Journal of the American Mosquito Control Association, 18(3):214–221, 2002

INVESTIGATION OF THE RELATIONSHIP BETWEEN *s*-METHOPRENE AND DEFORMITIES IN ANURANS

CLIVE A. HENRICK,[1] JINREN KO,[2] JACK NGUYEN,[2] JIM BURLESON,[2] GEORGE LINDAHL,[2] DOUGLAS VAN GUNDY[2] AND JULIE M. EDGE[3]

ABSTRACT. Evidence that amphibian deformities are on the rise has prompted a number of researchers to question a variety of natural and man-made substances, including chemicals such as *s*-methoprene (trademark Altosid®), an insect growth regulator applied to water bodies for mosquito control. Despite conclusions reached by the U.S. Environmental Protection Agency and a considerable body of scientific evidence demonstrating that it is unlikely that *s*-methoprene or any of its degradation products are causing the high rates of deformities found in Minnesota and other states, concerns persist about potential negative impacts of *s*-methoprene on nontarget species, specifically anurans. Water analyses in field and laboratory conditions and a comparison of reported Altosid use with reported frog deformities in Minnesota demonstrate that a connection between frog deformities and Altosid use is unlikely. These results indicate that factors other than *s*-methoprene and its degradation products are contributing to the recent outbreak of frog deformities.

KEY WORDS *s*-Methoprene, Altosid, frog deformities, amphibians, FETAX

INTRODUCTION

Recent reports of frog deformities found in Minnesota, coupled with evidence of a general decline in the amphibian population, have many researchers contemplating the potential cause or causes of these anomalies. Although amphibian deformities have been well documented around the world since the mid-1700s (Van Valen 1974), the number of occurrences apparently has increased in Minnesota over the past 5 years (Hileman 1996, 1998). Examination of historical and current data demonstrates that frog deformities have been more frequent, more varied, more severe, and more widely distributed in Minnesota in 1996 and 1997 than from 1958 to 1993 (Hoppe 1997).

Both natural and synthetic substances have been suspected, but determining the direct or indirect cause has proven difficult. The current situation is further complicated by the fact that amphibian deformity outbreaks have been documented in different parts of the world (Bishop 1947; Rostand 1958a, 1971; Woitkewitch 1961; Hebard and Brunson 1963; Merrell 1968–69; Van Valen 1974) long before many of the substances in question were synthesized. A number of causes are suspected, including amphibian parasites (e.g., trematodes), ultraviolet (UV) light, toxic chemicals, natural and unnatural retinoids, and agricultural chemicals (Rostand 1958b, Sessions and Ruth 1990, Bryant and Gardiner 1992, Grant and Licht 1995, Blaustein et al. 1997, Bruner et al. 1997, Dumont et al. 1997, Glaser and Bogart 1997, Hale 1997, Ouellet et al. 1997, Ovaska et al. 1997, Schoff and Holy 1997, Ankley et al. 1998, Britson and Threlkeld 1998, Burkhart et al. 1998, Gardiner and Hoppe 1998, Hirsch and Temple 1998, Huang et al. 1998, Jofre and Karasov 1998, La Clair et al. 1998, Rosenshield and Jofre 1998, Johnson et al. 1999, Sessions et al. 1999, Sparling 2000). Some of the specific pesticides considered include organophosphates, atrazine, polychlorinated biphenyls, glyphosate, and *s*-methoprene (Dumont et al. 1997, Glaser 1997, Hale 1997, Ankley et al. 1998, Britson and Threlkeld 1998, Hirsch and Temple 1998, Huang et al. 1998, La Clair et al. 1998, Rosenshield and Jofre 1998, Sparling 2000).

In an attempt to determine the cause of these malformations, Burkhart et al. (1998), representing the National Institute for Environmental Health Sciences and the Minnesota Pollution Control Agency (MPCA), conducted a series of studies to evaluate the cause of the frog deformities. They used the frog embryo teratogenesis assay—*Xenopus* (FETAX) to test pond water and groundwater samples from affected surface water sites and adjacent wells in Minnesota. The results showed significant mortality and developmental retardation in the test frogs. The screening assay also showed teratogenic activity and lethality associated with some surface water and groundwater sources. However in 1998, no specific compound or compounds were identified as the cause of malformations and mortality. The only conclusion from their work was that "something in the water" was causing deformities in anurans.

In the more recent analyses of suspected pond water and sediment samples collected from various sites in Minnesota by Burkhart and colleagues (Fort et al. 1999a, 1999b), they suggested that mixtures of both naturally occurring and man-made compounds were primarily responsible for the developmental toxicity observed with these samples on *Xenopus laevis.* The chemical entities identified included several herbicides and insecticides and their degradation products; however, *s*-methoprene and

[1] Zoecon Research and Development (Retired), 3177 Manchester Court, Palo Alto, CA 94303.
[2] Wellmark International, 12200 Denton Drive, Dallas, TX 75234.
[3] Fleishman-Hillard Inc., 2405 Grand Boulevard 700, Kansas City, MO 64108.

its degradation products were not detected in their study (Fort et al. 1999b; Burkhart, personal communication).

