from WTP is the result of new mutations resulting from contaminant exposure is that seven out of the eight heteroplasmic individuals were found at WTP. The remaining heteroplasmic frog was collected at the closest site to the west (Site 75). Our data suggest that there is a significant amount of gene flow between these two populations. Therefore, we cannot rule out the possibility that the single heteroplasmic frog collected away from WTP could have actually originated from or is a descendant of a female from WTP. If paternal leakage was the mechanism by which heteroplasmy arose one would expect to see this phenomenon in all populations at a comparable rate of occurrence. In fact, the observed frequencies of heteroplasmic frogs in Azerbaijan populations are significantly different from expected values under a random distribution model (χ^2, P=0.031). We cannot rule out the possibility that contaminants at WTP have somehow interfered with the normal processes that prevent paternal transmission of mtDNA. However, contaminants have not been shown to interfere with the expected exclusive maternal transmission of mtDNA. Thus, we conclude that paternal leakage is likely not the cause of the observed heteroplasmy in marsh frogs from Sumgayit. We hypothesize that observed heteroplasmy is the transitional state resulting from new mtDNA mutation. It is impossible to predict whether these heteroplasmic mutations will become full mutations or if they will be lost in future generations. Forster et al. (2002) showed that mutational heteroplasmy can continue through multiple generations in humans without reaching fixation. In fact, they found 23 heteroplasmic mutations yet failed to find a single full mutation.

We are unable to determine the number of mutational events that have led to these observed heteroplasmic frogs. We hypothesize that all of the individuals sharing a similar heteroplasmic condition are the result of single mutational events. We know that at least two independent events would be required to produce the two observed heteroplasmic forms. At least one other mutational event is required to account for the individual from Site 75 if this frog is not the result of gene flow with WTP. We believe the likelihood of migration to be much higher than another independent identical mutational event.

An increase in the frequency of heteroplasmy is sufficient to suggest that Sumgayit is acting as a source of new mutations. Evidence of genetic damage and even genomic instability in frogs from populations within Sumgayit provide us with evidence that the increased mutation rate at Sumgayit is likely an impact of contaminant exposure. While PAHs and mercury were linked to the elevated genetic damage in marsh frogs from Sumgayit, the complex mixture of contaminants found at WTP make it impossible to establish a causal relationship with any particular individual or group of contaminants and the elevated mutation rate.

In addition to the elevated contaminant concentrations found in Sumgayit, populations of marsh frogs in Sumgayit reveal the population genetic and evolutionary impacts of living in a contaminated environment in several ways. Genetic damage has been shown to be elevated in both the WTP and CAP in Sumgayit. WTP seems to be a source of new mutations as a result of an increased mutation rate. The Sumgayit region has reduced levels of genetic diversity which is detrimental to a population's potential to adapt and survive. Lastly, the Sumgayit region seems to act as an ecological sink. The effects we have documented at multiple levels of biological organization combine to provide real insight into the effects of long-term contaminant exposure on wildlife populations.

CHAPTER V

CONCLUSIONS

The chronic contaminant exposures to wildlife in Sumgayit clearly have an effect on the health of both individuals and populations. I have documented elevated environmental concentrations of many contaminants in Sumgayit, many of which are known to be genotoxic, clastogenic, or mutagenic. Sediment concentrations of both PAHs and mercury were associated with biomarkers of genetic damage. Both aquatic turtles and frogs revealed elevated levels of genetic damage at contaminated sites in Sumgayit. Not only are environmental contaminants impacting individuals, they are also affecting populations in a variety of ways. Population genetic analyses of marsh frogs suggest that populations from Sumgayit have reduced levels of genetic diversity. Additionally, the disproportionate frequencies of mtDNA heteroplasmy at WTP provide evidence of an increased mutation rate at this highly contaminated site in Sumgayit. Finally, regional migration estimates and diversity levels suggest that the Sumgayit region is acting as an ecological sink. The erosion of genetic diversity can reduce the ability of a population to adapt to changing environments or to deal with any type of challenge or stress. Population analyses provide a long-term view of contaminant effects. This perspective allows researchers to better understand the ultimate effects of contaminant exposure. While biomarkers can provide valuable information regarding the types of damage or alterations that individuals are experiencing, they lack the ability to estimate

the ultimate impact on populations. Population genetic analyses can provide a better understanding of the cumulative effects of chronic contaminant exposure.

