



Population Genetic Structure of Earthworms (*Lumbricus rubellus*) in Soils Contaminated by Heavy Metals

JOHN D. PELES,^{1,*} WILLIAM I. TOWLER² AND SHELDON I. GUTTMAN³

¹*Pennsylvania State University McKeesport, 4000 University Drive, McKeesport, PA 15132, USA*

²*Department of Biological Sciences, Marshall University, 400 Hal Greer Blvd., Huntington, WV 25755, USA*

³*Department of Zoology, Miami University, Oxford, OH, 45056, USA*

Accepted July 31, 2002

Abstract. The genetic structure of earthworm (*Lumbricus rubellus*) populations from plots ($N = 3$) contaminated by sewage sludge (containing Cd, Cu, Pb, Zn) and reference plots ($N = 3$) was compared. Earthworms ($N = 30\text{--}40$ per plot) were collected during May 2000 and electrophoretic analyses were used to assess genetic variation at three polymorphic loci (GPI, LAP, PGM). Allele and genotype frequencies for GPI and PGM differed significantly between sludge and reference populations. Sludge populations were characterized by a reduced frequency of the most common genotype and allele at these loci, and the presence of alleles not found in the reference population. Allele and genotype frequencies did not differ significantly among individual plots within the sludge or the reference population. These results suggest that certain alleles and genotypes may be more sensitive to the effects of heavy metals. Multi-locus heterozygosity was significantly higher in the sludge population compared to the reference population; the proportion of individuals heterozygous at two or more loci was more than 15% higher in the sludge population. Results demonstrate that measures of population genetic structure in earthworms may represent useful biological indicators for the assessment of impacted terrestrial habitats.

Keywords: earthworms; sewage sludge; heavy metals; genetic structure; biomarkers; heterozygosity

Introduction

During the past decade, the study of potential effects of toxicants on the genetic structure of natural populations has become an increasingly relevant aspect of ecotoxicology (Bickham and Smolen, 1994; Guttman, 1994; Bickham et al., 2000). Significant associations between changes in the population genetic structure of aquatic organisms and exposure to toxicants in natural systems have been documented in numerous investigations (see reviews by Guttman, 1994;

Gillespie and Guttman, 1999). The results of these studies suggest that significant changes in genotype/allele frequencies and overall genetic variation may occur in contaminated environments as a result of population bottlenecks and/or selection at certain loci (e.g., Gillespie and Guttman, 1989, 1993; Benton et al., 1994; Roark et al., 2001).

Although causative mechanisms are difficult to determine from field investigations, numerous laboratory studies have shown that organisms with certain allozyme genotypes vary in their resistance to various toxicants (see reviews by Guttman, 1994; Gillespie and Guttman, 1999; and recent studies by Duan et al., 2000a,b; Schlueter et al., 2000). Thus,

*To whom correspondence should be addressed: Tel.: 412-675-9484; Fax: 412-675-9166; E-mail: jdp12@psu.edu

there exists strong evidence for the selective effects of toxicants at certain loci (Gillespie and Guttman, 1999). Regardless of the mechanisms involved, measures of population genetic structure are regarded as useful biomarkers for monitoring the impact of toxicants on aquatic systems and the success of remediation efforts within these systems (Gillespie and Guttman, 1993; Guttman, 1994; Foré et al., 1995a,b).

Although population genetic structure represents a potentially useful tool for the assessment of environmental impacts, only two investigations to date have focused on genetic changes within terrestrial populations. Reding and Guttman (1991) found no significant associations between the genetic structure of periodical cicadas (*Magicicada cassini*) and radionuclide contamination. Tranvik et al. (1994) documented minor differences in the genetic structure of soil arthropods (*Orchesella bifasciata*) exposed to heavy metals. Thus, further investigation is needed concerning the genetic changes that occur in populations of terrestrial organisms exposed to toxicants.

This investigation focused on the potential influence of long-term exposure to heavy metals on the population genetic structure of a ubiquitously distributed terrestrial organism. Specifically, our purpose was to determine and compare (a) allele frequencies, (b) genotype frequencies, and (c) multi- and single-locus heterozygosity between populations of earthworms (*Lumbricus rubellus*) inhabiting soils contaminated by heavy metals from sewage sludge and those from uncontaminated reference soils. Earthworms are particularly relevant for the study of potential population genetic effects of exposure to heavy metals because of their important role as biomonitors of metal pollution in terrestrial systems (Morgan and Morgan, 1988).

