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The Impact of Phorate on the Genetic Diversity of Wetland Aquatic Invertebraes†

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Abstract

Impacts of the insecticide phorate on the genetic diversity of wetland invertebrates were investigated using field and laboratory studies in 1991. Electrophoretic methods were evaluated for revealing the impact of insecticides. Objectives were to determine the ability of electrophoresis to reveal the impact of phorate on invertebrates and to determine the influence of phorate on the genetic diversity in two common invertebrates. Amphipods, *Hyallela azteca* and mayflies, *Callibaetis ferrugineus* (Walsh) were placed in constructed mesocosms in wetlands and were exposed to varying amounts of phorate. Survivors and individuals from the parent population were genetically tested using cellulose acetate electrophoresis techniques. Allele frequencies were calculated for invertebrates in treatments and invertebrates from populations not exposed to phorate. Mortality of test invertebrates was significantly greater in phorate treatments than in controls ($F = 5.97$, $P = 0.019$). Chi-square analysis revealed differences in allele frequencies between the untreated populations and individuals of both species treated with phorate ($X^2 > 8.5$; $df = 1,2$; $P < 0.05$). In addition, phorate appeared to eliminate, or reduce the frequency of certain genotypes in both species. Results indicate phorate selected against sensitive individuals and electrophoresis was effective at detecting differences between untreated populations and invertebrates that survived treatments. Genetic techniques should enable wetland scientists to detect the effects of pollution on invertebrate populations by monitoring genetic composition.

Introduction

The Prairie Pothole region produces up to 75% of North American waterfowl (1) and produces important agricultural crops. Agricultural pesticides applied to adjacent cropland may enter wetlands and cause serious damage to wildlife and aquatic organisms. Pesticides entering wetlands can harm waterfowl directly by inhibition of the cholinesterase enzyme which causes nerve impulse failure (2) and indirectly by reducing the numbers and variety of important invertebrate prey. However, it is often difficult to confirm pesticide-induced population declines. Detecting the presence of pesticides in wetlands is difficult due to rapid sorption, volitalization and degradation.

Populations of organisms exposed to pollutants have less genetic variability than natural populations (3), and Lavie and Nevo (4) suggest using genetic structure to detect and monitor (marine) pollution. Natural populations of organisms usually exhibit a large amount of genetic variation (5) resulting from stochastic processes or natural selection of various types (6, 7). Electrophoresis has proven to be a reliable method for revealing the genetic composition of populations and Hebert et al. (8) used cellulose acetate electrophoresis techniques to detect genetic diversity in the sumac gall aphid, *Melaphis rhois*. Electophoretic research by Facemire (3) supported the assumptions that 1) an individual's response to pollutants is genetically controlled, 2) pollutants exert strong selective pressure against sensitive genotypes and 3) exposure to a pollutant changes the genetic compostion of a natural population. Genetic analysis may allow scientists to differentiate natural population declines from injury caused by pollutants.

The agricultural pesticide selected for our study was Phorate (Thimet, American Cyanamid, 0,0-diethyl S-[(ethylthio)methyl] phosphorodithiote, $C_7H_{17}O_2PS_5$, MW = 260.37). Phorate, an organophosphate soil insecticide registered for use on corn, is widely used in the Prairie Pothole Region (9) and according to Smith (2), has a high potential to move into wetlands via runoff.

The first objective was to evaluate the ability of electrophoretic analysis to reveal the impacts of phorate on mayflies, *Callibaetis ferrugineus* (Walsh) and amphipods, *Hyallela azteca*. The second objective was to determine the influence of phorate on genetic diversity in mayfly and amphipod populations.

Site Description

Three wetlands in east-central South Dakota (Kingsbury County) were studied in 1991. The Erwin wetland was located near Erwin, SD (T112N,R55W,

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Sec. 15SW). The Isaakson wetland was located about 8 km west of Lake Preston, SD (T110N, R55W, Sec. 31NE) and the Henry wetland was located near Lake Henry (T110N, R56W, Sec. 14NW). Study sites were palustrine, emergent wetlands (10) located on U.S. Fish and Wildlife Service Waterfowl Production Areas. The wetlands contained deep-marsh zones, areas with submergent vegetation, and were surrounded by emergent vegetation (9).

