Environmental and Molecular Mutagenesis 49:434–438 (2008)

Research Article

Genotoxicity Testing of the Herbicide Trifluralin and Its Commercial Formulation Treflan Using the Piscine Micronucleus Test

Serpil Könen and Tolga Çavaş*

Department of Biology, Faculty of Sciences and Letters, Mersin University, 33343 Mersin, Turkey

In this study, the genotoxic effects of a widely used herbicide, trifluralin, and its commercial formulation, Treflan, were evaluated using the micronucleus test in a commercially important fish species, *Oreochromis niloticus* (Nile Tilapia). Fish were exposed to 1, 5, and 10 μg/L doses of trifluralin and Treflan for 3, 6, and 9 days under laboratory conditions. Ethylmethanesulfonate, at a single dose of 10 mg/L, was used as positive control. Micronuclei were evaluated on the peripheral erythrocytes. Both Treflan and trifluralin treatments significantly increased the micronucleus frequencies in peripheral erythrocytes of *O. niloticus*. Furthermore, the genotoxicity of the active ingredient, trifluralin, was observed to be higher than that of the commercial formulation Treflan. Our results indicate that herbicide trifluralin has genotoxic potential in fish. Environ. Mol. Mutagen. 49:434–438, 2008.

Key words: trifluralin; treflan; genotoxicity; micronucleus test; *Oreochromis niloticus*

INTRODUCTION

Some pesticides are very reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA [Crosby, 1982]. Because large amounts of pesticides are released into the environment daily, they are of concern because of their potential toxicity, and any consequences of their potential genetic and related effects [Ribas et al., 1995]. Although understanding of the biological effects of currently used pesticides has increased in recent years, there are often incomplete, and sometimes contradictory, data on their genotoxicity [Dimitrov et al., 2006; Cavas and Konen, 2007].

Trifluralin is a selective preemergent dinitroaniline herbicide that is used to control annual grasses and broadleaf weeds on a variety of food crops, including fruit trees, nuts, vegetables, grains, cotton, soybeans, sunflowers, alfalfa, sugar beets, and peanuts [Worthing and Hance, 1991]. It also has many non-food uses including treatment of right-of-ways, ornamentals, cottonwood and poplar plantations, recreational lawns, and turf. Furthermore, trifluralin has pharmacological properties because it has been shown to inhibit the proliferation of parasitic protozoons such as *Plasmodium falciparum*, *Trypanosoma brucei*, and several species of *Leishmania* [Callahan et al., 1996]. Trifluralin was first registered in the United States in 1963. It is widely used in some countries, including Turkey, but is banned in several other countries, such as Norway, Denmark, and Sweden, because of its harmful effects. Currently, there are over 130 formulated products containing trifluralin in the world. Trifluralin may be released to the environment in fugitive emissions during its production and in wastewater effluent [US DHHS, 1993]. It is also released to the ambient environment during its application as an herbicide and is present in surface water as a result of agricultural runoff [Yockim et al., 1980]. Thus, assessment of genotoxic effects of this herbicide on aquatic organisms is crucial.

The genotoxic potential of trifluralin has been investigated by several researchers using different test systems and genetic endpoints [Ghiazza et al., 1984; Pilinskaia, 1987; Ribas et al., 1995; Gebel et al., 1997; Kaya et al., 2004]. However, the results that have been obtained are

Grant sponsor: Mersin University Research Fund; Grant number: BAP FBE BB (SK) 2006-4 YL.

*Correspondence to: Tolga Çavaş, Department of Biology, Faculty of Sciences and Letters, Mersin University, 33343 Mersin, Turkey.
E-mail: tcavas@mersin.edu.tr

Received 24 December 2007; provisionally accepted 18 February 2008; and in final form 6 March 2008

DOI 10.1002/em.20401

Published online 30 April 2008 in Wiley InterScience (www.interscience.wiley.com).

conflicting and there is no study dealing with the genotoxicity of this herbicide in aquatic organisms. The piscine micronucleus test system may play an important role in genetic toxicology in evaluating the genotoxic potentials of xenobiotics in the aquatic environment [Cavas and Ergene-Gozukara, 2005; Cavas, 2008]. The quantitative assessment of micronucleus-containing cells serves as an indicator for the induction of structural and/or numerical chromosomal aberrations (CA) [Al-Sabti and Metcalfe, 1995]. Many studies with pesticides show differences between active ingredients and their formulations with respect to toxicity and genotoxicity. Thus, in the present study, we aimed to comparatively evaluate the genotoxicity of herbicide trifluralin, and its commercial formulation Treflan, using the micronucleus test in the peripheral erythrocytes of fish *Oreochromis niloticus*. To our knowledge, this is the first study devoted to evaluation of genotoxic effects of trifluralin on fish.