Methods

Earthworms were sampled from 0.025 ha sludge-treated ($N=3$) and reference ($N=3$) plots at the Miami University Ecology Research Center (ERC) near Oxford, Butler County, OH. The plots were previously used in a long-term study examining the effects of nutrient enrichment on old-field succession (see Brewer et al., 1994 for a complete description of the history of these plots). From 1978 to 1988, sludge plots were treated annually ($8965 \text{ kg ha}^{-1} \text{ yr}$) with municipal sewage sludge, containing relatively high

concentrations of cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) (Levine et al., 1989), whereas reference plots remained untreated.

Concentrations of heavy metals were monitored yearly in each plot from 1978 to 1993. During this time period, concentrations of Cd, Cu, Pb, and Zn were several times higher in sludge-treated soils compared to reference soils and ranged from 1.3 to 2.7 ppm, 16.9 to 36.0 ppm, 23.1 to 48.0 ppm, and 81.0 to 140.5 ppm, respectively (Levine et al., 1989; Brewer and Barrett, 1995; Peles et al., 1998). In addition, earthworms from sludge-treated plots were also shown to concentrate heavy metals to levels well above those found in the soil (Kruse and Barrett, 1985; Levine et al., 1989; Brewer and Barrett, 1995). Thus, earthworm populations from sludge-treated plots have been exposed to elevated concentrations of Cd, Cu, Pb, and Zn for more than 20 years.

Earthworms ($N=30\text{--}42$ per plot) were collected during May 2000. Sludge-treated plots were interspersed with reference plots and distances between individual plots ranged from 16 to 72 m (Table 1). Each plot was divided into a 3×3 grid and samples were taken at nine locations with each sample being taken 4.1 m from the next nearest sample. At each location, blocks of soil (1000 cc) were taken from the surface and sorted by hand to collect all *L. rubellus* present. Earthworms were rinsed free of soil particles and frozen at -80°C until electrophoretic analyses.

The anterior two-thirds of each worm were homogenized on ice with an extraction buffer of 2% 2-phenoxyethanol. Homogenates were centrifuged and the resulting supernatants were used for electrophoresis. Three polymorphic loci—glucose phosphate isomerase (GPI; EC 5.3.1.9), leucine amino peptidase (LAP; EC 3.4.11.1) and phosphoglucosyltransferase (PGM; EC 2.7.5.1)—were identified following preliminary analyses of 20 loci. Genetic variation

Table 1. Matrix of geographic distances (m) between individual reference (R1, R2, R3) and sludge-treated plots (S4, S5, S6) at the Miami University Ecology Research Center

Plot	R1	R2	R3	S4	S5	S6
R1	***	32	51	32	32	45
R2		***	16	45	45	32
R3			***	51	68	51
S4				***	64	72
S5					***	32
S6						***

in GPI and LAP was examined using starch gel electrophoresis. GPI was resolved using a tris-citrate buffer (pH 6.7) and LAP was resolved using a lithium hydroxide buffer (pH 8.1). All electrophoretic and staining procedures followed those described by Harris and Hopkinson (1976). PGM was resolved with a tris-EDTA-malate (pH 7.4) buffer using cellulose acetate electrophoresis (Richardson, 1986).

BIOSYS-1 (Swofford and Selander, 1981) was used to determine mean number of alleles per locus, direct-count heterozygosity per locus, allele frequencies, and genotype frequencies, for populations from each individual plot, and for pooled samples of all sludge-treated plots (sludge population) and reference plots (reference population). χ^2 contingency analyses were used to compare allele and genotype frequencies within and between reference and sludge populations. BIOSYS-1 was also used to calculate modified Rogers' genetic distance values between plots; the correlation between genetic and geographic distance was examined by Pearson product-moment correlation analysis. Each individual was assigned to a multilocus heterozygosity (H) class (0, 1, ≥ 2 heterozygous loci) and the frequency of individuals in each of these classes was compared within and between populations using contingency χ^2 analysis. In addition, χ^2 was used to compare the frequency of heterozygotes at each individual locus within and between populations.