Environmental contamination in Azerbaijan is a serious concern for both humans and wildlife. The results presented here provide valuable data for use in developing more accurate risk assessments for animals and people. These results will be used to develop risk assessments for the refugees living within the industrial zone of Sumgayit, who are particularly at risk. By improving our understanding of the impacts of chronic contaminant exposure, I hope to aid in the development of methods by which people can reduce the health risks associated with life in Sumgayit.

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APPENDICES

Appendix A. Individual turtle biomarker data.

TAMU #	Species	Site	FPCV	MN/1000	Comments
AK20278	*Emys orbicularis*	Neftchala	5.04		
AK20279	*Emys orbicularis*	Neftchala	5.46		
AK20280	*Emys orbicularis*	Neftchala	5.12		
AK20282	*Emys orbicularis*	Neftchala	4.74		
AK20286	*Emys orbicularis*	Neftchala	4.93		
AK20287	*Emys orbicularis*	Neftchala	4.84		
AK20288	*Emys orbicularis*	Neftchala	4.90		
AK20289	*Emys orbicularis*	Neftchala	5.56		
AK20290	*Emys orbicularis*	Neftchala	5.43		
AK20291	*Emys orbicularis*	Neftchala	5.31		
AK20293	*Emys orbicularis*	Neftchala	4.74		
AK17353	*Emys orbicularis*	WTP	5.02	9.8	
AK17354	*Emys orbicularis*	WTP		9.0	
AK17355	*Emys orbicularis*	WTP	4.95	3.4	
AK17356	*Emys orbicularis*	WTP	4.62	4.4	
AK17357	*Emys orbicularis*	WTP		5.8	
AK17358	*Emys orbicularis*	WTP	4.57	3.4	
AK17359	*Emys orbicularis*	WTP	4.79	3.8	
AK17360	*Emys orbicularis*	WTP	4.62	7.2	
AK17361	*Emys orbicularis*	WTP	4.84	3.2	
AK17362	*Emys orbicularis*	WTP		3.2	
AK17363	*Emys orbicularis*	WTP	4.93	3.0	
AK17364	*Emys orbicularis*	WTP	4.75	5.2	
AK17365	*Emys orbicularis*	WTP	4.59	2.4	
AK17366	*Emys orbicularis*	WTP	4.61	3.6	
AK17367	*Emys orbicularis*	WTP	4.67	7.6	
AK17368	*Emys orbicularis*	WTP	4.68	4.0	
AK17369	*Emys orbicularis*	WTP	4.84	12.8	
AK17370	*Emys orbicularis*	WTP	4.73	9.0	
AK17371	*Emys orbicularis*	WTP	4.31	10.2	
AK17372	*Emys orbicularis*	WTP	4.48	4.6	
AK17373	*Emys orbicularis*	WTP	4.87	2.2	
AK17374	*Emys orbicularis*	WTP	4.67	19.2	

Appendix A. Continued.

TAMU #	Species	Site	FPCV	MN/1000	Comments
AK17375	*Emys orbicularis*	WTP	4.80	7.0	
AK17376	*Emys orbicularis*	WTP	4.80	2.8	
AK17377	*Emys orbicularis*	WTP	4.87	3.4	
AK17378	*Emys orbicularis*	WTP	5.04	8.0	
AK17379	*Emys orbicularis*	WTP	4.89	20.4	
AK17380	*Emys orbicularis*	WTP	5.12	20.2	
AK17381	*Emys orbicularis*	WTP		25.0	
AK17382	*Emys orbicularis*	WTP	4.62	24.4	
AK17383	*Emys orbicularis*	WTP	4.85	17.8	
AK17481	*Emys orbicularis*	Ali Bairamly	4.58	4.0	
AK17482	*Emys orbicularis*	Ali Bairamly	4.19	3.2	
AK17483	*Emys orbicularis*	Ali Bairamly	4.20	4.0	
AK17484	*Emys orbicularis*	Ali Bairamly	4.52	3.2	
AK17485	*Emys orbicularis*	Ali Bairamly	4.47	3.6	
AK17486	*Emys orbicularis*	Ali Bairamly	4.35	33.0	
AK17487	*Emys orbicularis*	Ali Bairamly	4.10	4.4	
AK17488	*Emys orbicularis*	Ali Bairamly	4.85	3.4	
AK17489	*Emys orbicularis*	Ali Bairamly	4.87	7.6	
AK17490	*Emys orbicularis*	Ali Bairamly	4.44	3.8	
AK17491	*Emys orbicularis*	Ali Bairamly	4.87	4.2	
AK17492	*Emys orbicularis*	Ali Bairamly	4.72	4.6	
AK17493	*Emys orbicularis*	Ali Bairamly	4.66	3.4	
AK17494	*Emys orbicularis*	Ali Bairamly	5.32	3.2	
AK20281	*Mauremys caspica*	Neftchala	4.79		
AK20283	*Mauremys caspica*	Neftchala	4.78		
AK20284	*Mauremys caspica*	Neftchala	4.79		
AK20285	*Mauremys caspica*	Neftchala	5.15		
AK20292	*Mauremys caspica*	Neftchala	4.78		
AK20294	*Mauremys caspica*	Neftchala	4.52		
AK20295	*Mauremys caspica*	Neftchala	4.54		
AK20296	*Mauremys caspica*	Neftchala	4.04		
AK20297	*Mauremys caspica*	Neftchala	3.84		
AK20298	*Mauremys caspica*	Neftchala	4.63		
AK17344	*Mauremys caspica*	WTP	4.12		
AK17345	*Mauremys caspica*	WTP	3.97		