MATERIALS AND METHODS

Fish and Chemicals

Nile Tilapia *Oreochromis niloticus* (L., 1758), (Pisces: Cichlidae) was chosen for this study because of its availability in most fish farms and markets in Turkey, and also due to its demonstrated sensitivity to genotoxic chemicals [Cavas and Ergene-Gozukara, 2003; Ergene et al., 2007]. Healthly specimens of *O. niloticus* with average weight and length of 5 ± 1 g and 5 ± 1 cm, respectively, were obtained from Cukurova University fish farm (Turkey). Before the experiments they were acclimated under laboratory conditions for 2 weeks at 25°C to 12/12 hr dark/light modes at a population density of 50 specimen per 90 L aquarium. Regular fluorescent light bulbs were used for lighting the laboratory environment during the experiments to prevent photodegradation of trifluralin in aquaria.

The herbicide Treflan consisted of trifluralin as the active ingredient (480 g/L); the other ingredients of Treflan are proprietary information and not available to the authors. Trifluralin (98% purity) (CAS number 1582-09-8) was purchased from Riedel-de Haën (Seelze, Germany). Ethylmethanesulfonate (EMS) and methanol were purchased from Sigma, St. Louis, MO. The chemical formula of Trifluralin is shown in Figure 1.

Three different concentrations of trifluralin were selected based on the limit value indicated as 11 μg/L according to Turkish Aquaculture Regulation [1995]. Immediately prior to treatment the trifluralin was dissolved in methanol to obtain three solutions of different concentrations and 100 μL of each solution was added to aquaria to obtain final trifluralin concentrations of 1, 5, and 10 μg/L. Treflan was diluted in various volumes of methanol to obtain three different dilutions. Aquaria were than treated with 100 μL of each dilution to obtain final concentrations of Treflan corresponding to 1, 5, and 10 μg/L trifluralin.

Experimental Design

The assays were carried out in aquaria containing 10 L of the test water. Five fish were used for each dose/duration group. Fish were exposed to the Treflan and Trifluralin for 3, 6, and 9 days. Dechlorinated tap water was used as a negative control. A further set of fish were treated with 10 mg/L EMS and 0.01% methanol (v/v) for 3, 6, and 9 days as positive and solvent controls, respectively. The test waters were renewed, and fresh herbicide and positive control were added every 72 hr to minimize changes due to metabolism by the fish, volatilization of less stable substances, and organism catabolites. At the end of exposure periods, blood samples were collected and processed for the micronucleus test.

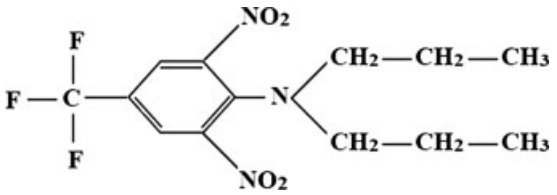

Fig. 1. Chemical structure of Trifluralin.

Analysis of Micronuclei

Peripheral blood samples were obtained from the caudal vein of the specimens and smeared onto precleaned slides. After fixation in pure ethanol for 20 min, the slides were allowed to air-dry and then the smears were stained with 5% Giemsa for 20 min. Three slides were prepared for each fish, and from each slide 1,500 erythrocytes were scored for the presence of micronuclei under 100× magnification. Nonrefractive, circular or ovoid chromatin bodies, smaller than the 1/3 of the main nucleus and displaying the same staining and focusing pattern as the main nucleus, were scored as micronuclei [Al-Sabti and Metcalfe, 1995]. Prior to analyses all slides were coded and scored blindly. The mean frequencies of MN were expressed as the number of MN per 1,000 erythrocytes (‰).