Despite research findings such as those of Burkhart, *s*-methoprene is a suspect in the frog deformity mystery because it is applied to water bodies where frogs spend their developmental period. *s*-Methoprene, trademark Altosid® (isopropyl (2*E*,4*E*)-(7*S*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate), is an insect growth regulator selectively applied to waterways by vector disease control operations to manage mosquito populations. The 1st formulation of Altosid, *rs*-methoprene (isopropyl (2*E*,4*E*)-(7*RS*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate), was registered for mosquito control with the U.S. Environmental Protection Agency (EPA) (1991, 2001) in 1975. The racemic product was subsequently replaced with the optically active *s*-isomer (Henrick et al. 1978).

Since the 1st synthesis of *rs*-methoprene in 1971 (Henrick et al. 1973, Henrick 1990), extensive toxicological and environmental studies have been conducted as part of the EPA registration process and as follow-up studies. These tests demonstrate its favorable toxicological profile (Wright 1976, Garg and Donahue 1989), short environmental persistence (Schaeffer and Dupras 1973; Quistad et al. 1975; Schooley et al. 1975a, 1975b; Quistad et al. 1976; Siddall 1976; Ross et al. 1994), and minimal impact on nontarget organisms (Miura and Takahashi 1973, Schooley et al. 1975a, Gradoni et al. 1976, Costlow 1977, Schooley and Quistad 1979, Batzer and Sjogren 1986, Bircher and Ruber 1988, Ross et al. 1994, Degitz et al. 2001).

Specific to anurans, Miura and Takahashi (1973) observed no mortality in tadpoles when treated with *s*-methoprene at 1,000 μg/liter (ppb), which is 167 times the maximum environmental concentration of 6 μg/liter for any liquid or solid formulations of Altosid (Ross et. al 1994). Simonin et al. (1992) also found no significant adverse impact on frogs and tadpoles in field studies with *s*-methoprene. In a more recent study, Degitz et al. (2001), on behalf of the EPA, evaluated the developmental toxicity of *s*-methoprene and its degradation products in amphibians. Their results demonstrated that *s*-methoprene and its degradation products were not potent development toxicants to *X. laevis*. Considering the concentration of *s*-methoprene applied in the field, they concluded that concern about methoprene-mediated developmental toxicity to amphibians was unwarranted.

Despite conclusions that *s*-methoprene does not represent an unacceptable risk to nontarget organisms from a considerable body of scientific evidence, concerns persist about potential negative impacts of *s*-methoprene on nontarget species, specifically anurans. Much of this concern has been fueled by an investigation (La Clair et al. 1998) that used FETAX to demonstrate *s*-methoprene degradation products could cause deformities in *X. laevis* with exaggerated doses (~15,000 times the maximum recommended application rate) in a laboratory setting.

The study by La Clair et al. (1998) compelled additional review of *s*-methoprene and its degradation products and their relationship to anuran deformities. As a result, this study was initiated to reconfirm how *s*-methoprene degrades under field conditions, determine whether *s*-methoprene and its degradation products occur in the Minnesota water bodies where frog deformities have been found, and to assess whether a correlation exists between reported Altosid use and frog deformities. Specifically, this paper provides a determination of the degradation process of *s*-methoprene in natural pond water, a test of water samples from field sites in Minnesota experiencing anuran deformities to determine whether *s*-methoprene and *s*-methoprene acid were present, and a comparison of nationally collected deformity data from treated and untreated counties in Minnesota to determine if any correlation exists between Altosid use and anuran deformities.

MATERIALS AND METHODS

Degradation in pond water

Two gallons of pond water samples were collected from Farmers Branch Park, Farmers Branch, TX, and stored at 4°C until treated with Altosid Liquid Larvicide (ALL). ALL (5% *s*-methoprene, EPA Reg. No. 2724-392) is a commercially available product manufactured by Wellmark International (Shaumburg, IL) for control of the emergence of adult mosquitoes. Analytical reference standards, prepared by Wellmark International and used in this study are as follows: 7-methoxycitronellal, 99%; 7-methoxycitronellic acid, 94%; *s*-methoprene acid, 96%; *s*-methoprene epoxides, 97.4%; *s*-methoprene, 96.89%; and (2*Z*,4*E*)-methoprene isomer, 95.81%. High-performance liquid chromatography (HPLC)–grade acetonitrile (ACN), water, methanol, hexane, and sulfuric acid were obtained from Fisher Scientific (Springfield, NJ). Dichloromethane was obtained from Burdick and Jackson (Muskegon, MI), and formic acid was from EM Science (Gibbstown, NJ). Gas-liquid chromatography–mass spectroscopy analyses were performed on a model 5973 Agilent Technologies (Palo Alto, CA) instrument. Liquid chromatography–mass selective detector analyses were performed on a model 1100 Agilent Technologies instrument. HPLC analyses were performed on a model 1090 Agilent Technologies instrument.

Determination: Determination was by gas chromatography–mass spectroscopy (GC-MS) with the following conditions. Column: DB-FFAP, 30 m × 0.25-mm inner diameter × 0.25-μm film (J and W Scientific, Folsom, CA). Temperature: initial temperature 100°C (1 min). Rate: 30°C/min. Final temperature: 250°C (15 min). Carrier: He ~ 45 cm/sec.

Injector: splitless mode (0.5-min delay). Acquisition mode: scan mode. Mass selective detector (MSD) temperature: 230°C. Scan mass: 40–460. Solvent delay: 3 min. Detector: electron impact.