Appendix A. Continued.

TAMU #	Species	Site	FPCV	MN/1000	Comments
AK17346	*Mauremys caspica*	WTP	4.53		
AK17347	*Mauremys caspica*	WTP	4.02		
AK17348	*Mauremys caspica*	WTP	3.73		
AK17349	*Mauremys caspica*	WTP	4.44		
AK17350	*Mauremys caspica*	WTP	4.50		
AK17351	*Mauremys caspica*	WTP	4.49		
AK17352	*Mauremys caspica*	WTP	3.93		Triploid

Appendix B. Individual frog haplotype and biomarker data.

TAMU #	Site	Haplotype	MN/1000	HPCV	Comments
AK20299	Neftchala	1			
AK20300	Neftchala	1			
AK20301	Neftchala	1			
AK20302	Neftchala	4			
AK20303	Neftchala	1			
AK20304	Neftchala	1			
AK20305	Neftchala	4			
AK20306	Neftchala	2			
AK20307	Neftchala	7			
AK20321	Neftchala	4			
AK20322	Neftchala	1			
AK20323	Neftchala				
AK17293	CAP	1	4.2	3.48	
AK17294	CAP	3	3.4	8.52	
AK17295	CAP	1	2.2	2.98	Triploid
AK17296	CAP	4	2.6	4.35	
AK17297	CAP	1	5.2	3.34	
AK17298	CAP	1	1	3.25	
AK17299	CAP	1	3.2	3.53	
AK17300	CAP	1	3	5.19	
AK17301	CAP	6		3.91	
AK17302	CAP	1	2.6	7.18	
AK17303	CAP	6	13.6	5.25	
AK17304	CAP	1	3		
AK17305	CAP	1	6.6	6.96	
AK17306	CAP	1	7.4	9.19	
AK17307	CAP	3	6	3.01	
AK17308	CAP	1	18	3.16	
AK17309	CAP	1	23.8	8.57	
AK17310	CAP	1	9.4		
AK17311	CAP	1	4.4		
AK17312	CAP	2		10.9	
AK17431	Alti-Agach	6	5.8		
AK17432	Alti-Agach	6	2.4		
AK17433	Alti-Agach	2	0.8		

Appendix B. Continued.

TAMU #	Site	Haplotype	MN/1000	HPCV	Comments
AK17434	Alti-Agach	6	1.8		
AK17435	Alti-Agach	2	1.8		
AK17436	Alti-Agach	4	2		
AK17437	Alti-Agach	9	1.2		
AK17438	Alti-Agach	2	1.4		
AK17439	Alti-Agach	6	1		
AK17440	Alti-Agach	2	3.4		
AK17441	Alti-Agach	6	2.2		
AK17442	Alti-Agach	6	1.4		
AK17443	Alti-Agach	6	3.6		
AK17444	Alti-Agach	6	3.6		
AK17445	Alti-Agach	4	4.2		
AK17446	Alti-Agach		4.6		
AK17447	Alti-Agach		2.4		
AK17448	Alti-Agach	6	3		
AK17449	Alti-Agach	2	1		
AK17450	Alti-Agach		4.2		
AK17451	Alti-Agach	6			
AK17452	Alti-Agach	15	1		
AK17453	Alti-Agach	3	2.2		
AK17454	Alti-Agach	1	2		
AK17455	Alti-Agach	9	1.8		
AK16874	11	6			
AK16875	11	6			
AK16876	11	6			
AK16877	11	3	5.7		
AK16878	11	6	4		
AK16879	11	6	3.7		
AK16880	11	6			
AK16881	11	6	8.3		
AK16882	11	1	6.3		
AK16883	11	6	7.7		
AK16884	11	6	4.7		
AK16885	11	6	5		
AK16886	11	1	4.3		

Appendix B. Continued.