Statistical Analysis

After assessing the normality of distribution of the micronuclei data with the Kolmogorov-Smirnov test, parametric tests were used to detect differences at the 0.05 level of significance. Differences between mean values were compared using one-way analysis of variance (ANOVA) and the Bonferroni tests. Dose-treatment interactions were tested using two-way ANOVA. All the data are expressed as the mean ± SE (standard error).

RESULTS

Micronucleus frequencies in peripheral erythrocytes of *O. niloticus* exposed to trifluralin, as well as parallel, negative, solvent and positive controls are summarized in Table I. As shown in the table, no significant increase in the solvent control group was observed, whereas significant increases in the positive control group with respect to the negative control group were observed ($P < 0.001$). Trifluralin treatment significantly increased the micronucleus frequencies in all treatment groups with respect to the solvent control group ($P < 0.01$). However dose-duration interactions were not found to be significant ($P > 0.05$).

Micronucleus frequencies in peripheral erythrocytes of *O. niloticus* exposed to Treflan are shown in the Table II. Treflan treatments significantly induced the micronucleus frequency in all experimental groups with respect to the solvent treatment group ($P < 0.05$) with the exceptions of 1 and 5 μg/L at the 3rd day ($P > 0.05$). Once again, dose-duration interactions were not found to be significant in Treflan exposed group ($P > 0.05$).

TABLE I. Micronucleus Frequencies in Peripheral Erythrocytes of *O. niloticus* Exposed to Trifluralin (‰ Mean ± SE)

				Trifluralin		
Treatment duration	Negative control	Solvent control	Positive control	1 μg/L	5 μg/L	10 μg/L
3 Days	3.06 ± 0.47	4.73 ± 1.16	23.33 ± 2.20***	18.73 ± 2.82***	16.60 ± 2.64**	21.60 ± 1.30***
6 Days	3.23 ± 0.72	5.66 ± 0.44	19.33 ± 1.90***	19.53 ± 2.79***	22.06 ± 3.44***	24.00 ± 3.36***
9 Days	3.40 ± 0.97	5.60 ± 0.52	22.93 ± 2.36***	16.46 ± 1.90**	20.13 ± 2.30***	18.13 ± 0.93***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. solvent control.

Frequencies of trifluralin induced micronuclei were generally higher than that of its commercial formulation Treflan. A sample photomicrograph, showing micronucleus formation in peripheral erythrocytes of *O. niloticus*, is demonstrated in Figure 2.

DISCUSSION

Acute toxicity data indicate that technical grade trifluralin containing *n*-nitrosodi-*n*-propylamine is moderately toxic to very highly toxic to freshwater fish and invertebrates and highly toxic to estuarine fish and invertebrates [US EPA, 1996]. To protect the aquatic environment from episodic inputs of high trifluralin concentrations, a maximum concentration for dissolved trifluralin of 1 μg/L has been proposed for freshwaters based on applying a safety factor of 10 to the 96 hr LC_{50} of 10 μg/L for rainbow trout to prevent short-term acute effects. [Mayer and Ellersieck, 1986]. Furthermore, a provisional environmental quality standard of 20 μg/L for total trifluralin expressed as a maximum concentration has been proposed by applying a safety factor of ∼100 to the acute 96 hr LC_{50} of 2.8 mg/L obtained for bluegill in the presence of suspended solids [Parka and Worth, 1965]. The EPA has established a Lifetime Health Advisory level of 5 μg/L for trifluralin in drinking water.

Trifluralin is classified by the US Environmental Protection Agency (EPA) as Group C, possible human carcinogen [US EPA, 1999]. IARC also evaluated technical-grade trifluralin and concluded that the evidence of carcinogenicity was inadequate inhumans and limited in animals (Group 3) [IARC, 1991].

