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# EVALUATION OF THE METHOXYTRIAZINE HERBICIDE PROMETON USING A SHORT-TERM FATHEAD MINNOW REPRODUCTION TEST AND A SUITE OF IN VITRO BIOASSAYS

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**Abstract**—Prometon is one of the most consistently detected herbicides in the U.S. environment. However, no previous assessment of the potential for prometon or related methoxytriazine herbicides to act as endocrine-disrupting chemicals has been conducted. This study used an array of in vitro bioassays to assess whether prometon, atraton, terbumeton, or secbumeton might act as potent (ant)agonists of the aryl hydrocarbon, estrogen, androgen, or glucocorticoid receptors or as aromatase inhibitors or inducers in vitro. Potential effects of prometon were also evaluated using a 21-d fathead minnow reproduction assay. Concentrations of methoxytriazines, as great as 1 mg/L (4.4 $\mu$M), did not induce significant dioxin-like responses in H4IIE-luc cells, estrogenic responses in MVLN cells, or androgen or glucocorticoid receptor–mediated responses in MDA-kb2 cells, nor did the methoxytriazines significantly affect aromatase activity in vitro. In the fathead minnow assay, exposure to 20, 200, or 1,000 $\mu$g prometon/L significantly reduced the weight of the male fat pad (an androgen-responsive tissue) relative to body weight. Exposure to 20 $\mu$g prometon/L significantly increased female plasma testosterone concentrations, but the effect was not observed at greater concentrations. Overall, prometon did not significantly reduce fecundity over the 21-d exposure, nor were other endpoints, including plasma vitellogenin and estradiol concentrations, brain and ovary aromatase activity, and male tubercle index, significantly affected. Evidence from our work suggests that prometon may cause subtle endocrine and/or reproductive effects in fathead minnows, but no clear mechanism of action was observed. The relevance of these effects to hazard assessment for the pesticide is uncertain.

**Keywords**—Endocrine-disrupting chemicals    Methoxytriazines    Secondary sex characteristics    Steroids    Reproduction

## INTRODUCTION

Prometon is a nonselective triazine herbicide widely used for bare-ground weed control around buildings, storage areas, fences, roadways, railroads, recreational areas, lumberyards, and rights-of-way (http://www.pramitol.com). When effectively applied, it usually inhibits plant growth for a year or more (http://www.pramitol.com). Like most triazine herbicides, prometon blocks electron transport within the Hill reaction of photosystem II, killing plants by inhibiting photosynthesis [1,2]. Unlike other triazines, such as atrazine and simazine, prometon is nonselective and kills virtually all nonwoody vegetation (http://www.pramitol.com). As a result, it is not widely registered for agricultural, home, or garden use and seldom appears on lists of herbicides in use [1], and loading into the environment is not well documented [3]. Prometon has, however, been widely detected in environmental samples [4–8].

Based on available data, prometon has low acute and chronic toxicity to vertebrates. No maximum contaminant load for prometon in U.S. drinking water has been established; how-ever, the lifetime human health advisory for prometon in drinking water is 100 $\mu$g/L [9]. Oral and dermal median lethal dose (LD50) and median lethal concentration (LC50) values for rats are around 2,000 mg/kg for prometon (http://www.pramitol.com). Acute and chronic toxicity of prometon to birds, fish, and aquatic invertebrates is also low (effect concentrations >5 mg/L; http://www.pramitol.com).

Despite the low toxicity of prometon, reason for concern may still exist about the herbicide because of its seemingly wide distribution in U.S. surface and groundwater [4–8]. Further, once in the environment, prometon can persist in soil and water for hundreds to thousands of days [1,10]. Therefore, indications exist that some level of exposure of humans and wildlife to prometon is likely. This is notable because a structurally similar chlorinated triazine, atrazine, may cause adverse effects on rodents and other animals through disruption of endocrine functions [11–20]. Hence, it is possible that prometon and/or other methoxytriazine herbicides could be environmental endocrine-disrupting chemicals (EDCs) of consequence. This study tested that hypothesis using a variety of in vitro bioassays as well as a fathead minnow 21-d reproduction test. Results of this investigation could help provide data for hazard assessments related to the use of methoxytria-

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zine herbicides, particularly prometon, in the United States and elsewhere.

