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Photoproducts and Metabolites of a Common Insect Growth Regulator Produce Developmental Deformities in *Xenopus*

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In the past decade, numerous sights across North America have been found which contain abnormal levels of deformed amphibians. This deformation was not limited to species, geography, or climate and spanned a wide range of phenotypes. This report begins to explain these findings by examining the effects of pesticide degradation in early amphibian development through directly exposing *Xenopus laevis* embryos to trace levels of synthetically prepared degradates. This method specifically determines the risk posed by each individual chemical resulting from the natural fate of the host pesticide. Through this approach, we find that while the common insect growth regulator, *S*-methoprene, poses minimal developmental determent, products of its reaction with sunlight, water, and microorganisms dramatically interfere with normal amphibian development. The addition of 1 μL/L of several of *S*-methoprene's degradates to the environment of developing embryos resulted in juveniles with deformation similar to that found naturally. Current developmental assessments examine only the risk posed by host pesticides. This study now suggest that the simple examination of the these materials is not sufficient, but now forward the need for critically examining the temporal relation between both development and chemical degradation.

Introduction

Although amphibians have lived on this planet for over 300 million years, nearly 120 times that of modern man, reports within the last three decades have shown that numerous amphibian species are either suffering from serious population loss or have disappeared altogether (*1*−*4*). The scope of this decline is not limited to specific species, geography, or life cycle and most likely arises from a collection of causes. In a large part, this loss results from the expansion of humankind, which has not only destroyed crucial habitat but also interfered with networks between neighboring communities, which rely on the missing colonies for procreation and food (*5*). In addition, this expanse has brought foreign species, disease, and pestilence into areas where the proper immune or evolutionary response has not developed. Pathogens have already been shown to contribute to the decline of the boreal toad (*Bufo borealis*) from the mountains of Colorado, the green toad (*Bufo viridis*) in Israel, the common European frog (*Rana temporaria*), and several tree frogs in Australia (*6*−*8*). However, the fact that several species, such as the Monte Verde Golden Toad and the Harlequin Frog, are disappearing from apparently pristine rain forest suggests additional causes (*9*). Recently, a collaborative effort between Lips and Green suggest that declines seen in a Panamanian rain forest arise from a mysterious protozoan (*10*). Although yet unknown, this pestilence may have become lethal due to nonnatural introduction or the fact that the immune system of the targeted amphibians was weakened due to chemical or environmental stimuli.

In 1994, Blaustein et al. reported that increased ultraviolet radiation, which is known to damage DNA by introducing a dimeric photocycloaddition, may be one factor contributing to these losses (*11*−*13*). They noticed that an increase in ultraviolet light, artificially created by placing a set of ultraviolet filters around developing embryos of three species in central Oregon, inhibited the development of those species lacking the appropriate level of photolyases, enzymes which repair light-induced DNA damage. Therefore, a sporadic increase in ultraviolet radiation would impose sudden and biased selection against species which lack sufficient protection from sunlight or mechanisms for its repair, ultimately leading to their eventual decline. In part, this explains why certain species are disappearing from areas where others survive. Yet, others believe that these declines may be due to acid rain, chemical pollution, or aquacultural practices, threats which may be enhanced by increased ultraviolet radiation (*1*−*4*).

Growing concern for this manifestation has been compounded by the recent finding of abnormal levels of deformed anurans in ponds and lakes in California, Iowa, Minnesota, Missouri, Ontario, Quebec, South Dakota, Texas, Vermont, and Wisconsin (*14*−*16*). These deformities have been observed in a number of species including American toads (*Bufo americanus*), North American bull frogs (*Rana catesbeiana*), American green frogs (*Rana clamitans*), gray tree frogs (*Hyla versicolor*), north American leopard frogs (*Rana pipiens*), mink frogs (*Rana septentrionalis*), wood frogs (*Rana sylvatica*), spring peepers (*Hyla crucifer*), Pacific tree frogs (*Hyla regilla*) and long-toed salamanders (*Ambystoma macrodactylum*), and spotted salamanders (*Ambystoma maculatum)* (*17*−*19*). The wide range of phenotypes, including supernumerary limbs (ectromelia and ectrodactyly), missing limbs, superfluous webbing about the limbs, anophthalmia, edema, melanism, missing or abnormal internal organs, and deformed jaws as well as missing or black eyes, suggests that these occurrences arise during development and are not attributed to heredity or preditorial attack. In 1990, Sessions and Ruth noticed that multilegged *H. regilla* and *A. macrodactylum* found in Santa Cruz county California suffered from infliction by a trematode (*20*). They theorized that these parasites bore into the limb bud region of the animal at the tadpole stage, thereby disrupting normal cellular organization and eventually resulting in abnormal limb development. Support for this theory came from the fact that similar supernumary limb patterns were produced by surgically implanting microbeads into the limb bud of healthy *Xenopus* tadpoles. In addition to infliction from parasites, developmental toxins including retinoic acids, viruses, overpopulation, hyper-regeneration due to injury, and nutritional deficiency, as well as exogenous abiotic factors, such as increased temperature, acidity, and ultraviolet light, have

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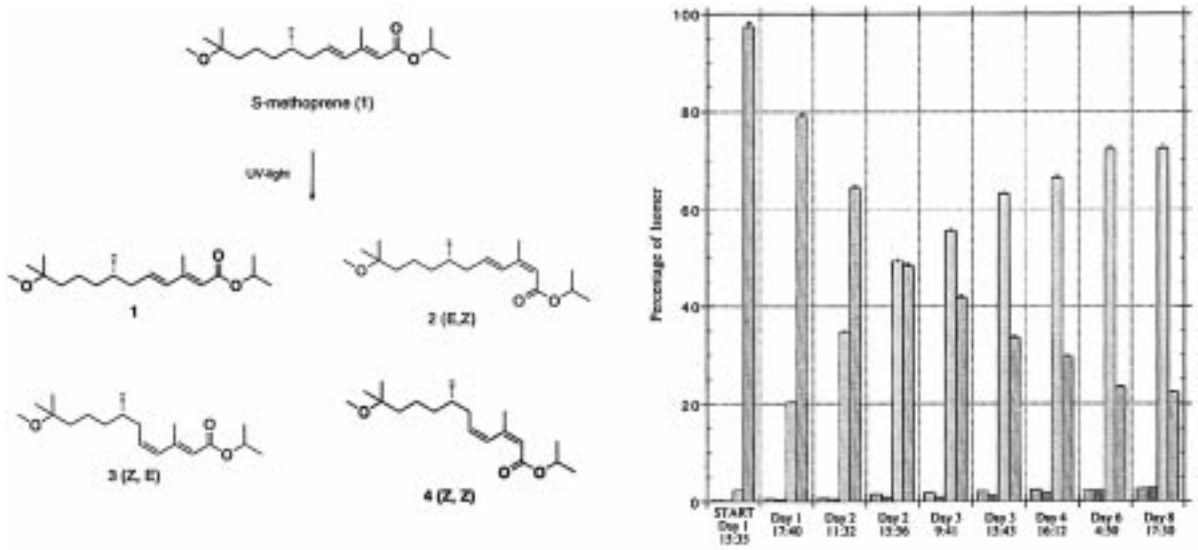