Results

The mean number of alleles per locus for all sludge-treated plots (S4 = 3.3, S5 = 3.7, S6 = 3.0) was 3.7 compared to 3.0 for reference plots (C1 = 3.0, C2 = 2.7, C3 = 2.7). Mean direct count heterozygosity for all sludge-treated plots (S4 = 29.4%, S5 = 28.9%, S6 = 26.5%) was 28.0% compared to 17.2% for reference plots (C1 = 11.2%, C2 = 22.7%, C3 = 16.0%). Modified Rogers' genetic distance (Table 2) was not significantly correlated with geographic distance ($r = 0.04$, $p = 0.88$).

Allele and genotype frequencies at the LAP locus did not differ significantly (range of χ^2 values = 1.22–5.71, $p > 0.05$ in all cases) among sludge-treated plots, among reference plots, or between pooled samples of sludge-treated plots and reference plots and will not be discussed further. Allele (reference $\chi^2 = 0.79$, $p = 0.67$; sludge $\chi^2 = 6.12$, $p = 0.19$) and

Table 2. Matrix of Rogers' modified genetic distances between earthworm (*L. rubellus*) populations from individual reference (R1, R2, R3) and sludge-treated plots (S4, S5, S6) at the Miami University Ecology Research Center

Plot	R1	R2	R3	S4	S5	S6
R1	***	0.07	0.06	0.13	0.16	0.11
R2		***	0.06	0.10	0.12	0.06
R3			***	0.12	0.16	0.100
S4				***	0.05	0.06
S5					***	0.07
S6						***

genotype (reference $\chi^2 = 0.84$, $p = 0.66$; sludge $\chi^2 = 5.02$, $p = 0.28$) frequencies for the GPI locus did not differ significantly among sludge-treated plots or among reference plots (Fig. 1). Likewise, allele (reference $\chi^2 = 8.71$, $p = 0.07$; sludge $\chi^2 = 6.81$, $p = 0.15$) and genotype (reference $\chi^2 = 8.12$, $p = 0.09$; sludge $\chi^2 = 3.41$, $p = 0.49$) frequencies for the PGM locus did not differ significantly among sludge-treated or reference plots (Fig. 2). Therefore, results regarding allele and genotype frequencies for GPI and PGM will be discussed on the basis of comparisons between pooled samples of all sludge-treated plots (sludge population) and reference plots (reference population).

Allele ($\chi^2 = 13.74$, $p = 0.001$) and genotype ($\chi^2 = 11.38$, $p = 0.003$) frequencies at the GPI locus differed significantly between sludge and reference populations (Fig. 1). The sludge population was characterized by (a) a reduced frequency of the GPI-A allele, (b) an increased frequency of GPI-B and (c) the occurrence of a third allele (GPI-C) that was not found in reference populations (Fig. 1A). The frequency of the most common homozygous genotype (GPI-AA) was 15% lower in the sludge population than in the reference population. In contrast, the frequency of the most common heterozygous genotype (GPI-AB) was nearly twice as high in the sludge population than in the reference population (Fig. 1).

Allele ($\chi^2 = 30.71$, $p < 0.0001$) and genotype ($\chi^2 = 17.59$, $p < 0.0005$) frequencies at the PGM locus differed significantly between sludge and reference populations (Fig. 2). The sludge population was characterized by (a) a reduced frequency of the most common allele (PGM-B), (b) an allele frequency for PGM-C that was twice as high as in the reference population, and (c) the presence of a PGM-D allele that was not found in the reference population (Fig. 2).