Materials and Methods

Before treatment, water from each wetland was analyzed by the University of Iowa Hygienic Laboratory for the presence of chemicals. Herbicides tested include Atrazine, Bladex, Dual, Lasso, and Treflan. Insecticides tested include Counter, Dyfonate, Lorsban, Thimet and Furadan. None of these pesticides were detected in any of the wetlands (9). Four mesocosms were constructed and placed about 3 m apart in each of the three wetlands described. Mesocosms consisted of a wood frame 1.2 m^2 by 0.9 m deep with clear plastic sides. The lower frame of each mesocosm was pushed into the bottom substrate to prevent contamination of other mesocosms. The upper frame of each mesocosm projected about 0.3 m above the water surface. Within each mesocosm, a 70 µm opening mesh basket was suspended below the water surface.

Amphipods were taken from a wetland located near Arlington, South Dakota (T110N, R52W, Sec. 20NW), while mayflies were taken from Isaakson marsh. The population of mayflies in Isaakson marsh and the population of amphipods in the Arlington wetland were termed the "parent population." Thirty individuals from each species were collected from the respective parent populations and genetically tested. A total of 600 amphipods and 600 mayflies were used for the mesocosm studies. Fifty amphipods and 50 mayflies were placed in each basket.

One mesocosm served as a control and three were treated with varying amounts of phorate. The label rate for agricultural uses of phorate varies from 560 to 3,360 g ha^{-1} (0.5 to 3 lbs ac^{-1}). We selected 560 g ha^{-1} as a benchmark test rate. Phorate was applied by spreading the granules evenly over the water surface within mesocosms. Initial trials using 280, 560 and 1,100 g ha^{-1} of phorate resulted in 100 % mortality of test invertebrates. Consequently, the amount of phorate applied to mesocosms was reduced to 9 g ha^{-1}, 18 g ha^{-1} and 34 g ha^{-1} (1.56, 3.13 and 6.25 % of the recommended field application, respectively). After 24 hr, we removed survivors and recorded mortality. Active invertebrates were placed in jars, and transported on ice to the laboratory (South Dakota State University) for genetic analysis.

Invertebrate samples were genetically analyzed using cellulose acetate methodologies described by Hebert and Beaton (11). Twelve whole invertebrates from samples were individually placed in wells on a Helena Laboratories Super Z sample well plate. A small amount of water was added to each well and invertebrates were crushed. Samples were applied to cellulose acetate gels using a Helena Laboratories Super Z applicator. Electric current (150 V) from a EC105 minicell power supply was applied for 30 min. Glucose-phosphate isomerase (GPI), phospho-glucomutase (PGM), isocitrate dehydrogenase (IDH), fumarate hydratase (FUM), and malate dehydrogenase (MDH) enzyme stains were prepared and one enzyme stain was applied to each cellulose acetate gel. Gels were left undisturbed for 5 min. and were then incubated at 60°C for 5 min. Excess stain was rinsed from the gels and gels were allowed to dry.

Cellulose acetate gels were scored and the genotype of each sample was determined. Loci were designated as 1 or 2 depending on mobility (e.g. most mobile = GPI_1, least mobile = GPI_2) and the most mobile allele was designated as A, and the second most mobile as B and so on (3). Allele and genotype frequencies for the parent population (expected) and treatment (observed) samples were calculated. A chi-square (X^2) test was used to determine goodness of fit.

Some cellulose acetate gels were incompletely resolved at certain loci and these were excluded in the final analysis. Samples may have degraded during transportation or storage. Consequently, data were available for only the parent populations, the Erwin treatments, and the 9 g ha^{-1} Isaakson treatment at certain loci.

Aquarium studies conducted in the SDSU Wildlife and Fisheries laboratory complimented the available mesocosm treatment data. Two hundred amphipods and 200 mayflies were collected from the parent populations for the laboratory studies. On 9 September 1991, fifty individuals were placed in each 38 liter (1300 cm^2) aquarium with the water in which they were collected. Each aquarium was then treated with 9 g ha^{-1}, 18 g ha^{-1} or 34 g ha^{-1} of phorate by spreading granules over the water surface. There was 100 % mortality of individuals for both species in the 18 g ha^{-1} and 34 g ha^{-1} aquarium treatments. Consequently we conducted an additional experiment with 4.3 g ha^{-1} of phorate (0.78% of the recommended field application) on 11 September 1991. After 24 hr, active survivors were frozen and stored for later genetic analysis.

Results and Discussion

Invertebrate survival data within mesocosms was analyzed using a one-way analysis of variance, which indicated reductions of amphipods in treatment

Table 1. Survival of mayflies, *Callibaetis ferrugineus*, and amphipods, *Hyallela azteca*, in control and treatment mesocosms 24 hr after application of phorate. Fifty individuals of each species were placed in each mesocosm before treatment.