FIGURE 1. Representation of the reaction of *S*-methoprene (t-ME) with sunlight. Structures of possible photoisomers from t-ME. (left) Percentages of each isomer collected at specific time intervals over an eight day period. *S*-Methoprene was suspended in *n*-heptane 5 nM, sealed 10 mL quartz glass tubes, and placed in direct sunlight beginning at 15:35 on April 21, 1997, and ending at 17:30 on April 29 in La Jolla, CA. (right) Calibration of the UV-intensity provided near this location during this period can be obtained from Biospherical Instruments Inc. (San Diego, CA). The data provided was determined by diluting 50-fold and comparing the GC retention times with that of synthetically prepared standards. Comparable results (within 1% deviation) were seen when run at 10-fold higher concentrations or while dissolved in ethanol or deionized water.

also been shown to produce deformities during amphibian development (*21*−*23*). Recently, studies in the St. Lawrence River Valley of Quebec have shown that the level of observed deformation increases near pesticide-exposed areas (*14*). Several insecticides, pesticides, and agricultural products, including oxamyl, dieldrin, malathion, lindane, and paraquat, have been shown to deter normal development in amphibian embryos (*24*−*29*). Often, detection of these materials in aquatic environments is complicated due to their metabolism, and therefore, the timing of their application with respect to amphibian egg laying becomes a crucial factor in assessing their potential risk. For a large part, amphibian development is directly related to the growth of food supplies such as algae and insects. Therefore, application of agents which target the growth of these species would be present during amphibian growth.

S-Methoprene, an insect growth regulator, was introduced in the late 1970s as a means of flea and mosquito control. Operating by mimicking natural juvenile hormone, JHIII, this material inhibits developing pupae from molting and passing into the adult stage. Since these processes are not expressed in vertebrates, it was thought that *S*-methoprene would pose little toxicity to amphibians and mammals (*30*). Hence, the oral LD50 for *S*-methoprene in rats has been reported to be greater than 34 600 mg/kg and between 5 000 and 10 000 mg/kg in dogs. Furthermore, no teratogenic effects have been seen in rats or rabbits at 500 mg/kg (*31*). To be effective, *S*-methoprene must applied at or before the larval stage and, therefore, is applied to aquatic environments at a time which often coincides with early amphibian development. Under normal sunlight and temperature, *S*-methoprene decomposes rapidly in aquatic surroundings. Studies conducted in model ponds have shown that the half-life of *S*-methoprene is approximately 30 h at 1 ppb and 40 h at 10 ppb (*32*). This disappearance is attributed to degradation by microorganisms and reaction with sunlight (*33*). When exposed to light of the appropriate wavelength, materials containing olefinic bonds undergo excitation from the ground state to a singlet excited state. This excited state is typically short lived and rapidly undergoes relaxation back to the ground state through pathways such as fluorescence. For a number of substances, the energy gained by photoexcitation can also be diverted into chemical isomerization (*34*). The ramifications of this process have been recognized by nature and are incorporated in such processes as vision. A recent study by Zaga et al. showed that ultraviolet light (UV-B) could convert the insecticide carbaryl to a more lethal photoproduct which induced greater mortality and inactivity in *Xenopus laevis* and *H. versicolor* embryos (*35*). This study suggests that other organic materials may also be altered by exposure to only a few hours of normal sunlight.

Results and Discussion

S-Methoprene absorbs light in the ultraviolet region ranging from 190 to 310 nm with an absorption maximum at 259 nm in *n*-heptane. Irradiation of *S*-methoprene (t-ME) in *n*-heptane, ethanol, or water with a 450 W Hanovia High-Pressure Mercury lamp led to a photostationary state containing a 52:49 mixture of t-ME and its 2-*cis*-4-*trans*-isomer (ct-ME), respectively (*36*). In addition to these products, trace amounts (∼1−2%) of the 2-*trans*-4-*cis*-isomer (tc-ME) and *all-cis*-isomer (c-ME) were also detected. In sunlight, *S*-methoprene underwent a similar photoisomerization, reaching a photostationary state containing 22:72:3:3 of t-ME:ct-ME:tc-ME:c-ME, respectively, after 6 days in local sunlight. The intensity of ultraviolet light near this location can be obtained from Biospherical Instruments Inc. (San Diego, CA). Significant amounts of ct-ME were present within a few hours of exposure to sunlight (Figure 1); therefore, environments treated with *S*-methoprene would also encounter ct-ME, tc-ME, and c-ME. In addition to isomerization, *S*-methoprene is subject to hydrolytic degradation to *all-trans*-*S*-methoprenic acid (t-MA) in aquatic environments as well as in cells. Recently, Mangelsdorf et al. showed that t-ME and t-MA bind to the retinoic X receptor, RXR, through mimicking the structure of natural retinoic acids (Figure 2) (*37*). Rotational flexibility about the C6−C7 bond allows t-MA to adopt conformations which mimic both 9-*cis*-retinoic acid (9c-RA) and *all-trans*-retinoic acid (t-RA) (Figure 3). By the same analogy, 2-*cis*-4-*trans*-*S*-methoprenic

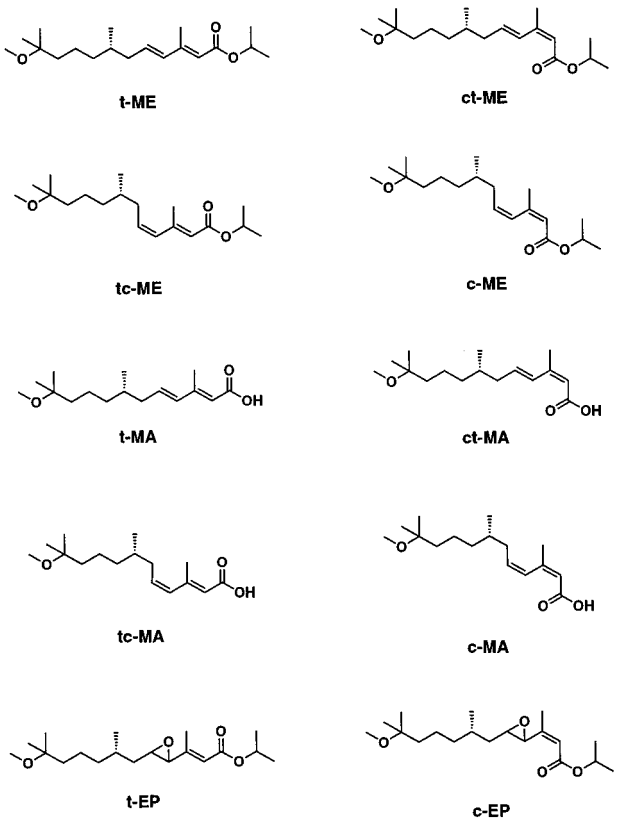

FIGURE 2. *S*-Methoprene (t-ME) and its metabolites. The photoisomers ct-ME, tc-ME and ct-ME. (top two rows) t-MA, ct-MA, tc-MA, and c-MA arise from hydrolysis of their parent compounds either by water in natural environments or enzymatically by esterases within microorganisms, plants or animals. (Rows 3 and 4) t-EP and c-EP are early products in the animal and plant metabolism of t-ME and ct-ME, respectively (*33*). (Bottom row) Samples of t-EP and c-EP synthesized herein consist of a 1:1 mixture of both epoxide diastereomers, *R*,*R*- or *S*,*S*- at C2−C3, and do not represent the diasteromeric mixture produced naturally.

acid (ct-MA), the hydrolytic product of the photoisomer ct-ME, would be capable of simultaneously mimicking 13-*cis*-retinoic acid (13c-RA) and 9-*cis*-13-*cis*-retinoic acid (9,13c-RA). t-RA, 9c-RA, 13c-RA, and 9,13c-RA are known vertebrate teratogens, producing heart, craniofacial, and limb malformations (*38*−*44*). In addition to isomerization and hydrolysis, *S*-methoprene is metabolized in animals, microorganisms, and plants through epoxidation of the C3−C4 olefin (*33*). This may happen either at the ester stage, converting t-ME to t-EP and ct-ME to c-EP, or after hydrolysis with the corresponding acids. Although *S*-methoprene has undergone significant testing, little is know about the teratogenic risk of its photoisomers or metabolites.