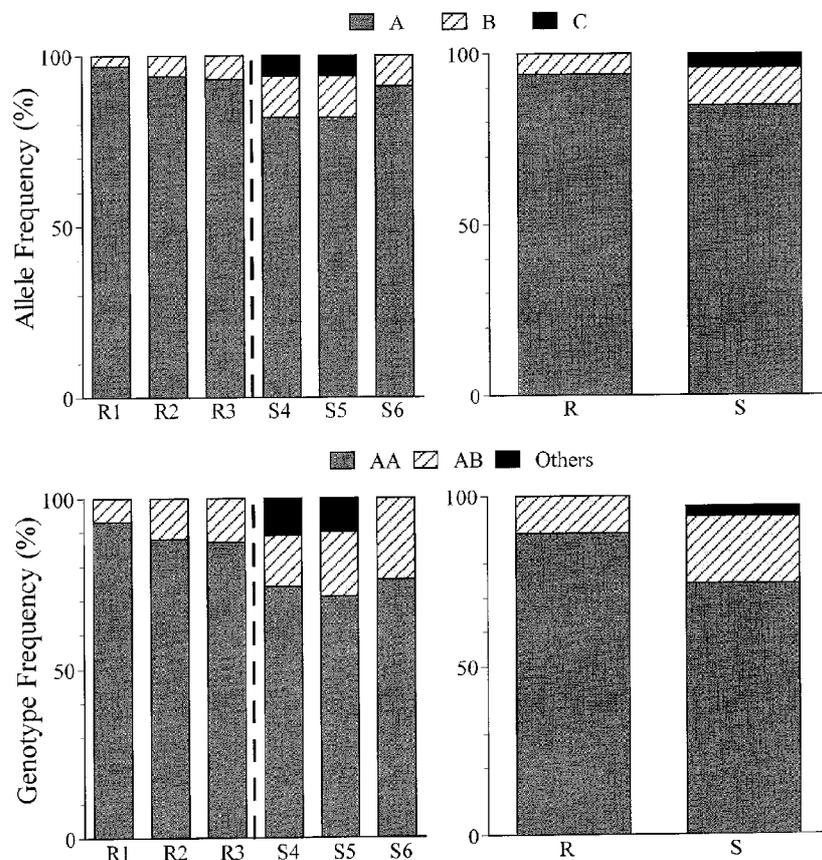


Figure 1. Allele and genotype frequencies of GPI for earthworms collected from reference plots (R1, R2, R3) and sludge-treated plots (S4, S5, S6) contaminated by heavy metals. Frequencies are presented for individual plots and for all reference (R) plots and all sludge (S) plots combined.

A rare fifth allele (PGM-E) occurred in one individual within the sludge population. The frequency of the most common homozygous genotype (PGM-BB) was 25% lower in the sludge population than in the reference population. In contrast, the frequency of genotypes containing the GPI-C and GPI-D alleles was much higher in the sludge population (Fig. 2).

The proportion of individuals in each multilocus heterozygosity class (Fig. 3) differed significantly between populations ($\chi^2 = 17.96$, $p < 0.0001$). Proportions of individuals in each multilocus heterozygosity class did not differ among individual plots within the sludge population ($\chi^2 = 1.49$, $p = 0.83$) or the reference population ($\chi^2 = 5.21$, $p = 0.27$). The proportion of individuals with heterozygous genotypes for both GPI (reference = 11.1%, sludge = 23.0%) and PGM (reference = 19.1%, sludge = 32.5%) was significantly higher in the sludge population compared to the reference population (GPI

$\chi^2 = 5.59$, $p = 0.02$; PGM $\chi^2 = 5.24$, $p = 0.02$). The proportion of heterozygous genotypes at the LAP locus did not differ significantly ($\chi^2 = 2.03$, $p = 0.15$) between the sludge and reference populations.

Discussion

It was hypothesized that the genetic structure of earthworm populations exposed to heavy metals from sewage sludge would differ significantly from that of the reference populations. Results regarding differences in genetic structure of sludge and reference populations at the GPI and PGM loci (Figs 1 and 2) supported this hypothesis. Furthermore, the fact that there were no differences in allele and genotype frequencies among individual plots within the reference or sludge populations (Figs 1 and 2) support the notion that these differences were due to exposure