Taxon	Site	Phorate Applied, g ha^{-1}				ANOVA	
		0 (Control)	9	18	34	F	Probability
		Survivors					
Callibaetis ferrugineus	Isaakson	1	0	0	0	5.97	0.0190
	Henry	27	27	0	0		
	Erwin	19	11	0	0		
Hyallela azteca	Isaakson	35	33	16	2	6.64	0.0150
	Henry	42	41	31	9		
	Erwin	50	50	37	24		

mesocosms (F = 6.64, P = 0.015; Table 1). Mayfly numbers were also reduced in treatment mesocosms with 100% mortality in the two strongest concentrations of phorate (F = 5.97, P = 0.019; Table 1). Only one mayfly was alive in the Isaakson control mesocosm 24 hrs after treatment. Contamination was possible, but unlikely due to the survival of amphipods in the same mesocosm. Mortality of mayflies in the Isaakson control mesocosm may have been due to handling stress.

Electrophoretic analysis of parent populations of both species revealed few heterozygotes. With A and B frequencies equal, AA, AB and BB genotypes would be expected at a ratio of 1:2:1, respectively. Only 11%

Table 2. Allele frequencies (locus 1) of the amphipod *Hyallela azteca* parent population (expected) and amphipods exposed to varying concentrations of phorate (observed). Most mobile allele is designated "A" and least mobile Allele is designated "B."

Date	Wetland Location	Phorate Treatment	Locus	Allele		Chi Square	Probability
				A	B		
day mo yr		— g ha^{-1} —					
19 07 91	Arlington	0††	FUM_1	0.25	0.75		
			PGM_1	0.21	0.79		
			IDH_1	------	------		
			MDH_1	0.21	0.79		
			GPI_1	------	------		
11 09 91	Aquarium	4.3	FUM_1	0.00	1.00	28.6	0.0001
			PGM_1	0.92	0.08	102.6	0.0001
			IDH_1	0.42	0.58	------	------
			MDH_1	0.21	0.79	0.0	1.0000
			GPI_1	------	------	------	------
09 09 91	Aquarium	9	FUM_1	0.00	1.00	28.6	0.0001
			PGM_1	0.75	0.25	58.4	0.0001
			IDH_1	------	------	------	------
			MDH_1	0.46	0.54	14.0	0.0001
			GPI_1	------	------	------	------

† FUM = fumarate hydratase, PGM = phospho-glucomutase, IDH = isocitrate dehydrogenase, MDH = malate dehydrogenase, GPI = glucose-phosphate isomerase.
†† Parent population.

Table 3. Allele frequencies (locus 2) of the amphipod *Hyallela azteca* parent population (expected) and amphipods exposed to varying concentrations of phorate (observed). Most mobile allele is designated "A" and least mobile Allele is designated "C."

Date	Wetland Location	Phorate Treatment	Locus	Allele A	Allele B	Allele C	Chi Square	Probability
day mo yr		— g ha^{-1} —						
19 07 91	Arlington	0††	FUM_2	0.08	0.92	0.00		
			PGM_2	0.21	0.70	0.09		
			IDH_2	0.32	0.68	0.00		
			MDH_2	1.00	0.00	0.00		
			GPI_2	0.17	0.75	0.08		
11 09 91	Aquarium	4.3	FUM_2	0.00	1.00	0.00	8.3	0.0039
			PGM_2	0.00	0.58	0.42	43.5	0.0001
			IDH_2	0.00	0.96	0.04	40.8	0.0001
			MDH_2	1.00	0.00	0.00	0.0	1.0000
			GPI_2	0.00	1.00	0.00	28.6	0.0001
09 09 91	Aquarium	9	FUM_2	0.59	0.41	0.00	58.4	0.0001
			PGM_2	0.00	0.62	0.38	39.4	0.0001
			IDH_2	0.00	1.00	0.00	38.1	0.0001
			MDH_2	0.80	0.20	0.00	22.0	0.0001
			GPI_2	0.00	1.00	0.00	28.6	0.0001
19 07 91	Isaakson mesocosm	9	FUM_2	0.00	0.82	0.18	26.6	0.0001
			PGM_2	------	------	------	-----	---------
			IDH_2	0.00	1.00	0.00	38.1	0.0001
			MDH_2	------	------	------	-----	---------
			GPI_2	0.29	0.54	0.17	9.8	0.0075
19 07 91	Erwin mesocosm	9	FUM_2	0.00	1.00	0.00	8.3	0.0039
			PGM_2	------	------	------	-----	---------
			IDH_2	------	------	------	-----	---------
			MDH_2	1.00	0.00	0.00	0.0	1.0000
			GPI_2	0.00	0.92	0.08	18.7	0.0001
23 07 91	Erwin mesocosm	18	FUM_2	0.00	1.00	0.00	8.3	0.0039
			PGM_2	------	------	------	-----	---------
			IDH_2	------	------	------	-----	---------
			MDH_2	1.00	0.00	0.00	0.0	1.0000
			GPI_2	0.17	0.83	0.00	8.4	0.0150
23 07 91	Erwin mesocosm	34	FUM_2	0.00	1.00	0.00	8.3	0.0039
			PGM_2	0.29	0.71	0.00	10.3	0.0058
			IDH_2	------	------	------	-----	---------
			MDH_2	1.00	0.00	0.00	0.0	1.0000
			GPI_2	0.21	0.79	0.00	8.5	0.0141