Frog embryo teratogenisis assay-*Xenopus* (FETAX) is a 96 h whole embryo developmental toxicity test that utilizes the embryos of the South African clawed frog, *X. laevis* (*45*−*47*). The outcome of this assay is expressed by the concentration required to induce 50% mortality (96 h LC50), the concentration required deform 50% of the individuals (96 h EC50) and the minimum concentration to inhibit growth (MCIG). From these variables, a teratogenic index (TI) is calculated by dividing the 96 h LC50 by the 96 h EC50. FETAX is applicable to aquatic toxicity assessments and is well suited for testing either pure compounds or complex mixtures, such as industrial effluents or mixtures found at hazardous waste sites. Recent modifications have been made to FETAX permitting routine testing of volatile organic compounds, soils, and sediments, therefore, suggesting the use of FETAX for deducing chemical causes of amphibian decline (*48*).

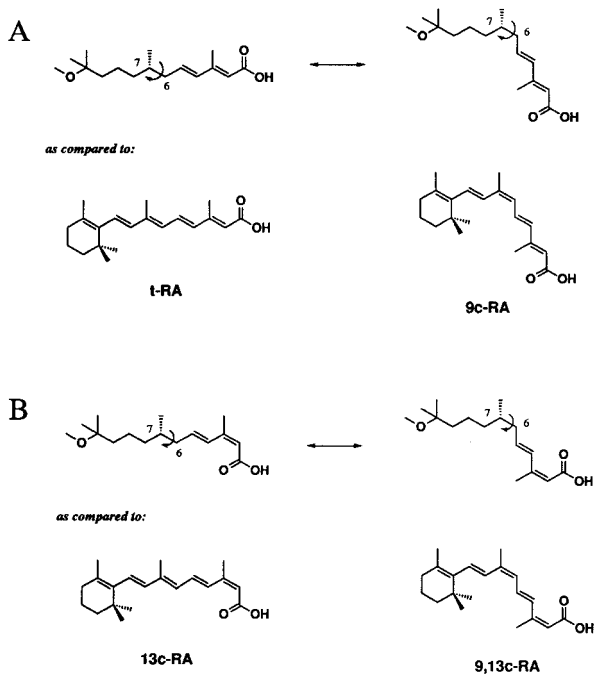

FIGURE 3. Rotational analysis of the major methoprenic acids allow these materials to exist in conformations which can mimic the structure of four known teratogenic retinoic acids (RA). (A) t-MA and (B) ct-MA.

Recently, FETAX was used to evaluate the developmental toxicity of t-RA, 9c-RA, and 13c-RA (*49*−*50*). 13c-RA demonstrated a 96 h LC50, 96 h EC50, and MCIG of 30.3, 3.3, and 12.3 μg/L (1 μg/L of t-MA corresponds to approximately 0.0096 μL/L), respectively, while t-RA gave a 96 h LC50, 96 h EC50, and MCIG of 375, 34, and 70 μg/L. The TI of 13c-RA was 12.7 while that of t-RA was 10.9. Nervous system and craniofacial defects were the most common malformations observed in the FETAX investigation of 13-*cis*-retinoic acid. 13c-RA was more embryo toxic and teratogenic than t-RA acid by a factor of approximately 10. This result is opposite of that found in many mammalian studies (*51*−*56*). However, differences in placental transfer, bioactivation, and species sensitivity play important roles in relative toxicity. The important point from these FETAX studies was the great sensitivity of *Xenopus* embryos to small amounts of retinoic acids as evidenced by high TIs and the severity of the observed malformations, thereby ranking these substances as strong developmental toxicants. Here, we describe the use of FETAX to determine the teratogenicity of metabolites and photoisomers of *S*-methoprene.

As suggested by previous testing on vertebrates (*29*−*30*), t-ME exhibited little mortality and malformation at concentrations below 15 μL/L, at which point it surpassed its water solubility even with added ethanol or DMSO. In contrast, a sample of t-ME resulting from a few hours of exposure to sunlight, containing 78% of t-ME, 20% of ct-ME, 1.5% of tc-ME, and 0.5% of c-ME, caused malformations at concentrations as low as 7.5 μL/L, a 2-fold decrease in effective concentration. At this concentration, this mixture produced 18% mortality and 12% malformity as compared to only 4% and 0% for nonphotolyzed *S*-methoprene. Hydrolysis to t-MA also enhanced the occurrence of deformities showing significant levels of deformities down to the 2.5 μL/L level and complete malformation at 15 μL/L. Photolysis of t-MA led to a mixture containing 69% of t-MA, 27% of ct-MA, 2% of c-MA, and 3% of tc-MA after 1 day in sunlight. This mixture showed increased malformation at 7.5 μL/L and obvious mortality at 10 μL/L when run without added cosolvent. The TI for this sample was 2.0, and its 96 h LC50 was 6.7 μL/L.

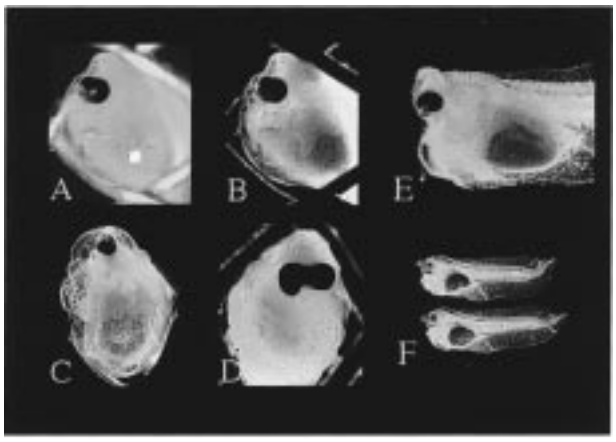

FIGURE 4. The effect of t-MA on the development of *Xenopus* embryos through 96 h. (A−D) Facial views of 96 h embryos exposed to 15 μL/L of t-MA. (E) Saggital view of head region of a 96 h embryo showing expanded brain vesicle and eye containing two lenses. The cement gland is displaced ventrally which is unusual for FETAX but common in these experiments. Heart and gut malformation are severe. (F) Saggital views of two 96 h embryos showing all of the abnormalities. A single dorsal flexure occurs near the end of the tail. Embryo length is greatly reduced.

Embryos were not as sensitive to the photolyzed mixture of methoprenic acids as they were to pure t-MA, as demonstrated by the fact that there was 97−100% malformation with pure t-MA and only 5−9% with the photolyzed mixture of acids at 5 μL/L. The 96 h LC50 of t-MA was over twice that of ct-MA, while the 96 h EC50 of t-MA was nearly four times less, suggesting that while ct-MA may be more toxic, the *all-trans*-isomer, t-MA, poses the greatest developmental risk. This was also supported by the decrease in t-MA's 96 h EC50 after exposure to sunlight. In addition to hydrolysis, t-ME is subject to oxidation to provide t-EP. t-EP as well produced effects at far lower concentrations than t-ME alone, with an 96 h LC50 and 96 h EC50 of 8 μL/L.