In addition to toxicity and carcinogenity, the genotoxicity of pesticides on non-target organisms and their influences on ecosystems are also of worldwide concern [Pimentel et al., 1998]. In the present study, we evaluated the genotoxic effects of herbicide trifluralin at the concentrations of 1, 5, and 10 μg/L in the fish *Oreochromis niloticus* using the micronucleus test. We observed significant increases in the frequencies of micronuclei in the peripheral erythrocytes of *O. niloticus* following treatment with trifluralin indicating the genotoxic potential of this herbicide on fish. The genotoxicity of trifluralin has been studied by several authors using different test organisms and test systems, and conflicting results have been obtained. For example, Pilinskaia [1987] investigated CA induced by pure trifluralin and its 25% emulsifiable concentrate formulation in human lymphocyte cultures in vitro and in mouse bone marrow cells in vivo, and found no clastogenic effects in either test system. However, Ribas et al. [1995] tested the genotoxicity of trifluralin in human peripheral lymphocytes using the single cell gel electrophoresis assay and reported an increase in the comet tail lengths, indicating DNA damaging effects of this herbicide. On the other hand, Ribas et al. [1996] evaluated sister chromatid exchanges (SCE), CA, and micronucleus formation induced by trifluralin in cultured human blood lymphocytes and reported that trifluralin treatment induced a slight but statistically significant increase in the frequency of SCE but no significant effects were observed in the CA and micronucleus assays. Similar results were also obtained by Ghiazza et al. [1984] who reported significant increases in SCE frequencies in human lymphocyte cultures treated with trifluralin. Furthermore, Gebel et al. [1997] reported that treatment with trifluralin

TABLE II. Micronucleus Frequencies in Peripheral Erythrocytes of *O. niloticus* Exposed to Treflan (‰ Mean ± SE)

				Treflan		
Treatment duration	Negative control	Solvent control	Positive control	1 μg/L[a]	5 μg/L[a]	10 μg/L[a]
3 Days	3.06 ± 0.47	4.73 ± 1.16	23.33 ± 2.20***	3.13 ± 0.33	9.60 ± 1.16*	8.40 ± 0.45*
6 Days	3.23 ± 0.72	5.66 ± 0.44	19.33 ± 1.90***	7.46 ± 0.90	8.86 ± 0.68*	13.93 ± 1.39**
9 Days	3.40 ± 0.97	5.60 ± 0.52	22.93 ± 2.36***	8.33 ± 0.56*	19.6 ± 2.14***	18.2 ± 1.06***

[a]Concentration of trifluralin.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. solvent control.

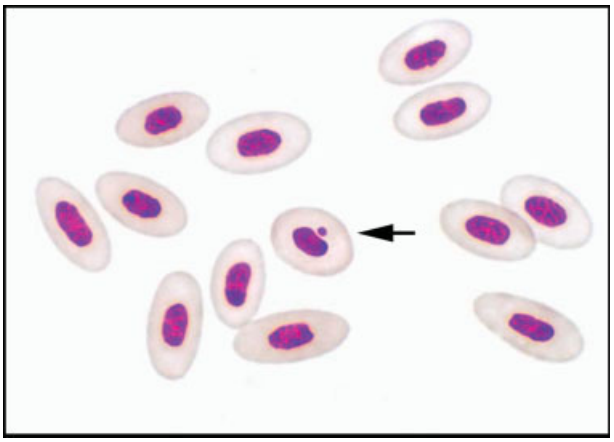

Fig. 2. Micronuclei in a blood smear from *O. niloticus* exposed to trifluralin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(99.5% purity) significantly increased the micronucleus frequency in mouse bone marrow.

The herbicide trifluralin penetrates into the seeds at the hypocotylous region and affects plant development by acting directly on cell division [Royal Society of Chemistry, 1989]. This herbicide is characterized as a microtubule-depolymerizing chemical. The trifluralin NO_2 group can be attached to tubulin molecules and prevent their polymerization and, consequently, the formation of microtubules [Morejohn and Fosket, 1991; Anthony and Hussey, 1999] Thus, they produce colchicine-like aneugenic effects in plants [Morejohn and Fosket, 1986]. However, it has been suggested that these chemicals show their adverse affects only on plant microtubules, but not on fungal or vertebral tubulins [Royal Society of Chemistry, 1989].

When using a herbicide in agriculture, not only the active ingredient but also the whole herbicide formulation, is applied. Results of many studies with pesticides show differences between active ingredients and their formulations with respect of toxicity and genotoxicity [Mayer and Ellersieck, 1986; Bolognesi et al., 1997; Zeljezic et al., 2006]. However, the identity of chemicals used as coformulants and surface-acting agents is often difficult to determine because of patent protection. At this point, comparative analysis of the genotoxicity of the active ingredient and the commercial product is often the most efficient way to assess the potential risks of these chemicals.