## MATERIALS AND METHODS

### Chemicals

Prometon (Chemical Abstract Service [CAS] 1610-18-0) and the structurally similar methoxytriazine herbicides atraton (gestamine; CAS 1610-17-9), terbumeton (CAS 33693-04-8), and secbumeton (CAS 26259-45-0), for use in the in vitro bioassays, were obtained from Accustandard (New Haven, CT, USA). All were 100% pure and dissolved in methanol (MeOH), except for atraton at 95.9% purity. In vivo exposures were conducted using technical-grade prometon obtained from Platte Chemical (Greenville, MS, USA; lot 0310770, purity 98.7%).

### Recombinant cell bioassays

The MVLN cells are human breast carcinoma cells stably transfected with a luciferase reporter gene under control of estrogen-responsive elements of the *Xenopus laevis* vitellogenin *A2* gene [21]. The H4IIE-luc (CALUX) cells are rat hepatoma cells stably transfected with a luciferase reporter gene under control of dioxin-responsive elements [22]. The cell lines were used to evaluate the ability of prometon, atraton, terbumeton, and secbumeton to bind to the estrogen receptor (ER) or aryl hydrocarbon receptor (AhR), respectively, and activate or inhibit reporter gene expression. Culturing conditions and assay procedures for both cell lines have been described previously [23,24]. In brief, cells for bioassay were plated into the 60 interior wells of 96-well culture plates (250 µl/well) at a density of approximately 15,000 (H4IIE-luc) to 30,000 (MVLN) cells per well. Cells were incubated overnight prior to dosing. A dilution series consisting of 12 concentrations ($5.64 \times 10^{-6}$–1.0 mg/L; equivalent to $2.5 \times 10^{-5}$–4.44 µM for prometeon, secbumeton, terbumeton and $2.7 \times 10^{-5}$–4.73 µM for atraton) of each test chemical, prepared by three-fold serial dilution in MeOH, was tested. Test and control wells were dosed with 2.5 µl of the appropriate test chemical or MeOH alone. Blank wells received no treatment. Each sample dilution, control, and blank was tested in triplicate. Luciferase assays were conducted after 72 h of exposure. Sample responses (relative luminescence units) were converted to relative response units expressed as a percentage of the maximum response observed for 17β-estradiol (E2; %-E2-max.) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; %-TCDD-max.) standard curves generated on the same day. This was done to normalize data for day-to-day variations in response magnitude and enhance the comparability of these results to other in vitro bioassay results reported in the literature [23,25].

The MDA-kb2 cells are human breast cancer cells stably transformed with the pMMTV.neo.luc reporter gene construct [26]. Reporter gene expression in this cell line can be activated by chemicals acting through either the androgen receptor (AR) or the glucocorticoid receptor (GR) [26]. Cells were maintained in L-15 media with phenol red (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37°C and atmospheric $CO_2$. For the bioassay, confluent plates of MDA-kb2 cells were trypsinized and diluted to a concentration of 200,000 cells/ml in media supplemented with 10% hormone-stripped (dextran-coated charcoal-filtered) FBS (Hyclone). Dilute cell solution (250 µl) was pipetted into each well of a 96-well ViewPlate (Perkin Elmer, Boston, MA, USA). Plates were incubated for 48 h and then dosed with 2.5 µl (per well) of the appropriate test compound (in MeOH) or MeOH only (solvent control). Blank wells received no treatment. Each chemical concentration, solvent control, and blank was tested in triplicate, using the same concentrations as for the MVLN and H4IIE-luc assays. Cells were exposed for 48 h and then visually examined for confluence, cytotoxicity, and contamination. Media were removed, and cells were washed three times with phosphate-buffered saline. Cells were lysed by freeze–thaw in 30 µl of ultrapure water per well. One hundred microliters of luciferase assay reagent [27] was automatically injected into each test well while the well was directly under the photomultiplier tube of the luminometer (Micro Lumi XS, Harta Instruments, Gaithersburg, MD, USA). Luminescence was measured using a 1,000-ms shutter speed. Data analysis was conducted as described for the MVLN and H4IIE-luc assays, using testosterone as a standard. For the purposes of this study, no attempts were made to distinguish possible AR- from GR-mediated responses.