Growth inhibition in FETAX is usually rated as the most sensitive endpoint. *S*-Methoprene did not affect embryonic growth with concentrations between 0.25 and 1.5 μL/L. However, a sample of photolyzed t-MA acid containing 69% of t-MA, 27% of ct-MA, 2% of c-MA, and 3% of tc-MA had an MICG of only 1.0 μL/L, 93.5% of control length, and at 15 μL/L, embryos were only 53.7% of the controls. This was an extreme reduction in embryo length and demonstrated severe defects. At 48 h of incubation, the mixture of methoprenic acids caused severe eye defects such that pigmentation was lacking, and often diffuse and the choroid fissure refused to close on the ventral aspect of the eye. The cement gland was displaced significantly from the cranial region compared to controls and mouth parts were not readily evident. The neural tube appeared damaged at an early stage of development. At 96 h of development, moderate concentrations of the mixture of methoprenic acids caused serious malformations. At this point, the defects of the eyes were accentuated further than what they were at 48-h (individuals A−D in Figure 4). Several individuals lacked proper ocular separation from the brain and displayed duplicate or single eyes. Cranial and facial defects were severe when compared with controls. Gut formations were common with a corkscrew shape, and coiling was often observed. Fin malformations were observed with occasional kinking of the spinal column. Similar deformities were also produced with the photoisomerized mixtures of t-MA as well as pure ct-MA. The limit of abnormality detection by FETAX is summarized in Table 1. Furthermore, the epoxide *S*-methoprene (t-EP) was also more teratogenic than t-ME, with an approximate 40% decreased in its 96 h EC50. The type of malformations from this epoxide included spinal curvature, abnormal and duplicate eye formation, optical edema, cranial, and facial defects (Figure 5).

The concentrations where malformations occurred, the type of malformations observed, and the severity of these malformations were consistent with previous FETAX studies on retinoic acids (*29*−*30*). De Young et al. found that 13c-RA caused loose gut coiling and musculoskeletal kinking at concentrations well below 2.0 μg/L (1 μg/L of *S*-methoprene is approximately 0.002 μL/L) and above 2.0 μg/L resulted in brain and eye malformations (*49*). Cyclopia, eye pigment ruptures, reduced eye size, edema, spinal kinking, and cranial-facial abnormalities were induced in concentrations of >10.0 μg/L. The abnormalities described above that were caused by 13c-RA and t-RA are pictured in Bantle et al. (*46*). Cyclopia is extremely rare in FETAX as is the formation of a second lens in a single eye. However, compounds formed by the degradation of methoprene through the interaction of UV light cause the exact same types of cranial-facial malformations as seen with retinoic acids and at similar concentrations. The complete absence of a mouth is also a very rare malformation observed in FETAX. Of the 167 different chemicals tested in FETAX to date, only the retinoic acids and the compounds reported here cause this defect (*57*). These same malformations were also observed in mammals treated with retinoic acids (*50*−*55*). The 96 h LC50 of 13c-RA was 30 μg/L (95% CI-25−38.7), the 96 h EC50 (malformation) was 3.0 μg/L (95% CI-2.0−3.3), and the TI was 12.7 (*48*). These concentrations are approximately 1000-fold lower than the concentrations that cause effects in this study (Table 2), which in part explains the fact that the TI of 13c-RA is more than four times higher than the highest compound tested in this study. The very characteristic types of malformations caused strongly indicate that the effects of the methoprene derivatives is the same as retinoic acids. It was clear that either ultraviolet light or hydrolysis leads to mortality and malformation far above that of undegraded *S*-methoprene. Cosolvents may have interacted slightly but not enough to change the conclusion that *S*-methoprene can degrade to compounds which were of high developmental toxicity. The greatest effects were seen with t-MA and ct-MA, where malformation was readily detected at concentrations above 2.5 μL/L. Studies are currently underway to synthesize and compare the two minor photoisomers.

Several questions must be addressed before the conclusions of this report can be linked to the deformities seen in the wild. First of all, these materials must be shown to exist at the levels mentioned in the areas where high level of deformities have been found. *S*-Methoprene is routinely applied to wetlands across the United States by vector control agencies in order to control mosquito populations. It is typically applied to these areas either by spraying Altosid, a commercial preparation which contains 5% t-ME, at a recommended dosage of 219−293 mL/ha, or by dropping a slow-release pellet form. When correctly applied to a 1 he pond with an average depth of 0.25 m, the concentration of *S*-methoprene lies between 0.0044 and 0.0060 μL/L or ∼14 and −20 nM. At this level, our data suggest that there would be little visible malformation at the tadpole stage. However, examination of the UV-absorption intensity of the material used in FETAX solution shows that a significant portion of the methoprene derivatives under the 1 μL/L level adheres to the surface of the container and often cannot be detected (Table 3). In a natural environment, the surface adhesion is reduced, and therefore, it is difficult to mimic the natural conditions in the laboratory. In addition, the level described above does not account for multiple treatments, potential bioaccumulation, improper over application, and local increased concentrations about the slow-release form, as well as potential synergistic activation by other endogenous

TABLE 1. Percent Mortality (Mort) and Percent Malformation (Malf) for t-ME, Photolyzed t-ME, t-MA, Photolyzed t-MA, ct-MA and t-EP[a]

added concentration (μL/L)	t-Me				photolyzed t-Me		t-Ma				photolysed t-Ma				ct-Ma		t-EP			
	Mort run 1	Mort run 2	Malf run 1	Malf run 2	Mort run 1	Malf run 1	Mort run 1	Mort run 2	Malf run 1	Malf run 2	Mort run 1	Mort run 2	Malf run 1	Malf run 2	Mort run 1	Malf run 1	Mort run 1	Mort run 2	Malf run 1	Malf run 2
control	0	1	0	1	0	1	0	0	2	1	16	3	0	3	10	0	2	0	0	0
0.01							2		6											
0.1							0		6											
0.5							0		4											
1	4	2	5	4	2	0	0	6	0	3		0		0	25	0	8	0	0	6
1.5		2		2	6	2	8		4											
2		2		4	0	4	8		4											
2.5		0		2	0	0	2	8	8	0	24	8	8	6			18	6	2	6
3.5		4		6	0	2														
5	2	0	10	8	2	6	2	6	100	97	30	2	9	5	26	3	4	0	4	18
7.5		4		0	18	12		28		100	60	4	92	39			30	10	26	29
10	4	0	16	6	18	32	64	62	100		22	20	85	83	74	46	100	2		33
15	6	50	60	44	4	86		34		100	76	80	100	100	100		100	34		33
20	4	88	90	100	100						62	10	100	100	100					

[a] Samples of t-ME, photolyzed t-ME, and t-EP contained 1% DMSO added to the stock FETAX solution to aid in solubility. The photolyzed sample of t-ME contained 78% of t-ME, 20% of ct-ME, 1.5% of tc-ME, and 0.5% c-ME. The photolyzed sample of methoprenic acids had 69% of t-MA, 27% of ct-MA, 2% of c-MA and 3% of tc-MA, as determined by GC analysis.