† FUM = fumarate hydratase, PGM = phospho-glucomutase, IDH = isocitrate dehydrogenase, MDH = malate dehydrogenase, GPI = glucose-phosphate isomerase.
†† Parent population.

of the individuals in the amphipod parent population exhibited heterozygosity. Of the mayfly parent population, 8 % were heterozygotes. In addition, 29 % of the mayflies in the Erwin control mesocosm were heterozygotes. Invertebrate survivors of phorate treatments were also low in heterozygosity. Eleven

Table 4. Allele frequencies (locus 1) of the mayfly *Callibaetis ferrugineus* (Walsh) parent population (expected) and those exposed to varying amounts of phorate (observed). Most mobile allele is designated "A" and least mobile allele is designated "B."

Date	Wetland Location	Phorate Treatment	Locus	Allele A	Allele B	Chi Square	Probability
day mo yr		— g ha^{-1} —					
22 07 91	Isaakson	0††	FUM_1	0.00	1.00		
			PGM_1	0.88	0.12		
			IDH_1	1.00	0.00		
			MDH_1	0.33	0.67		
			GPI_1	0.33	0.67		
23 07 91	Erwin	0	FUM_1	0.17	0.83	18.6	0.0001
			PGM_1	1.00	0.00	12.8	0.0004
			IDH_1	----	----	----	-----
			MDH_1	0.30	0.70	0.2	0.6479
			GPI_1	0.05	0.95	25.5	0.0001
11 09 91	Aquarium	4.3	FUM_1	0.75	0.25	120.0	0.0001
			PGM_1	1.00	0.00	12.8	0.0004
			IDH_1	0.75	0.25	28.6	0.0001
			MDH_1	0.00	1.00	39.5	0.0001
			GPI_1	0.00	1.00	39.5	0.0001
23 09 91	Erwin mesocosm	9	FUM_1	0.00	1.00	0.0	1.0000
			PGM_1	1.00	0.00	12.8	0.0004
			IDH_1	1.00	0.00	0.0	1.0000
			MDH_1	0.00	1.00	39.4	0.0001
			GPI_1	0.09	0.91	17.4	0.0001

† FUM = fumarate hydratase, PGM = phospho-glucomutase, IDH = isocitrate dehydrogenase, MDH = malate dehydrogenase, GPI = glucose-phosphate isomerase.
†† Parent population.

percent of treated amphipods and 18 % of treated mayflies were heterozygous. Non-conformity to the Hardy-Weinberg law suggests little genetic variability in the test invertebrate populations (3). A low occurrence of heterzygous individuals in the parent populations may be caused by inbreeding due to isolation.

Chi-square analysis of the allele frequency data revealed differences between the parent populations and individuals that survived phorate treatments ($X^2 > 8.4$; df = 1,2; $P < 0.05$). Phorate treatments appeared to reduce genetic variability in both taxa even further. At locus 1 and 2 of the 5 enzymes studied, there was often a reduction in allele frequency, or a total elimination of certain alleles. Of individuals in the *H. azteca* parent population, 79 % possessed the B allele at the PGM_1 locus; yet, 92 % of the survivors of the 4.3 g ha^{-1} aquarium treatment, 75 % of the survivors of the 9 g ha^{-1} aquarium treatment exhibited the A allele at that locus (Table 2). In the *H. azteca* parent population, there were 17 % A, 75 % B and 8 % C alleles at the GPI_2 locus, but no A or C alleles were present in the individuals that survived either of the phorate-aquarium treatments (Table 3).