Confirmation: Confirmation was by HPLC with UV detector at 264 nm with the following conditions. Column: Restek pinnacle ODS (Restek Corp., Bellefonte, PA) 250 × 4.6-mm C-18 with 5-μm particle size. Flow: 1 ml/min. Oven temperature: 40°C. Mobile phase: 0–14 min 55:45 ACN : water with 0.1% formic acid, 16–30 min 87 : 13 ACN : water with 0.1% formic acid. Liquid chromatography–mass spectroscopy atmospheric pressure ionization-electrospray (API-ES) with selected ion mode scanning.

Sample preparation and analysis: Four grams of ALL (lot 00315512, 4.96% *s*-methoprene) was weighed into a 500-ml class A volumetric flask. The flask was filled to the mark with HPLC-grade water and shaken for 1 h. Four milliliters of the above-prepared ALL solution was added to each of the three 1,500-ml pond water samples contained in separate clear beakers. This resulted in a concentration of 1 ppm of methoprene at time 0 (100 times the maximum recommended field rate). The 3 beakers were placed on the roof for atmospheric and sunlight exposure. The 1-day sample was collected after 24 h of exposure, and two 7-day samples were collected after 7 days of exposure. The minimum temperatures were in the upper 60°F range and the maximum temperatures were around 90°F during the 7-day period. The samples were extracted in the same fashion described under *s*-methoprene analysis below, except for the larger (1,500-ml) samples, and the solvents and NaCl were increased proportionally to 150 ml and 20 g, respectively. The extracts were evaporated to almost dryness and reconstituted in 2 ml of ACN for analysis by GC-MS.

Analysis of Minnesota pond water

All samples analyzed by ELAB Inc. (Ormond Beach, FL) and Wellmark were chilled and maintained between 1°C and 4°C for shipping and storage. The MPCA shipped the samples directly to both ELAB and Wellmark to conduct independent analyses. Wellmark prepared the *s*-methoprene (>95% purity) and *s*-methoprene acid (>95% purity) reference materials for both sets of tests. The HPLC-grade ACN, water, and methanol from Fisher Scientific and high-purity dichloromethane from Burdick and Jackson were used as solvents.

s-*Methoprene analysis:* The samples were analyzed according to Wellmark chemical analytical procedure 323B. Ten grams of NaCl was added to a 1,000-ml water sample (containing 75 ml of dichloromethane) in a 2-liter separatory funnel, which was shaken to dissolve the material. Twenty-five milliliters of dichloromethane was added and shaken for 3 min on a shaker. The organic layer was passed through 50 g of Na_2SO_4 into a 500-ml flask, and 100 ml of hexane was used to further extract *s*-methoprene. The aqueous layer then was discarded and the organic layer was passed through 50 g of Na_2SO_4 into the same 500-ml flask. The extract was then evaporated to near dryness with a rotary evaporator and transferred to a 125-ml flask. The residue from the flask was reconstituted into 2 ml of ACN for both HPLC and GC-MS analysis.

s-*Methoprene acid analysis:* The samples were extracted according to EPA method 3510 (separatory funnel liquid–liquid extraction). The 1,000-ml water samples were acidified in a separatory funnel with 1.5 ml of 1:1 sulfuric acid to reach pH 1–2. After acidification, 20 g of NaCl were added to each sample. Each sample was then extracted 3 times with 100 ml of dichloromethane, and the combined extract was dried through Na_2SO_4, evaporated to near dryness, and brought up to 1 ml with ACN for HPLC analysis. To convert the *s*-methoprene acid to its methyl ester for GC-MS analysis, 2 ml of diazomethane in ether was added to 0.5 ml of sample extract in ACN and shaken for 15 min. The solvent was evaporated by N_2 to near dryness, then the residue was reconstituted into 0.5 ml of ACN for HPLC analysis. The HPLC conditions were as follows. Column: Restek Pinnacle ODS, 5 μm, 250 × 4.6 mm. Mobile phase: for (*s*)-methoprene, 87:13 ACN : water; for *s*-methoprene acid, 87:13 ACN : water containing 0.5% acetic acid. Flow: 1 ml/min. Oven temperature: 40°C. Injection volume: 20 μl. Wavelength: 264 nm. The GC-MS conditions were as follows. For *s*-methoprene: 30-m × 0.25-mm × 0.25-μm SPB-608 column. Temperature 1: 80°C (2 min). Rate: 10°C/min. Final temperature: 250°C (10 min). Carrier: He ~ 38 cm/sec at 160°C. For *s*-methoprene acid methyl ester: 30-m × 0.25-mm × 0.25-μm DB-5 column, splitless injection. Temperature 1: 80°C (0 min). Rate: 4°C/min. Final temperature: 300°C.