In the present work, in addition to the trifluralin, the genotoxicity of a trifluralin-containing commercial formulation, Treflan, was also evaluated. We observed a significant increase in micronucleus formation as a result of Treflan treatment. Previous reviews of technical vs. formulation toxicity showed that approximately one-third of the considered formulated products were more toxic than the technical grade material [Mayer and Ellersieck, 1986; Murty, 1986]. Similarly, results of several studies indicated that pesticide formulations are more potent genotoxins than active ingredients alone in a variety of test systems [Bolognesi et al., 1997; Grisolia et al., 2004; Zeljezic et al., 2006]. On the other hand, Schmuck et al. [1994] performed a comprehensive toxicity test using 273 pesticide formulations and active ingredients on fish and reported that more than 75% of the formulated products did not show a higher toxicity than the active ingredients. In our study, comparison of active ingredient and commercial formulation revealed that the level of genotoxic damage induced by the active ingredient trifluralin was comparatively higher than that of commercial formulation Treflan.

It is known that the commercial forms of pesticides can be chemically different, particularly in terms of the solubility of the active pesticide; thus the nature of adjuvants and other additive substances in the formulation may affect sorption, biodegradation and toxicity of the product. Furthermore it was demonstrated that surfactants and other inert compounds can increase or decrease the toxicity of the formulations [Schmuck et al., 1994; Grisolia et al., 2004; Cox and Surgan, 2006]. Our data suggest that adjuvants in Treflan may reduce the genotoxic potential of trifluralin in fish as compared to pure trifluralin, perhaps as a result of more efficient and direct penetration of the pure product into the fish body.

In conclusion, our results demonstrated the genotoxic potential of the herbicide trifluralin and its formulation Treflan in fish, and the suitability of the piscine micronucleus test for assessment of aquatic genotoxicity of herbicides. Our results further indicate that genotoxicity tests of pesticides should be conducted with both active ingredient and commercial formulations to better understand the potential effects of products which may pose a risk to aquatic organisms. We suggest that these formulations should also be further examined using different genetic endpoints in fish and in other aquatic species.

REFERENCES

Al-Sabti K, Metcalfe CD. 1995. Fish micronuclei for assessing genotoxicity in water. Mutat Res 343:121–135.

Anthony RG, Hussey PJ. 1999. Dinitroaniline herbicide resistance and the microtubule cytoskeleton. Trends Plant Sci 4:112–116.

Bolognesi C, Bonatti S, Degan P, Gallerani E, Peluso M, Rabbonu R, Roggieri P, Abbondandolo A. 1997. Genotoxic activity of glyphosate and its technical formulation roundup. J Agric Food Chem 45:1957–1962.

Callahan HL, Kelley C, Pereira T, Grogl M. 1996. Microtubule inhibitors: Structure-activity analyses suggest rational models to identify potentially active compounds. Antimicrob Agents Chemother 40:947–952.

Cavas T. 2008. In vivo genotoxicity of mercury chloride and lead acetate: Micronucleus test on acridine orange stained fish cells. Food Chem Toxicol 46:352–358.

Cavas T, Ergene-Gozukara S. 2003. Micronuclei, nuclear lesions and interphase silver stained nucleolar organizer regions (AgNORs)

as cytogenotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent. Mutat Res 538:81–91.

Cavas T, Ergene-Gozukara S. 2005. Micronucleus test in fish cells: A bioassay for in situ monitoring of genotoxic pollution in the marine environment. Environ Mol Mutagen 46:64–70.

Cavas T, Konen S. 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagen 22:263–268.

Cox C, Surgan M. 2006. Unidentified inert ingredients in pesticides: Implications for human and environmental health. Environ Health Persp 114:1803–1806.

Crosby, DG. 1982. Pesticides as environmental mutagens. In: Fleck RA, Hollander A, editors. Genetic Toxicology: An agricultural Perspective. New York: Plenum Press. pp 201–218.

Dimitrov BD, Gadeva PG, Benova DK, Bineva MV. 2006. Comparative genotoxicity of the herbicides roundup, stomp and reglone in plant and mammalian test systems. Mutagen 21:375–382.

Ergene S, Cavas T, Celik A, Koleli N, Kaya F, Karahan A. 2007. Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): Genotoxic damage in relation to water pollution. Ecotoxicology 16:385–391.

Gebel T, Kevekortes S, Pav K, Edenharder R, Dunkelberg H. 1997. In vivo genotoxicity of selected herbicides in the mouse bone-marrow micronucleus test. Arch Toxicol 71:193–197.