### In vitro aromatase assays

Two types of in vitro aromatase assays were employed in this study. The first examined aromatase activity in the H295R human adrenocortical carcinoma cell line. The H295R cells were obtained from the American Type Culture Collection (ATCC no. CRL-2128) and maintained as described elsewhere [28]. Aromatase assays were conducted as described by Sanderson et al. [29] and Letcher et al. [30] with minor modifications. Briefly, for the bioassay, cells were seeded into the wells of a 24-well plate at a density of approximately 200,000 cells/ml, 1 ml per well, and incubated overnight. Wells were then dosed with 0.1% (v/v; 1 µl/1 ml) of test compound dissolved in MeOH and incubated for 24 h at 37°C. Seven dilutions of each chemical (threefold series; $1.37 \times 10^{-4}$–0.1 mg/L) were tested in triplicate, along with one MeOH control, one positive control (100 µM 8-bromo cAMP; Sigma, St. Louis, MO, USA), and one negative control (500 µM 4-androsten-4-ol-3,17-dione). Following exposure, medium was replaced with a reaction substrate solution of 54 nM $^3$H-androstenedione (Perkin Elmer NET-926; specific activity 25.3 Ci/mmol) dissolved in serum-free medium, and cells were incubated for 1.5 h at 37°C. Tritiated water was then separated from unreacted $^3$H-androstenedione by liquid:liquid extraction with chloroform followed by dextran-coated charcoal treatment of the aqueous fraction. Activity remaining in the aqueous fraction (tritiated water) was quantified by liquid scintillation counting.

In addition to the H295R assay, we examined the ability of the methoxytriazines to inhibit fathead minnow brain aromatase activity following in vitro exposure. A pool of fathead minnow brain postmitochondrial supernatant (S9 fraction) was prepared from $n = 8$ (mixed males and females) whole brains using methods described previously [31]. Five microliters of test chemical (in MeOH), MeOH only, or buffer were added to a 50-µl aliquot of the pooled homogenate followed by 150 µl of phosphate buffer (10 mM $K_2HPO_4$, 100 mM KCl, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, pH 7.4) containing 60 nM $^3$H-androstenedione and 1 mM β-NADPH (reduced nicotinamide adenine dinucleotide phosphate; Sigma N1630) to initiate the reaction. Heat-inactivated samples were used as blanks for determining nonspecific activity. One heat-inactivated sample and three active samples were run for each chemical, MeOH alone, and the buffer control. Each of the

four methoxytriazines was tested at a single concentration (10.8 μM for prometon, terbumeton, secbumeton; 11.5 μM for atraton). Samples were incubated for 90 min at 25°C and then analyzed as described elsewhere [31].

*Fathead minnow reproduction assay*

A solvent-free stock solution of prometon (200 mg/L) was prepared by dissolving technical-grade prometon in Lake Superior (USA) water. The stock solution was diluted in Lake Superior water and delivered to 20-L tanks containing 10 L water at a continuous flow of 46 ml/min to achieve nominal (target) test concentrations of 15, 50, 250, and 1,250 μg prometon/L over the course of the 21-d exposure period. Control tanks received Lake Superior water only, at the same flow rate. General water quality characteristics measured in the test system over the course of the study were hardness 46 mg/L as CaCO₃, alkalinity 40 mg/L as CaCO₃, pH 7.45 ± 0.24 (mean ± standard deviation [SD]), dissolved oxygen 5.55 ± 0.48 mg/L, and temperature 25.3 ± 0.3°C.

The basic experimental design was described by Ankley et al. [32] except that a paired rather than group spawning approach was employed [31,33]. Prior to starting chemical exposure, reproductively mature fish from an on-site culture unit were held in the test system, receiving clean Lake Superior water, for a 14-d acclimation period during which the fecundity of each pair was assessed daily. The animals were kept at 25°C under a 16:8-h light:dark cycle and fed adult brine shrimp twice daily. After 14 d, exposures were initiated using pairs of animals that had spawned successfully during acclimation. Eight pairs of fish allocated into four separate aquaria (two pairs per aquaria separated by a mesh divider) were exposed at each prometon concentration and control.

During the 21-d exposure, fecundity and fertility of the spawned eggs were recorded daily. Water samples (∼1 ml) were collected from each replicate tank two or three times per week, and prometon concentrations were measured (see the following discussion). At conclusion of the assay, the fish were anesthetized in buffered tricaine methanesulfonate (Finquel; Argent, Redmond, WA, USA). Blood was collected with a heparinized microhematocrit tube, and plasma was prepared by centrifugation. Plasma samples were stored with a protease inhibitor at −80°C prior to analyses. Following blood collection, whole-body wet weight was recorded. Gonads were then removed and weighed. Half the gonad tissue was fixed in 1% glutaraldehyde/4% formaldehyde in 0.1 M phosphate buffer [34], while the other half was snap-frozen in liquid nitrogen. Male tubercle development was scored as described elsewhere [35], and dorsal fat pads were removed from all males using a scalpel and weighed. Brains were removed from the fish and snap-frozen in liquid nitrogen. Pituitary glands were also removed and preserved in RNAlater® (Sigma) for analysis of gonadotropin β-subunit mRNA expression. The remainder of the fish carcass was frozen at −20°C until analyzed for tissue concentrations of prometon.