Correlation of anuran deformity vs. Altosid use in Minnesota

To assess the relationship between the presence of frog deformities with reported Altosid use, data from 60 Minnesota counties monitoring for anuran deformities, provided by the U.S. Geological Survey, North American Reporting Center for Amphibian Malformations (NARCAM) (Northern Prairie Wildlife Research Center 2001), were compared based on the reported use of Altosid in those counties during 1997 and 1998 (Metropolitan Mosquito Control District 1999). Seven treated and 53 untreated counties were compared. Only collections made in 1997 and 1998 were included, because data were not consistently collected across counties sampled in other years. The data were reviewed for the year, species, number of specimens per collection, and number of deformities per collection. A paired *t*-test was utilized with the null hypothesis

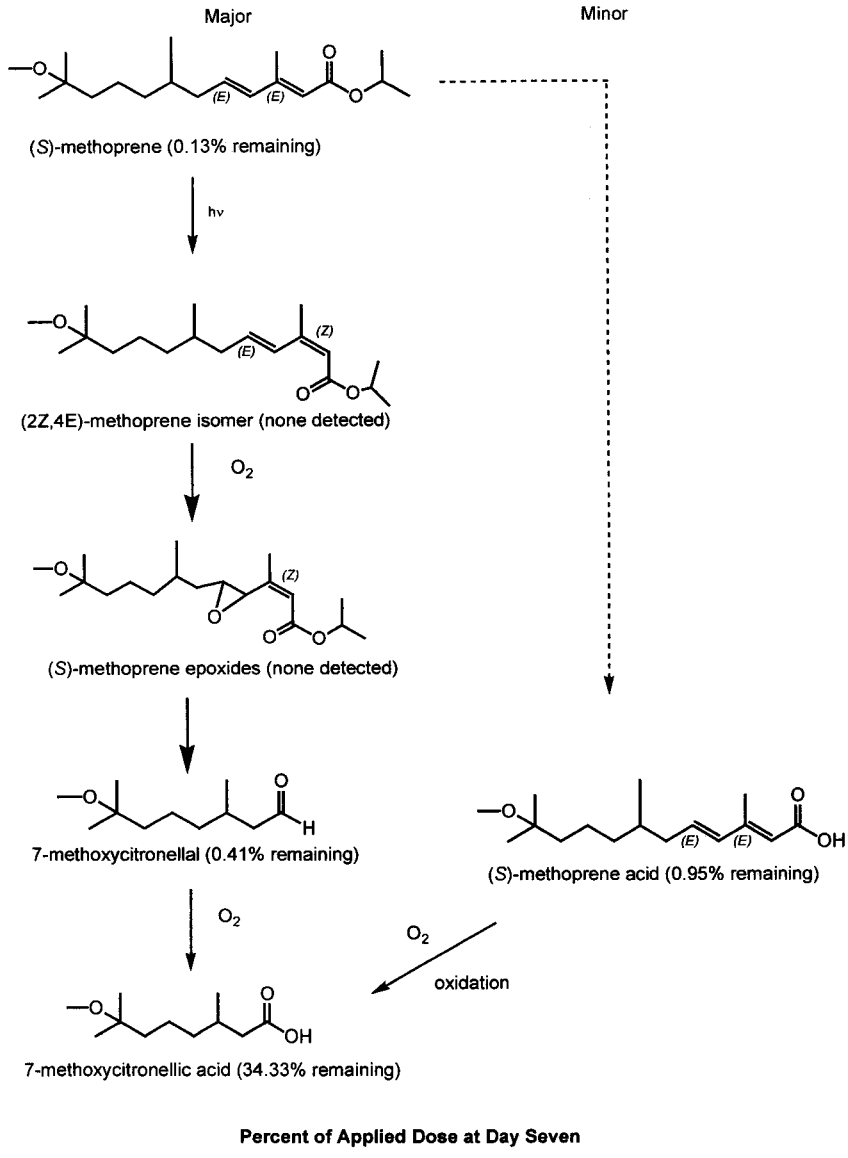

Fig. 1. Degradation pathway of *s*-methoprene.

stating that the difference between mean deformity rate of the 2 groups is equal to zero. The alternative hypothesis stated that the mean deformity rate was higher in treated counties versus untreated counties.

RESULTS

Degradation in pond water

Rapid degradation of *s*-methoprene occurred in the pond water samples. The major degradation product was 7-methoxycitronellic acid on day 1, with small quantities of the 5 other degradation products (Table 1 and Fig. 1). The only significant component remaining after day 7 was 7-methoxycitronellic acid. Only trace amounts of *s*-methoprene and *s*-methoprene acid were detected, whereas (2*Z*,4*E*)-methoprene isomer and *s*-methoprene epoxides were not detected on day 7.

Analysis of Minnesota pond water

s-Methoprene and *s*-methoprene acid were not found in any of the surface water and groundwater samples collected by MPCA and analyzed by ELAB and Wellmark. The detection limits and the results are shown in Tables 2 and 3. Recovery of *s*-methoprene acid from spiked laboratory water blanks at ELAB was 101% for *s*-methoprene at 10

Table 1. Degradation product recovery percentages and levels at day 1 and day 7.

Degradation products	Recovery (%)	Day 1 (μg/liter)[1] (ppb)	Day 7 (μg/liter)[1] (ppb)	NOEL[2] (ppb)
s-methoprene	85	26	1	
(2*Z*,4*E*)-methoprene isomer	85	37	ND[3]	
s-methoprene epoxides	100	3	ND	
7-methoxycitronellal	95	34	3	
7-methoxycitronellic acid	20	267	237	>10,000
s-methoprene acid	64	36	9	>1,250

[1] Corrected for recovery.
[2] NOEL, no-observable-effect level.
[3] ND, not detected, <0.1 μg.