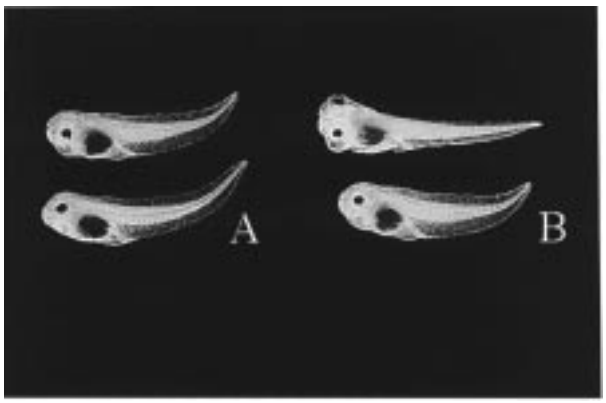

FIGURE 5. Abnormalities caused by the addition of t-EP up to 96 h of development. Embryos shown were exposed from the mid-blastula stage to a stock solution containing t-EP which was replenished daily. Note that the cement gland is displaced ventrally or missing. Edema is common at higher concentrations. Embryos were moderately malformed exhibiting reduced brains, misshapen eyes, poorly coiled guts and edema. (A) Embryos which were treated with 15 μL/L of t-EP. Note that the mouth is typically absent and the cement gland is displaced ventrally. (B) At 20 μL/L of t-EP, ocular edema was observed as well as stunting, misshapen eyes, poorly coiled guts and some upward dorsal tail flexure.

TABLE 2. TI, 96 h LC50, and 96 h EC50 for Samples of t-ME, Photolyzed t-ME, t-MA, Photolyzed t-MA, ct-MA and t-EP in *Xenopus* Development[a]

compd	96 h LC50 (μL/L)	96 h EC50 (μL/L)	TI
t-ME	24.5	13.2	1.9
photolysed t-ME	11	10.9	1
t-MA	9	2.9	3.2
photolysed t-MA	13.3	6.7	2
ct-MA	4	11	<1
t-EP	8	8	1

[a] The composition of the photolyzed samples is identical to that given in Table 1.

and exogenous materials. When added to pond water with normal turbidity, salinity, and pH, *S*-methoprene underwent rapid isomerization, and gradual hydrolysis and oxidation. While nearly all of the *S*-methoprene has disappeared in 380 h, a significant amount of t-ME and ct-ME was still present (Figure 7). Furthermore, *S*-methoprene is added to sprays against fleas which can be purchased in most grocery, pet, and pest-control stores. The level of this material present in these sprays is approximately 8 mM! At this level, a single recommended application to a 4.5 kg (10 lb.) pet could maximally transfer enough t-ME to contaminate 110 000 L at the 0.0015 μL/L and, therefore, allow this material to enter areas which may not be monitored by vector control agencies and state or local governments. Furthermore, t-ME is incorporated with a wide range of agricultural uses such as an additive to cattle feeds and grazing areas and tobacco, as well as certain grains.

So far, the deformities seen in the wild have been seen in anurans just prior to, undergoing, or past metamorphosis. As described, FETAX only examines the malformation visible up to the 96 h level. To determine the effects past metamorphosis, we allowed a portion of the living individuals to grow past development. While most treatments above 1 μL/L prevented the tadpoles from reaching metamorphosis, some individuals were able to undergo further development and were transferred after 96 h to clean containers loaded with nonmethoprene containing FETAX solution. After 3 months, several of these individuals suffered from structural defects, including black and missing eyes as well as apparent brain damage, and their development was clearly delayed. As shown in Figure 6, individuals B−D clearly possess incomplete eye structure and lack functioning lids and lenses. One further question that exists is whether the deformities seen here in *Xenopus* occur in anurans native to America. Although other studies have shown that individual species are not subject to the same level of developmental risk by chemical stimuli (*24*−*29*), the fact that all multicellular terrestrial organisms share a similar pattern of early development strongly suggests that it would be unlikely that these effects would not be apparent in other anurans.

Currently, few environmental protection agencies require a full examination of the products resulting from the natural degradation of a material added to the environment. This study provides an example of an insecticide which is rapidly converted to more detrimental materials. In so doing, this result suggests that criteria for analyzing the environmental impact of a chemical should be extended to consider carefully the products of its metabolism and environmental degradation in order to minimize future impact.

Experimental Section

Preparation of *S*-Methoprene Derivatives. *S*-Methoprene was obtained by extraction from Altosid or Zodiac Step 4

TABLE 3. Added versus Detected Levels of t-ME, t-MA, and ct-MA in FETAX Solution Containing 1 % DMSO[a]

	t-ME			t-MA			ct-MA		
added concentration (μM)	theoretical A_{max}	actual A_{max}	effective concentration (μM)	theoretical A_{max}	actual A_{max}	effective concentration (μM)	theoretical A_{max}	actual A_{max}	effective concentration (μM)
25	0.711	0.712	25	0.606	0.607	25	0.531	0.531	25
10	0.284	0.286	10	0.242	0.243	10	0.212	0.211	10.1
5	0.142	0.139	4.9	0.121	0.12	5	0.106	0.108	4.9
3	0.085	0.084	3	0.073	0.075	3	0.064	0.063	3
2	0.057	0.051	1.8	0.048	0.046	1.9	0.042	0.038	1.8
1	0.028	0.009	0.3	0.024	0.012	0.5	0.021	0.009	0.4
0.8	0.022	0.003	0.1	0.019	0.005	0.2	0.017	0.004	0.2
0.5	0.014	BL	BL	0.012	BL	BL	0.011	BL	BL
0.1	0.0029	BL	BL	0.0024	BL	BL	0.0029	BL	BL

[a] Each substance was added to five new 100 mL Schott Duran glass and 125 mL Nalgene bottles and shaken for 10 min. A 1 mL portion of these solution was transferred with a glass Pasteur pipet to a 0.5 cm quartz glass cuvette. Minimal differences (<2% deviation) were seen between the glass and plastic vessels and hence they were treated as one. Data presented are an average over the five runs in plastic and glass. The absorption intensity was collected using a HP8452 UV−visible spectrometer at 267 nm for t-ME, 260 nm for t-MA and 257 nm for ct-MA. BL indicates that the level seen was below that of background ($A \approx 0.002$).