For *H. azteca,* over 82 % of the allele frequencies of treatment comparisons were different from the parent population allele frequencies. The same tests of *C. ferrugineus* displayed similar results. Of the allele frequency comparisons, 80 % were significantly different from the parent population allele fequencies. At some loci, allele frequencies were reduced or eliminated. Although present in the parent population and the Erwin control mesocosm mayflies, alleles of the A type were eliminated in both phorate-aquarium treatments at the MDH_1, locus (Table 4). In the 4.3 g ha^{-1} phorate-aquarium treatment, C alleles were eliminated, B alleles were reduced and A alleles became more prevalent (Table 5).

Mayfly tests displayed similar results. Eighty percent of the allele frequencies of treatment

Table 5. Allele frequencies (locus 2) of the mayfly *Callibaetis ferrugineus* (Walsh) parent population (expected) and mayflies exposed to varying concentrations of phorate (observed). Most mobile allele is designated "A" and least mobile allele is designated "C."

Date	Wetland Location	Phorate Treatment	Locus†	Allele A	Allele B	Allele C	Chi Square	Probability
day mo yr		— g ha^{-1} —						
22 07 91	Isaakson	0††	FUM_2	0.67	0.33	0.00		
			PGM_2	0.17	0.66	0.17		
			IDH_2	1.00	0.00	0.00		
			MDH_2	0.33	0.67	0.00		
			GPI_2	0.38	0.62	0.00		
23 07 91	Erwin mesocosm	0	FUM_2	0.54	0.46	0.00	3.5	0.0601
			PGM_2	0.21	0.50	0.29	5.8	0.0562
			IDH_2	-----	-----	-----	-----	-------
			MDH_2	0.25	0.75	0.00	1.6	0.2125
			GPI_2	0.46	0.54	0.00	1.3	0.2517
11 07 91	Aquarium	4.3	FUM_2	1.00	0.00	0.00	39.5	0.0001
			PGM_2	0.42	0.38	0.00	33.3	0.0001
			IDH_2	1.00	0.00	0.00	0.0	1.0000
			MDH_2	0.00	1.00	0.00	39.5	0.0001
			GPI_2	0.21	0.79	0.00	7.0	0.0084
23 07 91	Erwin mesocosm	9	FUM_2	1.00	0.00	0.00	39.5	0.0001
			PGM_2	0.08	0.42	0.50	24.8	0.0001
			IDH_2	0.83	0.17	0.00	18.6	0.0001
			MDH_2	0.00	1.00	0.00	39.5	0.0001
			GPI_2	0.41	0.59	0.00	0.2	0.6643

† FUM = fumarate hydratase, PGM = phospho-glucomutase, IDH = isocitrate dehydrogenase, MDH = malate dehydrogenase, GPI = glucose-phosphate isomerase.
†† Parent population.

comparisons were different from the parent population allele frequencies. There was also a reduction in allele frequencies and an absence of alleles at certain loci. Although present in the parent population and the Erwin control mesocosm mayflies, alleles of the B type were eliminated at the FUM_2 locus in both aquarium treatments receiving phorate (Table 4). In the 4.3 g ha^{-1} phorate aquarium treatment, C alleles were eliminated, B alleles were reduced and A alleles became more prevalent (Table 5).

Electrophoresis effectively revealed differences between the parent populations (both species) and those invertebrates exposed to phorate. The observed changes appeared to be closely correlated with phorate treatments. Apparently, phorate selected against sensitive genotypes, reducing invertebrate numbers and genetic diversity within the populations. A reduction in genetic variability inhibits a population's ability to adapt to sudden environmental change (3).

The results correspond with findings by Facemire (3) of lesser genetic variability in the mayfly *Stenonema femoratum* exposed to industrial contaminants and to those of Lavie and Nevo (4) that zinc and copper pollution selected against sensitive genotypes in marine gastropods. This study documents the impact of the insecticide phorate on two common invertebrate species. Because dilute concentrations of phorate had such a dramatic effect on test invertebrates, precautions should be taken to prevent the entry of insecticides such as phorate into wetlands. In cases of contamination, wetland managers could use electrophoresis to reveal the extent of the impact. The amount of time required for invertebrate populations to recover from a contaminant impact is not known. Further research should be conducted to discover the response of invertebrate populations after an impact.

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