μg/liter and 74% for *s*-methoprene acid at 23 μg/liter. Recovery of *s*-methoprene and *s*-methoprene acid from the spiked sample matrix at ELAB was 114% for *s*-methoprene at 10 μg/liter and 90% for *s*-methoprene acid at 23 μg/liter. In the tests conducted by Wellmark, recovery of *s*-methoprene and *s*-methoprene acid from spiked water blanks was 80% for *s*-methoprene at 3 μg/liter and 45% for *s*-methoprene acid at 11 μg/liter.

Correlation of anuran deformity vs. Altosid use in Minnesota

Seven of the 60 counties (Anoka, Carver, Dakota, Hennepin, Scott, Ramsey, and Washington) reporting information on frog deformities use Altosid (Metropolitan Mosquito Control District 1999, Northern Prairie Wildlife Research Center 2001). Only 7 of the total 88 counties in Minnesota were reported to use Altosid (Metropolitan Mosquito Control District 1999). Five of the treated counties had malformations between 1997 and 1998, and 2 did not. From the *t*-test comparing 1997 and 1998 data, the resulting *P*-value was 0.6996, confirming the null hypothesis ($\mu_1 - \mu_2 = 0$) and demonstrating no statistically significant difference in frog deformity rates between Altosid-treated counties and untreated counties in Minnesota (Table 4).

DISCUSSION

The natural pond water analysis results demonstrate that *s*-methoprene and most of its degradation products degrade rapidly in pond water exposed to sunlight, mimicking conditions found in the practical field application of this larvicide. Furthermore, the step-wise degradation mechanism for *s*-methoprene and the identity of the degradation products, established by Schooley et al. (1975a, 1975b) and Quistad et al. (1975) with radiolabeled *rs*-methoprene, are confirmed based on the chemical entities and levels found on day 1 and day 7 after treatment. Even at 100 times the maximum field rate of application, concentrations found 7 days after application are in the single digit parts per billion range or at nondetectable levels. Recent research by Degitz et al. (2001) on the effects of *s*-methoprene degradation products on the early developmental stage of *Xenopus* with the FETAX methodology indicate a no observable-effect-level >10,000 ppb for 7-methoxycitronellic acid and >1,250 ppb for *s*-methoprene acid. The resultant risk quotients for those particular degradation products are >3,800 and >19,000 ppb, respectively. This supports the conclusion that the degradation products of *s*-methoprene have minimal potential for affecting anurans, even under considerable overdose situations.

In the Minnesota pond water analysis, both ELAB and Wellmark International confirmed that neither *s*-methoprene nor *s*-methoprene acid were found in the water samples from locations with high incidences of frog deformities established by FETAX. These results support the results of a 3rd independent laboratory (Hale 1997) on samples taken by MPCA in September 1997 from the same sites in Crow Wing County, MN. By using GC-ion trap MS and GC-flame ionization detection, Hale (1997) found no evidence of *s*-methoprene, *s*-methoprene acid, or any potential *s*-methoprene metab-

Table 2. Water sample testing methods.[1]

Compound	Test methods	Wellmark LOD (μg/liter)	ELAB LOD (μg/liter)
s-methoprene	Chemical analytical procedure 323B with GC-MS confirmation	0.1	0.2
s-methoprene acid	Modified EPA method 8141A with GC-MS confirmation, and EPA method 8270A	0.2	0.5

[1] LOD, limit of detection; GC-MS, gas chromatography–mass spectroscopy; EPA, U.S. Environmental Protection Agency.

Table 3. Water sample results.

Testing agency	Sample site ID[1]	s-Methoprene	s-Methoprene acid
Wellmark	CWB	ND[2]	ND
Wellmark	CWB-SW	ND	ND
Wellmark	CWR	ND	ND
Wellmark	DOR	ND	ND
Wellmark	DOR-SW	ND	ND
Wellmark	CUM	ND	ND
ELAB	CWB	ND	ND
ELAB	CWB-SW	ND	ND
ELAB	CWR	ND	ND
ELAB	DOR	ND	ND
ELAB	DOR-SW	ND	ND
ELAB	CUM	ND	ND

[1] Designated by Minnesota Pollution Control Agency.
[2] ND, not detected.

olites in the water samples at a limit of detection of 0.1–0.01 ppb. Similarly, Fort et al. (1999b) did not detect any *s*-methoprene or its degradation products in pond water or sediment samples collected from the same sites in 1999 (Burkhart, personal communication).

The comparison of anuran deformity rates in treated sites vs. untreated sites in Minnesota counties demonstrates that no difference is found between deformity rates when Altosid is in use. This evidence is further bolstered by the fact that only 8% of the counties in Minnesota use Altosid and have experienced frog deformities, leaving the majority of counties experiencing frog deformities without a direct link to Altosid. Evidence from other states with even heavier Altosid use (Florida and California [State of Florida 1998; Zanus Corporation, personal communication]) also supports this assertion that a correlation cannot be established between Altosid use and frog deformities at a county level.