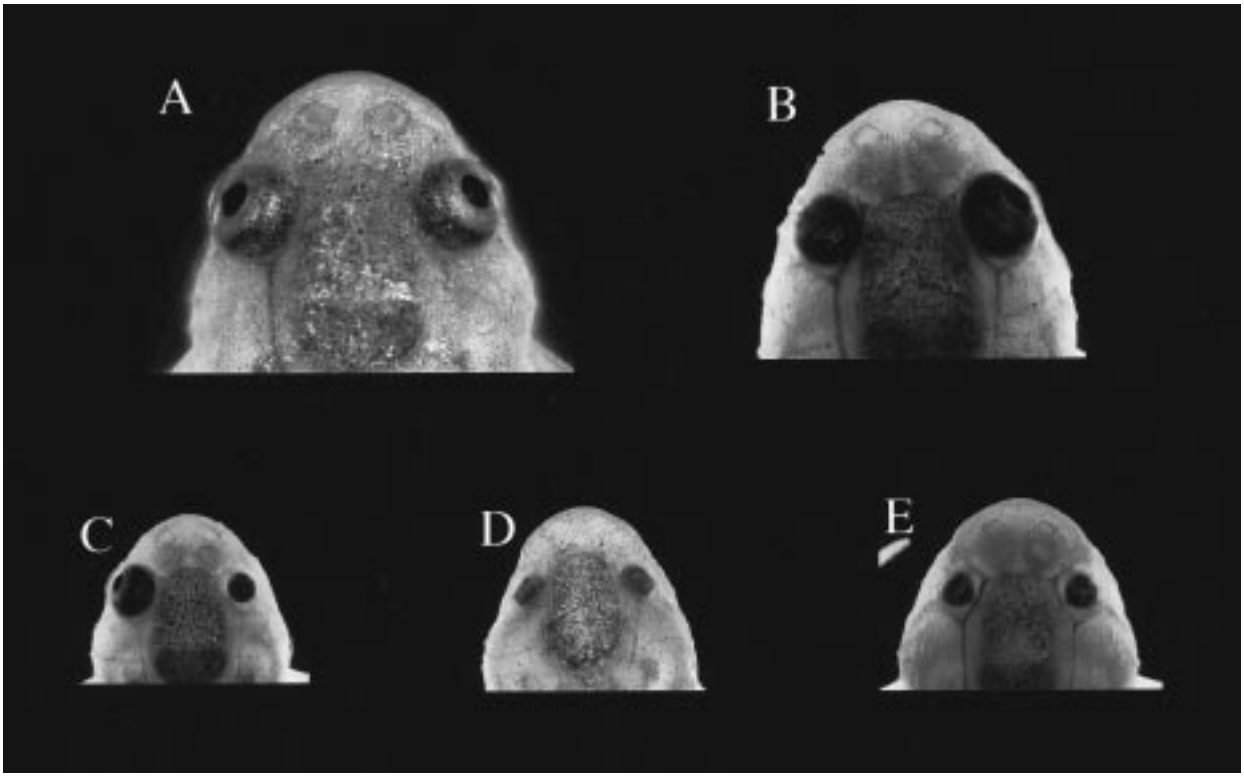

FIGURE 6. Malformations in juveniles caused by ct- and t-MA. Embryos were treated for 96 h and then allowed to continue development through metamorphosis. Eye malformations were extremely common. (A) Control, note generally larger size compared to the other individuals. (B) Juvenile with abnormal left eye and a darkly pigmented right eye. (C) Small juvenile with greatly reduced right eyes. (D) Small juvenile with both eyes malformed. (E) Juvenile with two abnormal eyes and poor lens development.

Flea Spray, purchased at local pest-control and pet stores, with diethyl ether and chromatographically purified. Photolysis of this material was performed in spectral grade *n*-heptane, which was placed in a quartz glass tubes and exposed to sunlight, as described below. Similar isomeric mixtures were also obtained when run in ethanol or suspended in water. The product composition was independent of concentration, showing little modification at concentrations as high as 30 mM. Samples were collected at different time intervals and resubjected to silica gel chromatography, and their composition was determined by gas chromatography. The other metabolites were either prepared by modification of *S*-methoprene or by independent chemical synthesis. Unless otherwise noted, all reagents and chemicals were purchased from commercial sources (Aldrich, Fisher, VWR, and ACROS chemical) and are used without further purification. Reactions were conducted under an argon atmosphere in rigorously dried glassware and magnetically stirred with a Teflon-coated stir bar. Anhydrous solvents were freshly distilled as follows: tetrahydrofuran from sodium benzophenone ketyl and methylene chloride from calcium hydride. Materials reacted under anhydrous conditions were dried extensively with toluene azeotrope prior to use. TLC on Merck Silica Gel DC 60 plates was routinely used to monitor reactions and stained with iodine absorbed on silica gel. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker MSL300 at 300 and 75 MHz, respectively. Chemical shifts (δ) are given in parts per million and coupling constants (J) in hertz. Gas chromatography was performed on a J & W Scientific DB-5 column using a HP 6890. Flash

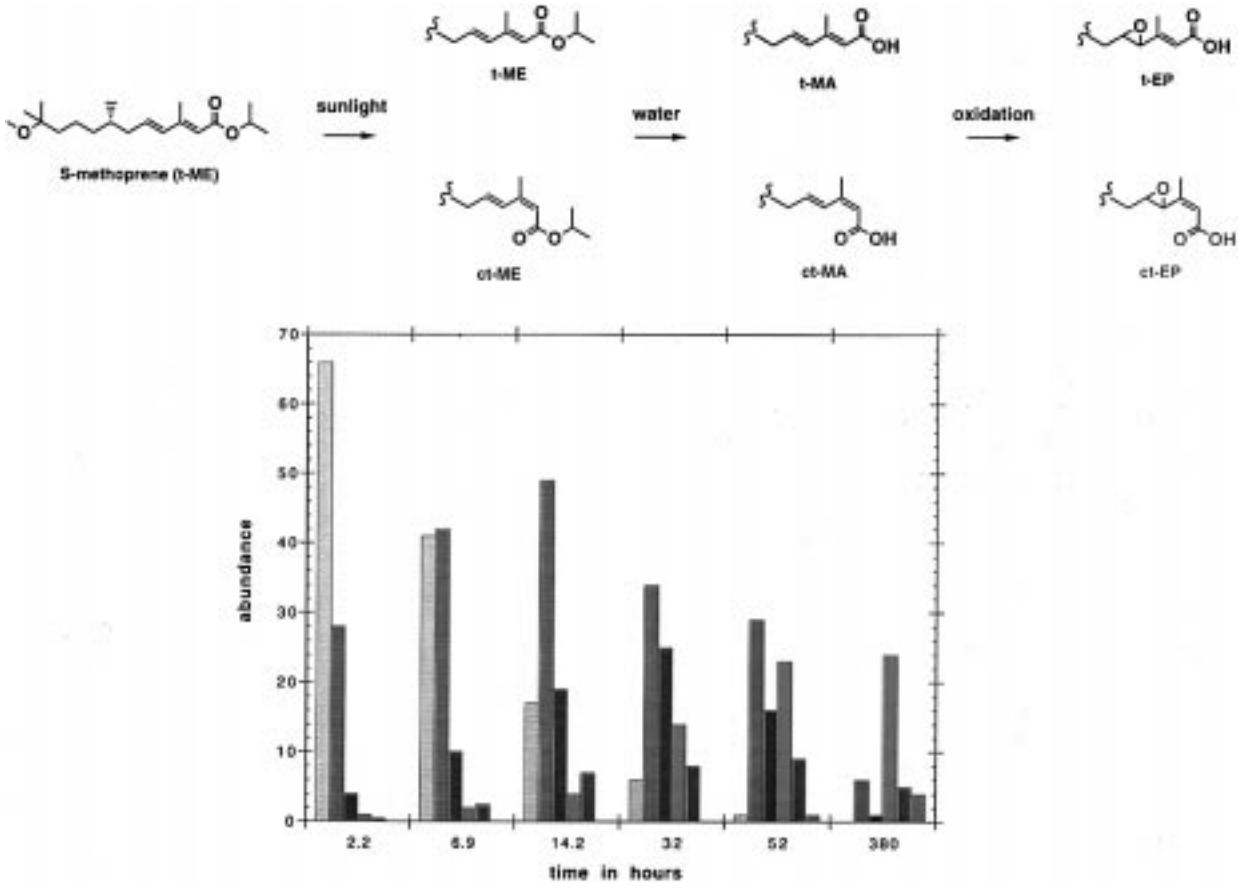

FIGURE 7. Decomposition of *S*-methoprene in pond water collected from three sights in San Diego county. The pH of these samples ranged from 7.4 to 7.8, the total amount of soluble materials in these samples ranged from 0.5 to 1 mg/mL, and the turbidity ranged from 0.2 to 1 ft. visibility at homogeneity. For each run, 30 mL of fresh Altosid was added per 10 L of pond water, and this solution was placed in a new 17 in. × 20 in. × 6 in. Nalgene dish located in partial shade in La Jolla, CA, beginning 11:40 am on July 3, 1997. At the indicated intervals, 250 mL samples were removed saturated with sodium chloride and extracted with 50 mL of *n*-heptane. A portion of this solution was directly examined by GC and the remainder monitored by ^{1}H NMR spectroscopy (not shown). The data presented is an average over five runs from each sight, and at each point the deviation was within 5%.

chromatography was performed on Mallinckrodt SilicAR 60 silica gel using a gradient from hexane to the solvent listed. The final chromatography was run on silica gel which was extensively washed with pesticide-grade hexane and ethyl acetate. All materials including *S*-methoprene were purified by the same procedure prior using the same batch of silica gel and solvent quality to minimize selective contamination.