TAMU #	Site	Haplotype	MN/1000	HPCV	Comments
AK16887	11	6	2.7		
AK16888	11	6	3.7		
AK16889	11	1			
AK16890	11	3			
AK16891	11	1	4.3		
AK16892	11	6	2.3		
AK16893	11	1			
AK16894	11	1	4.3		
AK16895	11	6			
AK16896	11	6	1.7		
AK16897	11	1	2.3		
AK17412	107	1	0.6		
AK17413	107	4	0.6		
AK17414	107	1	0.8		
AK17415	107	6	0.6		
AK17416	107	2	1		
AK17420	108		6.8		
AK17421	108	10	3		
AK17456	Ali Bairamly		0.8	3.49	
AK17457	Ali Bairamly	4	3.2	2.36	
AK17458	Ali Bairamly	4	1.6	3.56	
AK17459	Ali Bairamly	12	0.8	3.13	
AK17460	Ali Bairamly	1	1.4	3.34	
AK17461	Ali Bairamly		0.8	3.14	
AK17462	Ali Bairamly	13	5	3.74	
AK17463	Ali Bairamly	1	4.6	3.76	
AK17464	Ali Bairamly	4	1.2		
AK17465	Ali Bairamly	13			
AK17466	Ali Bairamly	11	1	5.03	
AK17467	Ali Bairamly	13	1.4		
AK17468	Ali Bairamly	7	0.8	3.44	
AK17469	Ali Bairamly	1	0.6	2.98	
AK17470	Ali Bairamly	1	0.8	4.66	
AK17471	Ali Bairamly	4	2.4		
AK17472	Ali Bairamly	10	0.2	3.19	

Appendix B. Continued.

TAMU #	Site	Haplotype	MN/1000	HPCV	Comments
AK17473	Ali Bairamly	2	0.4		
AK17474	Ali Bairamly	1	2.8	3.98	
AK17475	Ali Bairamly	1	0.6	4.34	
AK17476	Ali Bairamly	4	1.2		
AK17477	Ali Bairamly	1	2.6		
AK17478	Ali Bairamly	1	1.6		
AK17479	Ali Bairamly	2			
AK17480	Ali Bairamly	4	0.8		
AK16898	12	1	4		
AK16899	12	1	1		
AK16900	12	1	1.3		
AK16901	12	1			
AK16902	12	1	2.7		
AK16903	12	1	4.3		
AK16904	12	1	3.3		
AK16905	12	1	3.7		
AK16906	12	1	1.7		
AK16907	12		3		
AK17287	12	2	4.2		
AK17288	12	2	5		
AK17289	12	1	1.2		
AK17290	12	4	3.4		
AK17291	12	1	2.4		
AK17292	12	1	4.2		
AK16908	75	1			
AK16909	75	1	5.7		
AK16910	75	14	2.3		
AK16911	75	8	1.7		
AK16912	75	1	1.3		
AK16913	75	1	1.3		
AK16914	75	6	1.7		
AK16915	75	1	3.3		
AK16916	75	1	2.7		
AK16917	75	8	2		
AK16918	75	1	3.7		

Appendix B. Continued.

TAMU #	Site	Haplotype	MN/1000	HPCV	Comments
AK16919	75	1	4.3		
AK16920	75	1	4.7		
AK16921	75	1	20.7		
AK17422	75	1/5	0.6		
AK17423	75	1	1.2		
AK17424	75	1	2		
AK17425	75	1	3.8		
AK17426	75	14	1.4		
AK17427	75	8	4		
AK16922	76	1	3.7		
AK16923	76	1	7.7		
AK16924	76	3	5.7		
AK16925	76	1	9.3		
AK16926	76	1	2.3		
AK16927	76	1	4.3		
AK16928	76	1	9.7		
AK16929	76	1	11		
AK16930	76	1	8.7		
AK16931	76	1			
AK16932	76	4			
AK16933	76		6.3		
AK16934	76	1			
AK16935	76	1	3.7		
AK16936	76	1	5.3		
AK20308	WTP	6			
AK20309	WTP	4			
AK20310	WTP	6			
AK20311	WTP				
AK20312	WTP	1			
AK20313	WTP	6			
AK20314	WTP	1			
AK20315	WTP	14			
AK20316	WTP	14			
AK20317	WTP	1			
AK20318	WTP				

Appendix B. Continued.