Plasma vitellogenin (VTG) concentrations were determined using an enzyme-linked immunosorbent assay with a fathead minnow polyclonal antibody [35]. Plasma sex steroid concentrations (estradiol [E2] and testosterone) were determined by radioimmunoassay [35]. Brain aromatase activity was determined by measuring release of tritium-labeled water from the C1 carbon of tritiated androstenedione [36], with an assay optimized for fathead minnow postmitochondrial supernatant preparations [37]. Total RNA was extracted from female pi-

tuitary samples from the control, 50-, and 1,250-μg prometon/L (nominal) treatments using RNeasy micro kits (Qiagen, Valencia, CA, USA), and luteinizing hormone–like and follicle-stimulating hormone–like β-subunit (LHβ and FSHβ) pituitary transcript levels were quantified by quantitative real-time polymerase chain reaction using methods detailed elsewhere [38]. Gonad tissues preserved for histological analyses were embedded in paraffin, sagittally sectioned at 3 to 5 μm in a stepwise fashion, and stained with hematoxylin and eosin. Two sections, one taken at 250 μm and another at 500 μm, were assessed for each testis sample. A total of six sections, one at 250 μm, one at 500 μm, one at 800 μm, and three around 1,000 ± 10 μm, were assessed from each ovary sample (for details, see Leino et al. [34]). Gonad histology was evaluated for all males ($n = 8$ per treatment) and surviving females ($n = 8, 8,$ and 5, respectively) from the control and the 250- and 1,250-μg/L treatments.

Exposure was verified through analysis of prometon concentrations in both water samples collected throughout the exposure period and in fish carcasses collected at test termination. Water samples (1 ml) were placed in crimp-top amber vials and immediately analyzed for prometon by reverse-phase high-pressure liquid chromatography using an Agilent (Wilmington, DE, USA) model 1100 high-pressure liquid chromatograph with a capillary pump, chilled autosampler (4°C), heated column compartment (25°C), and a diode-array detector. An aliquot of sample (20 μl) was injected onto a Zorbax (Agilent) SB-C18 column (2.1 × 75 mm) and eluted isocratically with 60% acetonitrile/water at a flow rate of 0.4 ml/min. Prometon concentrations were determined using the response at a wavelength of 220 nm and an external standard method of quantitation. No prometon was detected in the control tanks ($n = 28$) or procedural blanks ($n = 7$). The mean (±SD) recovery of prometon in the spiked matrix samples was 113 ± 9.9% ($n = 7$), and the mean (±SD) percentage agreement among duplicate samples was 98 ± 1% ($n = 25$). The analytical quantification limit was 1 μg/L.

Tissue samples were homogenized and extracted in acetonitrile (5 ml/g of tissue) using a high-speed tissue homogenizer operated at 12,000 rpm for 3 to 5 min. After settling, a 5-ml aliquot of the supernatant was transferred to a clean tube, chilled in a freezer at −20°C for at least 2 h, and then centrifuged at 3,000 rpm for 20 min at 4°C. The supernatant was removed and concentrated under nitrogen to approximately 1.0 ml and, as before, chilled and centrifuged. The resulting supernatant was transferred to a clean tube and diluted to 5 ml with 10% MeOH in water for high-pressure liquid chromatographic analysis using the method described previously. No prometon was detected in method blanks. The limit of detection was estimated to be 300 ng/g, and mean percent recovery of prometon spiked samples was 77 ± 11% ($n = 12$). The percent agreement between duplicate analyses of the same tissue was 92 ± 3% ($n = 5$).