***All-trans-S*-Methoprene Epoxide (t-EP).** Solid *m*-chloroperoxybenzoic acid (1.87 g of 50% w/w pure, remainder *m*-chlorobenzoic acid) was added to t-ME (1.65 g, 5.32 mmol) and sodium bicarbonate (3.85 g, 45.8 mmol) in dry methylene chloride (100 mL) cooled with a dry ice acetone bath to −78 °C. After 1 h, this solution was warmed to room temperature and kept there for 18 h. The resulting gelatinous mixture was diluted with saturated sodium bicarbonate (100 mL) and extracted with methylene chloride (5 × 50 mL). The organic fractions were combined and washed with saturated sodium bicarbonate (100 mL), brine (100 mL), dried with Na_2SO_4, and purified using flash chromatography (two repetitions with 20% ethyl acetate/hexane) to yield 1.21 g (69%) of t-EP (GC analysis showed the presence of two diastereomers in a 49:51 ratio with an overall purity >99%). ^{1}H ($CDCl_3$): δ 5.83 (s, 1H), 4.97 (dddd, J = 6.2 Hz, 1H), 3.10 (s, 3H), 3.02 (d, J = 1.7 Hz, 1H), 3.00 (d, J = 1.6 Hz, 1H), 2.77 (m, 1H), 2.01 (s, 3H), 1.62 (m, 2H), 1.55−1.12 (m, 7H), 1.19 (d, J = 6.2 Hz, 6H), 1.07 (s, 6H), 0.91 (d, J = 6.5 Hz, 6H), 0.90 (d, J = 6.4 Hz, 6H). ^{13}C ($CDCl_3$): 14.0, 19.6, 20.0, 21.2, 21.2, 21.8, 24.9, 30.9, 31.4, 37.2, 37.7, 39.3, 40.0, 40.1, 49.1, 58.9, 60.5, 61.0, 67.0, 74.5, 116.7, 165.8.

***All-trans-S*-Methoprenic Acid (t-MA).** A 6 M aqueous solution of sodium hydroxide (3.0 mL) was added to *S*-methoprene (1.25 g, 4.03 mmol) in a mixture of tetrahydrofuran (40 mL) and methanol (30 mL) over 15 min. After 20 h, the crude reaction was diluted with 1 M sodium hydroxide (20 mL) and extracted with hexane (3 × 50 mL). The aqueous phase was collected and the pH adjusted to 3 with ice cold 3 M hydrochloric acid and then extracted with methylene chloride (5 × 50 mL). The organic fractions were combined, washed with brine (50 mL), dried with Na_2SO_4, concentrated, and directly submitted to flash chromatography (two repetitions with 25% ethyl acetate/hexane) to yield 641.2 mg (59%) of t-MA (>99.5% purity by GC analysis). ^{1}H ($CDCl_3$): δ 6.12 (t, J = 6.2 Hz, 1H), 6.10 (s, 1H), 5.69 (s, 1H), 3.15 (s, 3H), 2.26 (s, 3H), 2.16 (m, 1H), 2.00 (m, 1H), 1.56 (m, 1H), 1.49−1.17 (m, 4H), 1.12 (s, 6H), 0.86 (d, J = 6.6 Hz, 1H). ^{13}C ($CDCl_3$): 14.1, 19.6, 21.3, 25.0, 33.2, 33.2, 37.2, 40.0, 40.6, 40.9, 49.1, 74.7, 117.0, 134.7, 137.2, 155.0, 172.4.

2-*cis*-4-*trans*-*S*-Methoprene Acid (ct-MA) and 2-*cis*-4-*trans*-*S*-Methoprene (ct-ME) were prepared using the methods described by Henrick (*35*). Flash chromatography (10% ethyl acetate/hexane) afforded ct-MA. ^{1}H ($CDCl_3$): δ 7.50 (d, J = 15.8 Hz, 1H), 6.14 (td, J = 7.4, 15.8 Hz, 1H), 5.60 (s, 1H), 3.15 (s, 3H), 2.19 (m, 1H), 2.10−1.92 (m, 2H), 2.00 (s, 3H), 1.65−1.02 (m, 6H), 1.12 (s, 6H), 0.87 (d, J = 6.6 Hz, 1H). ^{13}C ($CDCl_3$): 19.7, 21.3, 25.0, 25.6, 32.8, 33.2, 37.3, 40.0, 40.7, 40.9, 49.1, 74.7, 115.7, 128.7, 139.0, 153.7, 171.1. ct-ME was prepared from ct-MA by formation of its acid chloride with thionyl chloride in *N*,*N*-dimethylformamide followed by

interception with 2-propanol, as described by Henrick (I). Again, these materials were readily purified by flash chromatography (10% ethyl acetate/hexane) to afford ct-ME (>99.5% purity by GC analysis): ^{1}H ($CDCl_3$). δ 7.52 (d, J = 15.8 Hz, 1H), 6.09 (td, J = 7.4, 15.8 Hz, 1H), 5.54 (s, 1H), 5.00 (dddd, J = 6.2 Hz, 1H), 3.15 (s, 3H), 2.19 (m, 1H), 2.11−1.94 (m, 2H), 1.94 (s, 3H), 1.43−1.02 (m, 6H), 1.24 (d, J = 6.2 Hz, 6H), 1.09 (s, 6H), 0.85 (d, J = 6.6 Hz, 1H). ^{13}C ($CDCl_3$): 19.6, 21.1, 21.3, 21.8, 24.9, 33.2, 37.2, 37.3, 40.0, 40.9, 49.1, 66.7, 74.6, 116.2, 128.8, 137.7, 150.8, 165.9.