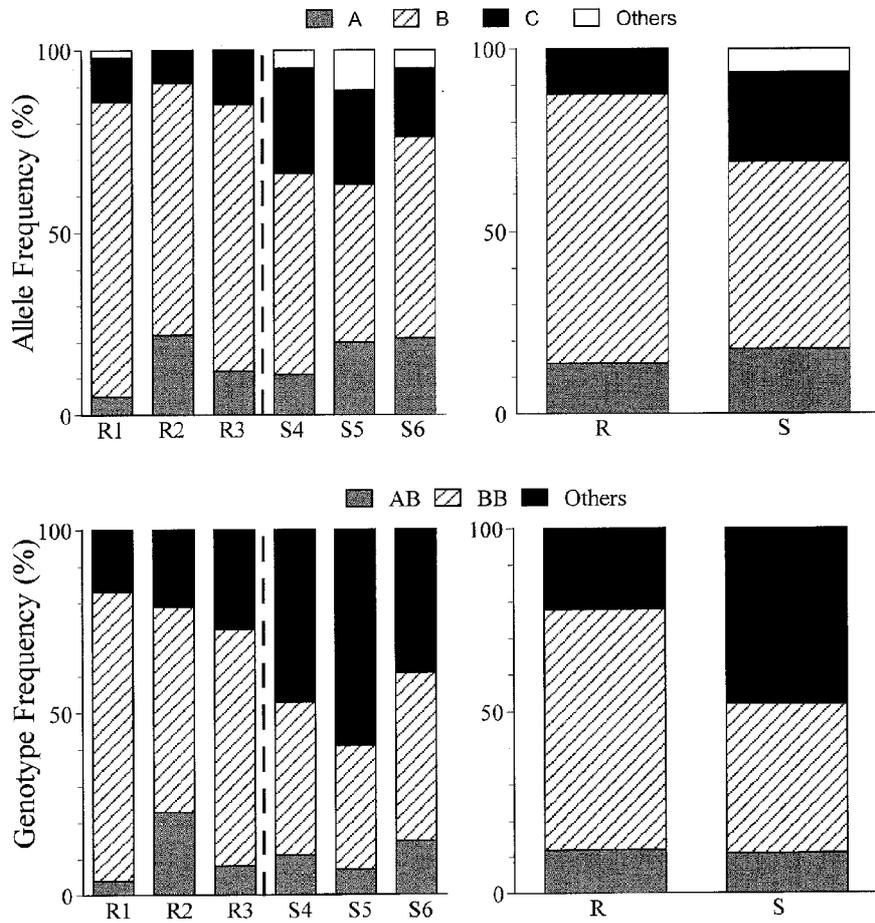


Figure 2. Allele and genotype frequencies of PGM for earthworms collected from reference plots (R1, R2, R3) and sludge-treated plots (S4, S5, S6) contaminated by heavy metals. Frequencies are presented for individual plots and for all reference (R) plots and all sludge (S) combined.

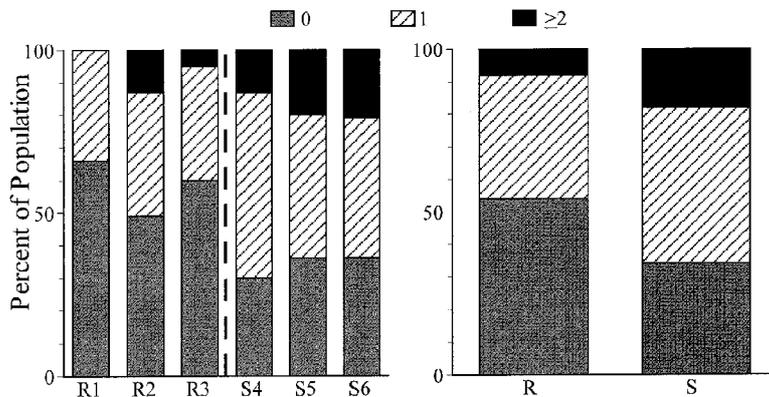


Figure 3. Percent of individuals from reference plots (R1, R2, R3) and sludge-treated plots (S4, S5, S6) contaminated by heavy metals in each of three multi-locus heterozygosity classes (0, 1, 2 + loci). Frequencies are presented for individual plots and for all reference (R) plots and all sludge (S) plots combined.

to metals and not simply due to random variation. Results of this investigation were similar to those of Benton et al. (1994), who found significant differences in allele and genotype frequencies of GPI between populations of mosquitofish (*Gambusia affinis*) and freshwater snails (*Heliosoma trivolvis*) from reference sites and aquatic habitats contaminated by heavy metals.