*Data analysis*

A Kolmogorov–Smirnov test was used to test data for normality. Where appropriate, one-way analysis of variance was used to test for differences across all treatment groups. A nonparametric Kruskal–Wallis test was used for data that did not meet parametric assumptions. Dunnett's post hoc test was used to determine which treatment(s) were different from the control. Correlation analysis was based on Pearson correlation coefficients. Differences were considered significant at $p ≤$
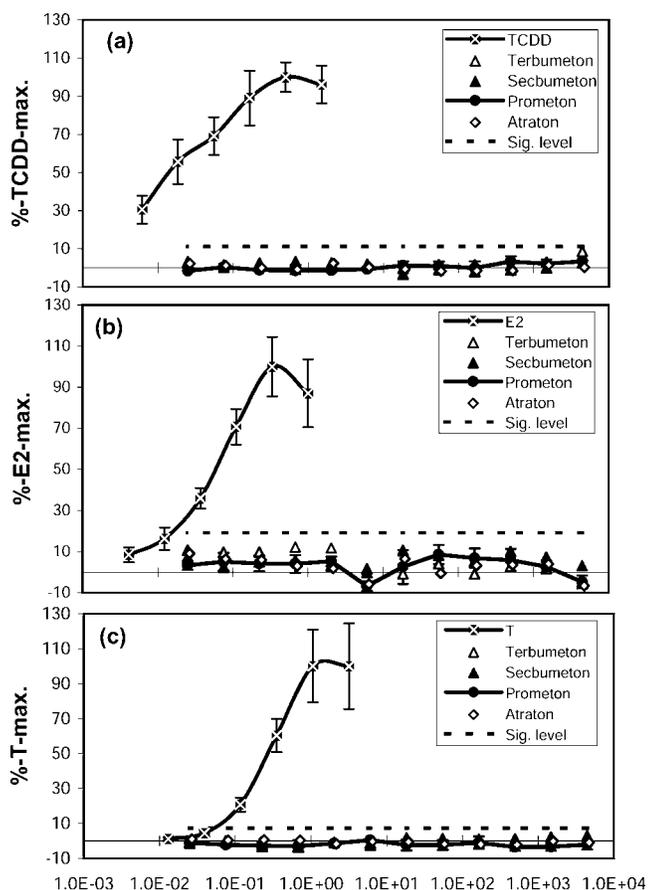
Fig. 1. Luciferase reporter gene induction in (**a**) H4IIE-luc (CALUX), (**b**) MVLN, and (**c**) MDA-kb2 recombinant cell bioassays following exposure to methoxytriazine herbicides and positive control standards (i.e., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] for H4IIE-luc, 17β-estradiol [E2] for MVLN, and testosterone [T] for MDA-kb2). Response magnitude presented as a percentage of the maximum response observed for the standard chemical. The dashed line (Sig. level) equals 3 SD above the mean solvent control response (set to 0%-max.). Error bars = standard deviation.



Fig. 2. Aromatase activity in (**a**) H295R human adenocarcinoma cells and (**b**) fathead minnow brain postmitochondrial supernatant following in vitro exposure to four methoxytriazine herbicides. (**a**) Asterisks indicate statistically significant difference from methanol (MeOH) control (analysis of variance, $p \leq 0.05$). 8Br- = 8-bromo cAMP positive control (100 μM). 4AD = 500 μM 4-androsten-4-ol-3,17-dione negative control. Atraton concentrations were 4.7E+02, 1.6E+02, 5.3E+01, 1.8E+01, 5.8E+00, 1.9E+00, and 6.5E-01 nM. Concentrations listed on the x-axis apply for terbumeton, secbumeton, and prometon. (**b**) Atraton was tested at 11.5 μM; terbumeton, secbumeton, and prometon were tested at 10.8 μM. No statistically significant differences were observed among treatments (analysis of variance, $p > 0.05$).

0.05. All statistical analyses were conducted using SAS® 9.0 (SAS Institute, Cary, NC, USA).

## RESULTS

### *In vitro bioassays*

None of the methoxytriazine herbicides caused significant dioxin-like responses in the H4IIE-luc cell bioassay, estrogen-like responses in the MVLN cell bioassay, or AR- or GR-mediated responses in the MDA-kb2 cell bioassay at the concentrations tested (up to 4.4–4.7 μM or ~1 mg/L; Fig. 1). Based on microscopic inspection, no evidence of cytotoxicity was observed at any of the concentrations of methoxytriazine herbicides tested.