However, considering the amount and resolution of the data available from NARCAM, the only conclusion that can be made from this aspect of the analysis is that no effect of Altosid was detectable. Although improved resolution of the data (e.g., actual site comparison vs. county-level data and prevalence of deformities in counties with Altosid use) would help solidify these conclusions, the water analyses, coupled with the correlation analysis, further reinforce the unlikelihood of a link between *s*-methoprene and its potential degradation products and anuran deformities in the field. Finally, the more recent 2-year study conducted by Johnson et al. (2001) confirms this conclusion. They found no difference in anuran deformities in ponds treated with and without Altosid in Wright County, MN, a county with reported frog deformities (Northern Prairie Wildlife Research Center 2001) where Altosid is not used operationally by the Metropolitan Mosquito Control District (1999).

In conclusion, neither *s*-methoprene nor its degradation products have been found in water or sediment samples from locations with high incidences of frog deformities, and no significant association has been found between the locations of Altosid use and reported frog deformities. The analyses conducted as part of this paper confirm the findings of a large, diverse body of literature indicating that *s*-methoprene has negligible or no effect on anurans even at 100 times the maximum recommended field rate. Thus, *s*-methoprene and its degradation products are unlikely to be contributors to the recent outbreak of frog deformities.

Table 4. Comparison of documented malformations in treated and untreated counties in Minnesota.[1]

Variable[2]	*n*	Mean	SD	Minimum	Maximum
T 97/98	7	3.5529	4.6713	0.0000	11.110
U 97/98	53	3.1628	3.2887	0.0000	13.460

[1] $P = 0.6996$.
[2] T 97/98, counties treated in 1997 and 1998. U 97/98, counties untreated in 1997 and 1998.

REFERENCES CITED

Ankley GT, Tietge JE, DeFoe DL, Jensen KM, Holcombe GW, Durhan EJ, Diamond SA. 1998. Effects of ultraviolet light and methoprene on survival and development of *Rana pipiens*. *Environ Toxicol Chem* 17:2530–2542.

Batzer DP, Sjogren RD. 1986. Potential effects of Altosid (methoprene) briquet treatments on *Eubranchipus bundyi* (Anostraca: Chirocephalidae). *J Am Mosq Control Assoc* 2:226–227.

Bircher L, Ruber E. 1988. Toxicity of methoprene to all stages of the salt marsh copepod, *Apocyclops spartinus* (Cyclopoida). *J Am Mosq Control Assoc* 4:520–523

Bishop DW. 1947. Polydactyly in the tiger salamander. *J Hered* 38:290–293.

Blaustein AR, Kiesecker JM, Chivers DP, Anthony RG. 1997. Ambient UV-B radiation causes deformities in amphibian embryos. *Proc Natl Acad Sci USA* 94: 13735–13737.

Britson CA, Threlkeld ST. 1998. Agrichemical, methyl mercury, and UV radiation effects on southern leopard frog (*Rana utricularia*) and gray treefrog (*Hyla chrysoscelis*) tadpoles [abstract]. In: *Midwest Declining Amphibian Conference* 1998 March 19–20; Milwaukee, WI. Milwaukee, WI: Milwaukee Public Museum. http://www.mpm.edu/collect/vertzo/herp/Daptf/MWabst.html

Bruner M, Jones T, Hull M, Rao M, Dumont J, Bantle J. 1997. Evaluation of ground and surface water toxicity downgradient from a municipal landfill [abstract]. In: *SETAC 18th Annual Meeting* 1997 November 16–20; San Francisco, CA. Pensacola, FL: Society of Environmental Toxicology and Chemistry. p 83. Abstract number 470.

Bryant SV, Gardiner DM. 1992. Retinoic acid, local cell–cell interactions, and pattern formation in vertebrate limbs. *Dev Biol* 152:1–25.

Burkhart JG, Helgen JC, Fort DJ, Gallagher K, Bowers D, Propst TL, Gernes M, Magner J, Shelby MD, Lucier G. 1998. Induction of mortality and malformation in *Xenopus laevis* embryos by water sources associated

with field frog deformities. *Environ Health Perspect* 106:841–848.

Costlow JD. 1977. The effect of juvenile hormone mimics on development of the mud crab, *Rhithropanopeus harrisii* (Gould). In: Vernberg FJ, Calabrese A, Thurberg FP, Vernburg WB, eds. *Physiological responses of marine biota to pollutants* New York, NY: Academic Press. p 439–457.

Degitz SJ, Durhan EJ, Kosian PA, Ankley GT, Tietge JE. 2001. Developmental toxicity of methoprene and its degradation products in *Xenopus laevis*. *Toxicologist* 60(1):333.

Dumont J, Bantle J, La Clair J, Fort D, Propst T, Faukner B, Stover E. 1997. Methoprene exposed to UV light causes developmental toxicity in *Xenopus* embryos [abstract]. In: *SETAC 18th Annual Meeting* 1997 November 16–20; San Francisco, CA. Pensacola, FL: Society of Environmental Toxicology and Chemistry. p 83. Abstract number 468.

Fort DJ, Propst TL, Stover EL, Helgen JC, Levey RB, Gallagher K, Burkhart JG. 1999a. Effects of pond water, sediment, and sediment extracts from Minnesota and Vermont, U.S.A., on early development and metamorphosis of *Xenopus*. *Environ Toxicol Chem* 18:2305–2315.

Fort DJ, Rogers RL, Copley HF, Bruning LA, Stover EL, Helgen JC, Burkhart JG. 1999b. Progress toward identifying causes of maldevelopment induced in *Xenopus* by pond water and sediment extracts from Minnesota, U.S.A. *Environ Toxicol Chem* 18:2316–2324.