TAMU #	Site	Haplotype	MN/1000	HPCV	Comments
AK20319	WTP	6			
AK20320	WTP	2			
AK16853	WTP	6	12.3		
AK16854	WTP	1	13.7		
AK16855	WTP	1/5	10		
AK16856	WTP	8	7.3		
AK16857	WTP	5	12.3		
AK16858	WTP	1			
AK16859	WTP	5	23		
AK16860	WTP	6			
AK16861	WTP	1	16.7		
AK16862	WTP	6	7		
AK16863	WTP	1	15.3		
AK16864	WTP	1			
AK16865	WTP	1			
AK16866	WTP	6	12		
AK16867	WTP	1			
AK16868	WTP	1	20		
AK16869	WTP	4			
AK16870	WTP	2	6.7		
AK16871	WTP	6	5.3		
AK16872	WTP	4/15	4		
AK16873	WTP	1	4.3		
AK17313	WTP	1/5	27.2	4.07	
AK17314	WTP	6	8.4	2.81	
AK17315	WTP	2	5.4	3.3	
AK17316	WTP	1/5	16.4	3.52	
AK17317	WTP	1	3	2.51	
AK17318	WTP	6	3.6	2.42	
AK17319	WTP	1	7.6	2.98	
AK17320	WTP	6	13.2	2.8	
AK17321	WTP	6	3.4	3.63	
AK17322	WTP	4	10.2	3.23	
AK17323	WTP	1	3	3.05	
AK17324	WTP	3	13.2	3.47	

Appendix B. Continued.

TAMU #	Site	Haplotype	MN/1000	HPCV	Comments
AK17325	WTP	4/15	1.4	2.98	
AK17326	WTP	6	0.6	3.31	
AK17327	WTP	1	3.8	2.52	
AK17328	WTP	1	4.4	7.84	
AK17329	WTP	1/5	2.4	3.45	
AK17330	WTP	3	0.6	5.62	
AK17331	WTP	1/5	14.2	6.72	
AK17332	WTP	6	10.2	6.85	

Appendix C. Individual frog carcass organochlorine (ppb) and mercury (ppm) concentrations, and weigh (g).