Relatively little data exist regarding levels of heavy metals at which toxic responses of earthworms would be expected to occur. In addition, the toxicity of metals to soil organisms is expected to vary depending on soil conditions and the chemical form of metals present in the soil (Edwards and Bohlen, 1996). Levels of heavy metals to which earthworms in our study were exposed are several times lower than any which have previously been shown to cause toxic effects (see review by Edwards and Bohlen, 1996). Thus, we suggest that the apparent effects of heavy metals on the population genetic structure of *L. rubellus* resulted from chronic exposure to sublethal concentrations of Cd, Cu, Pb, and Zn.

The results of numerous laboratory investigations provide strong evidence that genetic structure may be altered as a result of selection by heavy metals on certain allozyme genotypes (Gillespie and Guttman, 1999). For example, significant differences in survival have been observed among individuals of several aquatic species with different genotypes for GPI, PGM, and other enzymatic loci when exposed to Cu (Lavie and Nevo, 1982; Chagnon and Guttman, 1989; Gillespie and Guttman, 1989; Schlueter et al., 1997). Differential survival among allozyme genotypes has also been shown to occur when aquatic organisms are exposed to other heavy metals, including cadmium and zinc (Gillespie and Guttman, 1999).

We suggest that the observed patterns of differences in population genetic structure, when considered in the context of previous laboratory studies, are likely the result of increased sensitivity of certain alleles or genotypes to heavy metals. For example, the frequency of the most common genotypes for GPI was reduced by 15% in the sludge population compared to the reference population (Fig. 1B), whereas the most common PGM genotype was 25% lower (Fig. 2B). In addition, the sludge population was characterized by alleles that did not occur in the reference population as well as a higher frequency of other alleles (Figs 1A and 2A). These findings suggest that certain genotypes and/or alleles

for GPI and PGM may be more sensitive to heavy metals.

It is not possible to ascertain the exact causative mechanisms between heavy metals and changes genetic structure at the GPI and PGM loci. However, both GPI and PGM have previously been shown to be affected by metal ions (Milstein, 1961; Viarengo, 1985). This effect may occur as a result of direct effects of metals on protein structure or competition of metals with Mg (Milstein, 1961). In either of these scenarios, certain allozyme genotypes may be less sensitive to the effects of metals. Given the important roles of PGM (glycogenolysis) and GPI (glycolysis) in metabolism, the presence of these less sensitive genotypes may then provide a selective energetic advantage to the organism.

Several field investigations have documented significant decreases in heterozygosity within aquatic populations exposed to toxicants (Gillespie and Guttman, 1999). Gillespie and Guttman (1999) hypothesized that individuals with homozygous genotypes would be more fit than heterozygotes, thus leading to a reduction in heterozygosity over time. However, our results demonstrated that earthworms from soils that had been contaminated with heavy metals for more than 20 years had a greater heterozygosity than reference populations. In fact, the percentage of earthworms in the highest heterozygosity class (Fig. 3) was nearly four times higher in the sludge population (19.3%) compared to the reference population (4.9%). Duan et al. (2001) found that certain alleles/genotypes in *Hyallela azteca* were differently affected by Cu, Cd, and Zn. Thus, in the sludge plots, heterozygosity may be selectively advantageous to individuals that are exposed to multiple toxicants. Further investigation is necessary to elucidate the effects of heavy metals on heterozygosity in terrestrial organisms.

Previous investigations have documented correlations between population genetic structure of aquatic organisms and measures of water quality. For example, Gillespie and Guttman (1993) demonstrated significant correlations between water quality parameters and allele and genotype frequencies at the GPI locus in spotfin shiners (*Notropis spilopterus*). Foré et al. (1995a,b), found that genetic structure of *Pimephales notatus* and *Campostoma anomalum* was more sensitive to changes in water quality than traditional indices used by the US EPA. To our knowledge, this investigation is the first to document

a significant association between exposure to contaminants and changes in the genetic structure of populations within terrestrial systems.