Ghiazza G, Zavarise G, Lanero M, Ferraro G. 1984. Sister chromatid exchanges induced in human lymphocyte chromosomes by trifluralin, atrazine and simazine. Boll Soc Ital Biol Sper 11:2149–2153.

Grisolia CK, Bilich MR, Formigli LM. 2004. A comparative toxicologic and genotoxic study of the herbicide arsenal, its active ingredient imazapyr, and the surfactant nonylphenol ethoxylate. Ecotoxicol Environ Saf 59:123–126.

IARC 1991. Trifluralin. International Agency for Research on Cancer Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 53. Lyon, France: IARC.

Kaya B, Marcos R, Yanikoglu A, Creus A. 2004. Evaluation of the genotoxicity of four herbicides in the wing spot test of *Drosophila melanogaster* using two different strains. Mutat Res 557:53–62.

Mayer FL, Ellersieck MR. 1986. Manual of Acute Toxicity: Interpretation and Data Base for 410 Chemicals and 66 Species of Freshwater Animals. Washington DC: United States Department of the Interior, U.S. Fish and Wildlife Service. Resource Publication 160.

Morejohn LC, Fosket DE. 1986. Tubulins from plants fungi and protests: A review. In: Shay JW, editor. Cell and Molecular Biology of the Cytoskeleton. New York: Plenum Publishing Corp. pp 257–329.

Morejhon LC, Fosket DE. 1991. The biochemistry of compounds with anti-microtubule activity in plant cell. Clin Pharmacol Therapeut 51:217–230.

Murty AS. 1986. Toxicity of Pesticides to Fish, Vol. 2. Boca Raton FL: CRC Press.

Parka SJ, Worth HM. 1965. The effects of trifluralin to fish. Proc Southern Weed Conf 18:469–473.

Pilinskaia MA. 1987. Evaluation of the cytogenetic effect of the herbicide treflan and of a number of its metabolites on mammalian somatic cells. Tsit Genet 21:131–135.

Pimentel D, Greiner A, Bashore T, 1998. Economic and environmental costs of pesticide use. In: Rose J, editor. Environmental Toxicology: Current Developments. UK:Gordon and Breach Science Publishers. pp 121–187.

Ribas G, Frenzilli G, Barale R, Marcos R. 1995. Herbicide-induced DNA damage in human lymphocytes evaluated by the single-cell gel electrophoresis (SCGE) assay. Mutat Res 344:41–54.

Ribas G, Surralles J, Carbonell E, Xamena N, Creus A.Marcos R. 1996. Genotoxic evaluation of the herbicide trifluralin on human lymphocytes exposed in vitro. Mutat Res 371:15–21.

Royal Society of Chemistry 1989. The Agrochemicals Handbook. Cambridge: Dialog Information Services, File 306.

Schmuck R, Pfliiger W, Grau R, Hollihn U, Fischer R. 1994. Comparison of short-term aquatic toxicity: Formulation vs. active ingredients of pesticides. Arch Environ Contam Toxicol 26:240–250.

Turkish Aquaculture Regulation 1995. Published In official gazette. Numbered 22223 and dated 10th March, 1995.

U.S. Department of Health and Human Services. 1993. Hazardous substances data bank national toxicology information program. Bethesda, MD: National Library of Medicine.

U.S. Environmental Protection Agency. 1999. Integrated Risk Information System (IRIS) on Trifluraline. Washington DC: National Center for Environmental Assessment, Office of Research and Development.

U.S. Environmental Protection Agency. 1996. Reregistration Eligibility Decision (RED): Trifluralin. Washington, DC:USEPA. EPA 738-R-95–040.

Worthing CR, Hance RJ. 1991. The Pesticide Manual, A World Compendium, 9th ed. British Crop Protection Council: Alton Hampishire, UK. pp 851–852.

Yockim RS, Isensee AR, Walker EA. 1980. Behavior of trifluralin in aquatic model ecosystems. Bull Environ Contam Toxicol 24:134–141.

Zeljezic D, Garaj-Vrhovac V, Perkovic P. 2006. Evaluation of DNA damage induced by atrazine and atrazine-based herbicide in human lymphocytes in vitro using a comet and DNA diffusion assay. Toxicol Vitro 20:923–935.

Accepted by—
A. Kligerman