TAMU #	Site	Lindane	Heptachlor	Aldrin	Dieldrin	DDE	Endrin	DDT	Methoxychlor	Hg	Weight
AK17293	CAP									4.9	
AK17294	CAP									9.81	
AK17295	CAP									5.33	
AK17298	CAP									9.88	
AK17302	CAP									9	
AK17307	CAP									4.65	
AK17309	CAP									12.6	
AK17312	CAP									5.02	
AK16877	11	20	< D.L.	< D.L.	< D.L.	27	< D.L.	20	< D.L.		9
AK16878	11	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		10.4
AK16879	11	113	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	20	< D.L.		9.8
AK16881	11	13	< D.L.	< D.L.	< D.L.	20	< D.L.	13	< D.L.		16.4
AK16882	11	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		7.1
AK16883	11	12	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		8.8
AK16884	11	< D.L.	< D.L.	< D.L.	< D.L.	9	< D.L.	11	< D.L.		8.3
AK16885	11	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		24.6
AK16886	11	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	22	< D.L.		4
AK16887	11	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		7.2
AK16888	11	13	< D.L.	< D.L.	< D.L.	18	< D.L.	< D.L.	< D.L.		8.3
AK16891	11	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		6.6
AK16892	11	< D.L.	< D.L.	< D.L.	< D.L.	7	< D.L.	< D.L.	< D.L.		10.6
AK16894	11	64	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		11.2
AK16896	11	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		4.2
AK16897	11	22	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	30	< D.L.		5.2
AK16898	12	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		4.6
AK16899	12	< D.L.	5	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		24.1
AK16900	12	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		8.6
AK16902	12	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		2.7
AK16903	12	< D.L.	131	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		10
AK16904	12	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	50	< D.L.		9.3
AK16905	12	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		9.5
AK16906	12	7	5	< D.L.	< D.L.	10	< D.L.	19	< D.L.		23.4
AK16907	12	< D.L.	< D.L.	< D.L.	< D.L.	8	5	< D.L.	< D.L.		26.6
AK16909	75	165	< D.L.	< D.L.	< D.L.	250	< D.L.	317	< D.L.		7.8
AK16910	75	29	< D.L.	< D.L.	< D.L.	38	47	23	< D.L.		11.9
AK16911	75	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	91	< D.L.		2.5
AK16912	75	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	59	< D.L.		4.8
AK16913	75	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		14.6
AK16914	75	< D.L.	< D.L.	< D.L.	< D.L.	57	36	< D.L.	< D.L.		6.9
AK16915	75	42	< D.L.	< D.L.	< D.L.	272	15	98	< D.L.		6.3
AK16916	75	17	< D.L.	< D.L.	< D.L.	21	< D.L.	< D.L.	< D.L.		11.2
AK16917	75	< D.L.	< D.L.	< D.L.	< D.L.	319	91	229	< D.L.		10
AK16918	75	616	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	72	< D.L.		3.2
AK16919	75	< D.L.	< D.L.	< D.L.	< D.L.	32	< D.L.	44	< D.L.		5
AK16920	75	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		1.5
AK16921	75	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		5.9
AK16922	76	25	5	< D.L.	< D.L.	< D.L.	10	< D.L.	< D.L.		39.2
AK16923	76	8	< D.L.	< D.L.	< D.L.	< D.L.	22	< D.L.	< D.L.		31.7
AK16924	76	6	< D.L.	< D.L.	< D.L.	< D.L.	13	< D.L.	< D.L.		27.3
AK16925	76	< D.L.	< D.L.	< D.L.	< D.L.	6	30	< D.L.	< D.L.		33.1
AK16926	76	7	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		18.1
AK16927	76	10	< D.L.	< D.L.	< D.L.	< D.L.	16	< D.L.	< D.L.		25.1
AK16928	76	5	8	< D.L.	< D.L.	8	28	10	10		23.5
AK16929	76	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	27	< D.L.	< D.L.		15.5
AK16930	76	7	< D.L.	< D.L.	< D.L.	< D.L.	32	< D.L.	< D.L.		21.6
AK16933	76	< D.L.	6	< D.L.	7	10	28	< D.L.	< D.L.		19
AK16935	76	5	7	< D.L.	< D.L.	< D.L.	28	< D.L.	< D.L.		30.8

Appendix C. Continued.

TAMU #	Site	Lindane	Heptachlor	Aldrin	Dieldrin	DDE	Endrin	DDT	Methoxychlor	Hg	Weight
AK16936	76	7	9	< D.L.	< D.L.	13	82	13	< D.L.		17.8
AK16853	WTP	22	< D.L.	< D.L.	< D.L.	24	< D.L.	22	< D.L.		7.8
AK16854	WTP	< D.L.	< D.L.	< D.L.	< D.L.	156	< D.L.	140	< D.L.		14.6
AK16855	WTP	< D.L.	< D.L.	< D.L.	< D.L.	19	8	20	< D.L.		9
AK16856	WTP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		8.6
AK16857	WTP	< D.L.	< D.L.	< D.L.	< D.L.	82	< D.L.	32	< D.L.		5.5
AK16859	WTP	9	< D.L.	< D.L.	< D.L.	85	< D.L.	13	20		7
AK16861	WTP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		4.9
AK16862	WTP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	11	< D.L.		20.9
AK16863	WTP	2	< D.L.	< D.L.	< D.L.	9	< D.L.	5	< D.L.		23.9
AK16866	WTP	< D.L.	< D.L.	< D.L.	< D.L.	31	< D.L.	< D.L.	< D.L.		8.1
AK16868	WTP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		7.9
AK16870	WTP	38	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		3.2
AK16871	WTP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.		3.4
AK16872	WTP	< D.L.	< D.L.	< D.L.	< D.L.	59	< D.L.	< D.L.	< D.L.		10
AK16873	WTP	42	< D.L.	< D.L.	< D.L.	79	< D.L.	89	< D.L.		3.4
AK17313	WTP									1.91	
AK17317	WTP									1.91	
AK17318	WTP									1.12	
AK17321	WTP									0.97	
AK17328	WTP									2.32	
AK17330	WTP									1.75	
AK17331	WTP									0.96	
AK17332	WTP									2.77	

< D.L. represents samples below method detection limit.

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