We suggest that the allele and genotype frequencies at certain enzymatic loci in earthworms have the potential to serve as useful bioindicators of environmental impacts in terrestrial systems. Monitoring changes in genetic structure of earthworms may also provide a useful tool for assessing the success of remediation efforts in impacted habitats. Earthworms are an especially useful organism for the development of genetic biomarkers given their ubiquitous distribution and the current use of these organisms as monitors of environmental contaminants, such as heavy metals (Fordham and Wilber, 1992; Morgan et al. 1992; Edwards and Bohlen, 1996). Future studies should be designed to (a) identify sensitive loci in laboratory investigations, and (b) examine changes in the genetic structure of these loci within natural populations exposed to a wide range of toxicants.

Acknowledgements

Funding for this investigation was provided by a Faculty Research Development Grant awarded to J. D. P. by the Pennsylvania State University. Logistical support for the sampling of earthworm populations was provided by R. F. Stander. Assistance in electrophoretic analyses was provided by the laboratory staff of S. I. Guttman at Miami University.

References

- Benton, M.J., Diamond, S.J. and Guttman, S.I. (1994). A genetic and morphometric comparison of *Helisoma trivolvis* and *Gambusia holbrooki* from clean and contaminated habitats. *Ecotoxicol. Environ. Safety* **29**, 20–37.
- Bickham, J.W. and Smolen, M.J. (1994). Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. *Environ. Health Perspect.* **102**, 25–28.
- Bickham, J.W., Sandhu, S., Heber, P.D.N., Chikhi, L. and Athwal, R. (2000). Effects of chemical contaminants on genetic diversity in natural populations: Implications for biomonitoring and ecotoxicology. *Mutat. Res.* **463**, 33–51.
- Brewer, S.R. and Barrett, G.W. (1995). Heavy metal concentrations in earthworms following long-term nutrient enrichment. *Bull. Environ. Contamin. Toxicol.* **54**, 120–7.
- Brewer, S.R., Barrett, G.W. and Benninger-Truax, M. (1994). Mechanisms of ecosystem recovery following 11 years of nutrient enrichment in an old-field community. In S.M. Ross (ed.), *Toxic Metals in Soil-Plant Systems*, pp. 276–301. John Wiley: New York.
- Chagnon, N.L. and Guttman, S.I. (1989). Differential survivorship of allozyme genotypes in mosquitofish populations exposed to copper or cadmium. *Environ. Toxicol. Chem.* **8**, 319–26.
- Duan, Y., Guttman, S.I., Oris, J.T. and Bailer, A.J. (2000a). Genotype and toxicity relationships among *Hyallela azteca*. I. Acute exposure to metals or low pH. *Environ. Toxicol. Chem.* **19**, 1414–21.
- Duan, Y., Guttman, S.I., Oris, J.T., Huang, X. and Burton, G.A. (2000b). Genotype and toxicity relationships among *Hyallela azteca*. II. Acute exposure to fluoranthene-contaminated sediment. *Environ. Toxicol. Chem.* **19**, 1422–6.
- Duan, Y., Guttman, S.I., Oris, J.T. and Bailer, A.J. (2001). Differential survivorship among allozyme genotypes of *Hyallela azteca* exposed to cadmium, copper, zinc, or low pH. *Aquat. Toxicol.* **54**, 15–28.
- Edwards, C.A. and Bohlen, P.J. (1996). *Biology and Ecology of Earthworms*, 3rd edn. London: Chapman and Hall.
- Fordham, C.L. and Wilber, C.G. (1992). Effects of composted sewage sludge on the earthworm *Lumbricus terrestris*. In P.W. Greig-Smith, H. Becker, P.J. Edwards and H. Heimback (eds), *Ecotoxicology of Earthworms*, pp. 239–44. Adover, UK: Intercept Ltd.
- Foré, S.A., Guttman, S.I., Bailer, A.J., Altfater, D.J. and Counts, B.V. (1995a). Exploratory analysis of population genetic assessment as a water quality indicator. I. *Pimephales notatus*. *Ecotoxicol. Environ. Safety* **30**, 24–35.
- Foré, S.A., Guttman, S.I., Bailer, A.J., Altfater, D.J. and Counts, B.V. (1995b). Exploratory analysis of population genetic assessment as a water quality indicator. II. *Campostoma anomalum*. *Ecotoxicol. Environ. Safety* **30**, 36–46.
- Gillespie, R.B. and Guttman, S.I. (1989). Effects of contaminants on the frequencies of allozymes in populations of the central stone-roller. *Environ. Toxicol. Chem.* **8**, 309–17.
- Gillespie, R.B. and Guttman, S.I. (1993). Correlations between water quality and frequencies of allozyme genotypes in spotfin shiner (*Notropis spilopterus*) populations. *Environ. Pollut.* **81**, 147–50.
- Gillespie, R.B. and Guttman, S.I. (1999). Chemical-induced changes in the genetic structure of populations: Effects on allozymes. In V.E. Forbes (ed.), *Genetics and Ecotoxicology*, pp. 55–77. New York: Taylor and Francis.
- Guttman, S.I. (1994). Population genetic structure and ecotoxicology. *Environ. Health Persp.* **102**, 97–100.
- Harris, H. and Hopkinson, D.A. (1976). *Handbook of Enzyme Electrophoresis in Human Genetics*, New York: American Elsevier Publ.
- Kruse, E. and Barrett, G.W. (1985). Effects of municipal sludge and fertilizer on heavy metal accumulation in earthworms. *Environ. Pollut.* **38**, 235–44.
- Lavie, B. and Nevo, E. (1982). Heavy metal selection of phosphoglucose isomerase allozymes in marine gastropods. *Marine Biol.* **71**, 17–22.
- Levine, M.B., Hall, A.T., Barrett, G.W. and Taylor, D.H. (1989). Heavy metal concentrations during ten years of sludge treatment to an old-field community. *J. Environ. Qual.* **18**, 411–8.
- Milstein, C. (1961). Inhibition of phosphoglucosmutase by trace metals. *Biochem. J.* **79**, 591–6.