Gardiner DM, Hoppe D. 1998. Deformed frogs: developmental and molecular mechanisms [abstract]. In: *Midwest Declining Amphibian Conference* 1998 March 19–20; Milwaukee, WI. Milwaukee, WI: Milwaukee Public Museum. http://www.mpm.edu/collect/vertzo/herp/Daptf/MWabst.html

Garg RC, Donahue WA. 1989. Pharmacologic profile of methoprene, an insect growth regulator, in cattle, dogs, and cats. *J Am Vet Med Assoc* 194:410–412.

Glaser J, Bogart J. 1997. Longterm effects of aerially sprayed glyphosate on percent deformities and reproductive success of *Rana sylvatica* [abstract]. In: *SETAC 18th Annual Meeting* 1997 November 16–20; San Francisco, CA. Pensacola, FL: Society of Environmental Toxicology and Chemistry. p 83. Abstract number 469.

Gradoni L, Bettini S, Majori G. 1976. Toxicity of Altosid to the crustacean, *Gammarus aequicauda*. *Mosq News* 36(3):294–297.

Grant KP, Licht LE. 1995. Effects of ultraviolet radiation on life history stages of anurans from Ontario, Canada. *Can J Zool* 73:2292–2301.

Hale R. 1997. Transport and fate of contaminants in aquatic environments. Paper presented at NIEHS Frog Workshop Meeting, 1997 December 4–5; Research Triangle Park, NC. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

Hebard WB, Brunson RB. 1963. Hind limb anomalies of a western Montana population of the Pacific tree frog, *Hyla regilla* Baird and Gicard. *Copeia* 1963:570–572.

Henrick CA. 1990. This week's citation classic. *Curr Contents Agric Biol Environ Sci* 21:14.

Henrick CA, Anderson RJ, Staal GB, Ludvik GF. 1978. Insect juvenile hormone activity of optically active alkyl (2*E*,4*E*)-3,7,11-trimethyl-2,4-dodecadienoates and of arylterpenoid analogues. *J Agric Food Chem* 26: 542–550.

Henrick CA, Staal GB, Siddall JB. 1973. Alkyl 3,7,11-trimethyl-2,4-dodecadienoates. A new class of potent insect growth regulators with juvenile hormone activity. *J Agric Food Chem* 21:354–359.

Hileman B. 1996. Frog deformities pose a mystery. *Chem Eng News* November 18:24.

Hileman B. 1998. The puzzle of frog deformities. *Chem Eng News* May 25:40–41.

Hirsch R, Temple S. 1998. A methodology to assess exposure to organophosphate insecticides in amphibian species [abstract]. In: *Midwest Declining Amphibian Conference* 1998 March 19–20; Milwaukee, WI. Milwaukee, WI: Milwaukee Public Museum. http://www.mpm.edu/collect/vertzo/herp/Daptf/MWabst.html

Hoppe DM. 1997. Historical observations and recent species diversity of deformed anurans. In: *Summary of Workshop on Central North American Amphibian Deformities. North American Amphibian Monitoring Program III* 1997 November 14–February 14; Duluth, MN. Laurel, MD: U.S. Geological Survey. http://www.im.nbs.gov/naamp3/abstracts/4dfa.html

Huang Y, Karazov WH, Patnode KA, Jefcoate CR. 1998. Exposure of northern leopard frogs in the Green Bay ecosystem to polychlorinated hydrocarbons as measured by direct chemistry and hepatic P450 activity. *Environ Toxicol Chem* 18:2123–2130.

Jofre M, Karasov WH. 1998. The direct effect of ammonia on three species of midwestern North American anuran amphibians. *Environ Toxicol Chem* 18:1806–1812.

Johnson CM, Johnson LB, Murphy J, Beasley V (Natural Resources Research Institute). 2001. *Evaluation of potential effects of methoprene and* Bti *on anuran malformations in Wright County, Minn* Duluth, MN: NRRI Technical Report NRRI/TR-2001/01. 34 p. Available from Natural Resources Research Institute, Univ. of Minnesota, Duluth, 5013 Miller Trunk Highway, Duluth, MN 55811-1442.

Johnson PTJ, Lunde KB, Ritchie EG, Launer AE. 1999. The effect of trematode infection on amphibian limb development and survivorship. *Science* 284:802–804.

La Clair JJ, Bantle JA, Dumont J. 1998. Photoproducts and metabolites of a common insect growth regulator produce developmental deformities in *Xenopus*. *Environ Sci Technol* 32:1453–1461.

Merrell DJ. 1968–69. Natural selection in a leopard frog population. *J Minn Acad Sci.* 35:86–89.

Metropolitan Mosquito Control District. 1999. *Operational review plans for 1999* St. Paul, MN: Metropolitan Mosquito Control District.

Miura T, Takahashi RM. 1973. Insect development inhibitors. 3. Effects on nontarget aquatic organisms. *J Econ Entomol* 66:917–922.

Northern Prairie Wildlife Research Center. 2001. North American Reporting Center for Amphibian Malformations. Jamestown, ND: Northern Prairie Wildlife Research Center. http://www.npwrc.usgs.gov/narcam

Ouellet M, Bonin J, Rodrigue J, DesGranges J, Lair S. 1997. Hindlimb deformities (ectromelia, ectrodactyly) in free-living anurans from agricultural habitats. *J Wildl Dis* 33:95–104.