2-*cis*-*S*-Methoprene Epoxide (c-EP). *m*-Chloroperoxybenzoic acid (1.85 g of 50% w/w pure, remainder *m*-chlorobenzoic acid) was added to t-ME (1.19 g, 3.83 mmol) and sodium bicarbonate (3.38 g, 45.8 mmol) in dry methylene chloride (80 mL) cooled with a dry ice acetone bath to −78 °C. After 1 h, this solution was warmed to room temperature, where it was kept for 18 h. The resulting gelatinous mixture was diluted with saturated sodium bicarbonate (80 mL) and extracted with methylene chloride (5 × 40 mL). The organic fractions were combined and washed with saturated sodium bicarbonate (80 mL) and brine (80 mL), dried with Na_2SO_4, and purified using flash chromatography (20% ethyl acetate/hexane) to yield 748.1 mg (60%) of t-EP (GC analysis showed the presence of two diastereomers in a 53:47 ratio with an overall purity >99%). ^{1}H ($CDCl_3$): δ 5.82 (d, J = 1.1 Hz, 1H), 5.01 (dddd, J = 6.2 Hz, 1H), 4.37 (d, J = 10.4 Hz, 1H), 3.14 (s, 3H), 2.90 (m, 1H), 2.01 (s, 3H), 1.62 (m, 2H), 1.55−1.12 (m, 7H), 1.19 (d, J = 6.2 Hz, 6H), 1.07 (s, 6H), 0.91 (d, J = 6.5 Hz, 6H), 0.90 (d, J = 6.4 Hz, 6H). ^{13}C ($CDCl_3$): 18.5, 19.6, 19.9, 21.2, 21.3, 21.9, 23.6, 24.9, 30.9, 31.4, 37.4, 37.7, 39.6, 39.9, 40.0, 49.1, 56.4, 56.9, 57.0, 57.1, 67.2, 68.1, 74.6, 120.2, 129.8, 165.5.

Photolyzed Mixtures from t-ME or t-MA. A sample of *S*-methoprene (0.9−1.4 g) or t-MA (0.9−1.4 g) was dissolved in spectroscopic grade *n*-heptane and ethanol or suspended in deionized water (33 mL solvent/g of *S*-methoprene), placed in a 50 mL quartz glass tube, sealed, and placed in direct sunlight. At defined periodic intervals, samples of these materials were removed and a small portion of this material was diluted 10-fold with *n*-heptane and examined by gas chromatography. Twenty repetitions of each experiments were monitored and the results are tabulated (Figure 1). The crude isolates were concentrated and repurified by flash chromatography (10% ethyl acetate/hexane) immediately prior to FETAX use, to minimize risk from external contamination.

Teratogenic Assays. All assays were conducted using the established protocol of FETAX. The FETAX procedure has been extensively reviewed and conducted without modification (*45*−*50*). Materials to be assayed for developmental toxicity were suspended in FETAX solution containing 625 mg of NaCl, 96 mg of $NaHCO_3$, 30 mg of KCl, 15 mg of $CaCl_2$, 60 mg of $CaSO_4 \cdot 2H_2O$, and 75 mg of $MgSO_4$ in 1 L of deionized or distilled water. *Xenopus* eggs were reared in house at Oklahoma State University using a static population of adults. The low solubility of t-ME, ct-ME, tc-ME, and c-ME, ~1.4 ppm in water, required the use of 1% ethanol or DMSO as a cosolvent for homogeneity. t-MA, ct-MA, tc-MA, and c-MA were sufficiently soluble in FETAX solution and did not require the use of cosolvent under the 20 μL/L level. The difficulties inherent in the use of cosolvents have been previously described, and every attempt was made to use minimal amounts (*44*). Each analysis was compared to a control with and without cosolvent. The use of different cosolvents was also instrumental in eliminating concerns over solvent-based interactions. Materials to be tested were added at the given concentration to FETAX solution and an aliquot of these stock solutions was first presented to *Xenopus* embryos between the early blastula (stage 7.5) to mid-gastrula (stage 11.5) stages, as characterized by Nieuwkoop-Faber (*58*). The media surrounding the developing *Xenopus* was changed daily and replaced with the stock methoprene-containing FETAX solution. Dead embryos were removed daily and counted. FETAX was performed in both glass and plastic dishes in order to ascertain surface adherence. Twenty-five embryos were placed in each glass dish containing 10 mL of the FETAX solution, while in plastic, 20 embryos/dish and 8 mL were employed. Standard methods were used in the performance of FETAX, and all runs were collected when there was no interaction to the cosolvent, as determined by controls. Mortality and malformation data are reported as a percent response (Table 1). The concentrations of the methoprene derivatives in these FETAX solutions was shown to be lower than that added spectroscopically using a Hewlett-Packard UV−visible spectrometer. Five 100 mL samples of methoprene-treated FETAX solutions ranging from 25 to 0.5 μL/L were prepared in both 100 mL Schott Duran glass and 125 mL Nalgene bottles. A 1 mL portion of this material was removed with a glass Pasteur pipet and transferred to a 0.5 mL quartz glass cuvette. Both glass or plastic provided nearly identical outcomes and were therefore combined (Table 3).

Acknowledgments

This work is dedicated to the memory of Joseph La Clair for whom without his life long encouragement this effort would have never been realized. We thank A. R. Blaustein, B. Blumberg, D. M. Gardiner, M. G. Finn, R. A. Lerner, K. Lips, A. Macedo, M. Ouellet, S. Sessions, M. E. Thurman, and D. B. Wake for their support and comments.

Note Added in Proof

In November 1997, we collected water and sediment samples from three sights in the Lake Champlain basin of Vermont which contained abnormal levels of deformed Rana pipiens. We found traces of ct-ME using GC and GC-MS in hexane extracts of sediment collected from one of these sights. While the level found in this sample was considerably less than that required for deformation in *Xenopus*, the fact that ct-ME was found several months after application suggests that greater levels were present at this sight. Full details of this work have been communicated elsewhere (La Clair, J. J.; Bantle, J. A.; Dumont, J.; Levey, R. *Nature* **1998**, in review). One should be aware that the metabolites discussed here are not the only retinoids added either naturally or unnaturally to the environment. For instance, vitamin A, a biosynthetic precursor of *all-trans*-retinoic acid, is naturally required for vertebrate development and can enter agricultural environments unnaturally through animal feeds [for references on the effects of vitamin A in development, see (a) Zile, M. H. *J. Nutrit.* **1998**, *128* (2 Suppl. S), 455S; (b) Bavik, C.; Ward, S. J.; Ong, D. E. *Mech. Dev.* **1997**, *69*, 155; (c) Dickman, E. D.; Thaller, C.; Smith, S. M. *Development* ***1997***, *124*, 3111; (d) Tzimas, G.; Collins, M. D.; Burgin, H.; Hummler, H.; Nau, H. *J. Nutrit.* **1996**, *126*, 2159; (e) Maden, M.; Gale, E.; Kostetskii, I.; Zile, M. H. *Curr. Biol.* **1996**, *6*, 417; (f) Chen, Y. P.; Dong, D.; Kostetskii, I.; Zile, M. H. *Dev. Biol.* **1996**, *173*, 256; (g) Kraft, J. C.; Kimelman, D.; Juchau, M. R. *Drug Metab. Disposit.* **1995**, *23*, 72, 83]. The fact that PCBs have also been shown to alter retinoid metabolism even furthers the importance to critically evaluate the retinoid properties of nature's synthetic chemicals [for references on PCBs and retinoid regulation, see (a) Palace, V. P.; Klaverkamp, J. F.; Baron, C. L.; Brown, S. B. *Aquat. Toxicol.* **1997**, *39*, 321; (b) Murk, A. J.; Boudewijn, T. J.; Meininger, P. L.; Bosveld, A. T. C.; Rossaert, G.; Ysebaert, T.; Meire, P.; Dirks, S. *Arch. Environ. Contam. Toxicol.* **1996**, *31*, 128; (c) Gilbert, M. L.; Cloutier, M. J.; Spear, P. A. *Aquat. Toxicol.* **1995**, *32*, 177; (d) Morse, D. C.; Brouwer, A. *Toxicol. Appl. Pharmacol.* **1995**, *131*, 175; (e) Zile, M. H.;

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