- Morgan, J.E. and Morgan, A.J. (1988). Earthworms as biological monitors of cadmium, copper, lead, and zinc in metalliferous soils. *Environ. Pollut.* **54**, 123–38.
- Morgan, J.E., Morgan, A.J. and Corp, N. (1992). Assessing soil metal pollution with earthworms: Indices derived from regression analyses. In P.W. Greig-Smith, H. Becker, P.J. Edwards and H. Heimback (eds), *Ecotoxicology of Earthworms*, pp. 233–7. Andover, UK: Intercept Ltd.
- Peles, J.D., Brewer, S.R. and Barrett, G.W. (1998). Heavy metal accumulation by old-field plant species during recovery of sludge-treated ecosystems. *Am. Midl. Nat.* **140**, 245–51.
- Reding, M.E. and Guttman, S.I. (1991). Radionuclide contamination as an influence on the morphology and genetic structure of periodical cicada (*Magicicada cassini*) populations. *Am. Midl. Nat.* **126**, 322–37.
- Richardson, B. (1986). *Allozyme Electrophoresis: A Handbook for Animal Systematics and Population Studies*, San Diego: Academic Press.
- Roark, S.A., Andrews, J.F. and Guttman, S.I. (2001). Population genetic structure of the western mosquitofish, *Gambusia affinis*, in a highly channelized portion of the San Antonio River in San Antonio, TX. *Ecotoxicology* **10**, 223–7.
- Schlueter, M.A., Guttman, S.I., Oris, J.T. and Bailer, A.J. (1997). Differential survival of fathead minnows, *Pimephales promelas*, as affected by copper exposure, prior population stress, and allozyme genotypes. *Environ. Toxicol. Chem.* **16**, 939–47.
- Schlueter, M.A., Guttman, S.I., Duan, Y., Oris, J.T., Huan, X. and Burton, G.A. (2000). Effects of acute exposure to fluoranthene-contaminated sediment on the survival and genetic variability of fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* **19**, 1011–18.
- Swofford, D.L. and Selander, R.K. (1981). BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Hered.* **72**, 281–3.
- Tranvik, L., Sjørgen, M. and Bengtsson, G. (1994). Allozyme polymorphism and protein profile in *Orchesella bifasciata* (Collembola): indicative of extended metal pollution?. *Biochem. Systemat. Ecol.* **22**, 13–23.
- Viarengo, A. (1985). Biochemical effects of trace metals. *Mar. Pollut. Bull.* **16**, 153–8.