Ovaska K, Davis TM, Novales Flamarique I. 1997. Hatching success and larval survival of the frogs *Hyla regilla* and *Rana aurora* under ambient and artificially enhanced solar ultraviolet radiation. *Can J Zool* 75:1081–1088.

Quistad GB, Schooley DA, Staiger LE, Bergot BJ, Sleight BH, Macek KJ. 1976. Environmental degradation of the

insect growth regulator methoprene. IX. Metabolism by bluegill fish. *Pest Biochem Physiol* 6:523–529.

Quistad GB, Staiger LE, Schooley DA. 1975. Environmental degradation of the insect growth regulator methoprene (isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate). III. Photodecomposition. *J Agric Food Chem* 23:299–303.

Rosenshield M, Jofre M. 1998. Effects of PCB 126 on green frog (*Rana clamitans*) and northern leopard frog (*Rana pipiens*) hatching success, development and metamorphosis [abstract]. In: *Midwest Declining Amphibian Conference* 1998 March 19–20; Milwaukee, WI. Milwaukee, WI: Milwaukee Public Museum. http://www.mpm.edu/collect/vertzo/herp/Daptf/MWabst.html

Ross DH, Judy D, Jacobson B, Howell R. 1994. Methoprene concentrations in freshwater microcosms treated with sustained-release Altosid formulations. *J Am Mosq Control Assoc* 10:202–209.

Rostand J. 1958a. *Les anomalies des amphibians anoures* Paris, France: Sedes.

Rostand J. 1958b. Effet tératogène des rayons ultraviolets sur les larves d'amphibiens. *C R Hebd Seances Acad Sci* 247:972–974.

Rostand J. 1971. *Les étangs a monstres. Histoire d'une recherche (1947–1970)* Paris, France: Stock.

Schaeffer CH, Dupras EF Jr. 1973. Insect development inhibitors. 4. Persistence of ZR-515 in water. *J Econ Entomol* 66:923–925.

Schoff PK, Holy JM. 1997. Cellular methods for determining causes of amphibian malformations: use of cell culture models [abstract]. In: *SETAC 18th Annual Meeting* 1997 November 16–20; San Francisco, CA. Pensacola, FL: Society of Environmental Toxicology and Chemistry. p 82. Abstract number 466.

Schooley DA, Bergot BJ, Dunham LL, Siddall JB. 1975a. Environmental degradation of the insect growth regulator methoprene (isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate). II. Metabolism by aquatic microorganisms. *J Agric Food Chem* 23: 293–298.

Schooley DA, Creswell KM, Staiger LE, Quistad GB. 1975b. Environmental degradation of the insect growth regulator methoprene (isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate). IV. Soil metabolism. *J Agric Food Chem* 23:369–373.

Schooley DA, Quistad GB. 1979. Metabolism of insect growth regulators in aquatic organisms. In: Khan MAQ, Lech JH, Menn JJ, eds. *Pesticide and xenobiotic metabolism in aquatic organisms* American Chemical Society Symposium Series 99. Columbus, OH: American Chemical Society. p 161–176.

Sessions SK, Franssen RA, Horner VL. 1999. Morphological clues from multilegged frogs: are retinoids to blame? *Science* 284:800–802.

Sessions SK, Ruth SB. 1990. Explanation for naturally occurring supernumerary limbs in amphibians. *J Exp Zool* 254:38–47.

Siddall JB. 1976. Insect growth regulators and insect control: a critical appraisal. *Environ Health Perspect* 14: 119–126.

Simonin HA, Paul EA, Foley RE, Symula J, Martin T, Jackling S. 1992. The effects of several mosquito larvicides on non-target organisms [abstract]. In: *SETAC 13th Annual Meeting* 1992 November 9–12; Cincinnati, Ohio. Pensacola, FL: Society of Environmental Toxicology and Chemistry. p 247. Abstract number WP6A11.

Sparling DW. 2000. Field evidence for linking Altosid applications with increased amphibian deformities in southern leopard frogs. *J Iowa Acad Sci* 107(3):90–91.

State of Florida, Department of Agriculture and Consumer Services, Pesticide Consumption. 1998. *Mosquito control monthly report September 1998* Tallahassee, FL: Department of Agriculture and Consumer Services, Pesticide Consumption.

U.S. Environmental Protection Agency. 1991. *R.E.D. facts: methoprene 1991* Report 738-F-91-104. 6 p. Available from U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/oppsrrd1/REDs/factsheets/0030fact.pdf

U.S. Environmental Protection Agency. 2001. *Update of the March 1991 methoprene R.E.D. fact sheet.* 9 p. Available from U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/pesticides/biopesticides/factsheets/fs105401.pdf

Van Valen L. 1974. A natural model for the origin of some higher taxa. *J Herpetol* 8:109–121.

Woitkewitch AA. 1961. Le dévelopment des extrémités surnuméraires chez les amphibians. *Bull Biol France Belg* 95:569–600.

Wright JE. 1976. Environmental and toxicological aspects of insect growth regulators. *Environ Health Perspect* 14:127–132.