In vitro treatment with prometon concentrations as great as 0.44 μM (0.1 mg/L) did not cause significant induction or inhibition of aromatase activity in H295R human adenocarcinoma cells (Fig. 2a). Terbumeton, secbumeton, and atraton exposure also had no significant effect on aromatase activity in H295R cells. Similarly, at concentrations of 10.5 to 11.8 μM, none of the methoxytriazine herbicides significantly in-
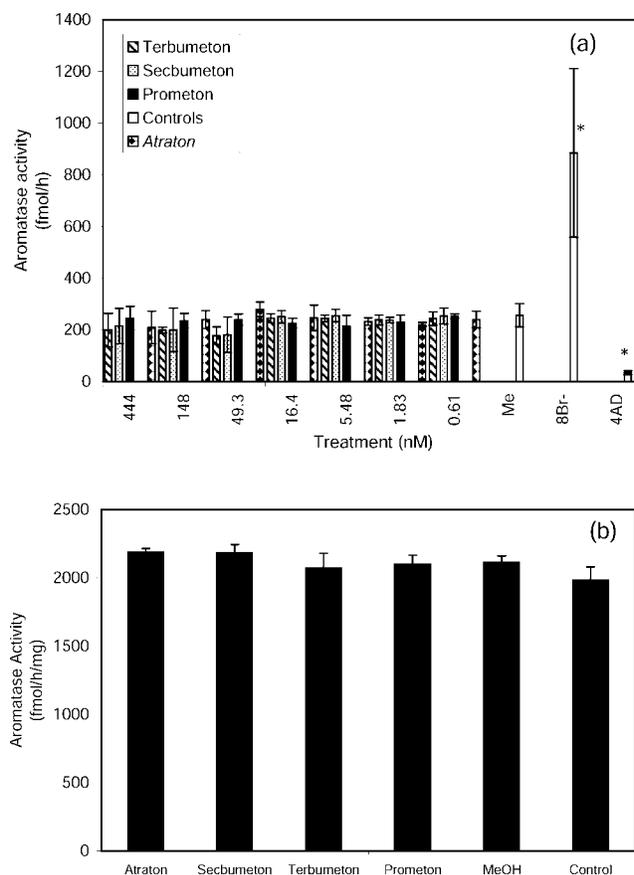
hibited aromatase activity in fathead minnow brain postmitochondrial supernatant following in vitro exposure (Fig. 2b).

### *Fathead minnow reproduction assay*

Prometon water concentrations were generally well maintained over the course of the 21-d reproduction assay. We found no detectable (≤1 μg/L) prometon in the control water. Measured prometon concentrations in the 15-μg/L treatment tanks were, on average, 130% of nominal and varied up to 11% over the course of the exposure (Table 1). Measured prometon concentrations in the higher treatments were, on average, 80 to 92% of nominal and varied less than 5% over the duration of the exposure (Table 1). No significant differences were observed in prometon concentrations among replicate tanks within a treatment.

Despite the lack of differences in waterborne concentrations among replicate exposure tanks, considerable variability was observed in prometon tissue concentrations in the fish. Prometon in the tissue of females exposed to the two highest concentrations varied up to threefold within a treatment, resulting in estimated bioconcentrations factors ranging from 1.3

Table 1. Exposure verification; measured concentrations of prometon in water (μg/L) over the course of the 21-d exposure and prometon concentrations measured in fathead minnow tissue (sans plasma, gonad, brain, and fat pad; ng/g wet wt)[a]

| Nominal | Water concn. measured (mean ± SD) | Male tissue (range; $n = 3$) | Female tissue (range; $n = 3$) | BCF (range) |
|---|---|---|---|---|
| Control | ND (<1 μg/L) | ND (<300) | ND (<300) | NA |
| 15 μg/L | 19.6 ± 2.2 μg/L | ND (<300) | ND (<300 ng/g) | NA |
| 50 μg/L | 46.1 ± 2.3 μg/L | ND (<300) | <300–385 | Up to 7.7 |
| 250 μg/L | 199 ± 11 μg/L | 626–1,047 | 640–2,040 | M: 2.5–4.2 F: 2.6–8.2 |
| 1,250 μg/L | 999 ± 35 μg/L | 1,898–2,207 | 1,624–4,250 | M: 1.5–1.8 F: 1.3–3.4 |

[a] SD = standard deviation; BCF = bioconcentration factor; ND = not detected (i.e., below detection limit: 1 μg/L for water, 300 ng/g for tissue); NA = not applicable; M = male range; F = female range.

up to 8.2 (Table 1). Concentrations detected in male tissues were similar to those detected in females, but less variability was observed among the fish analyzed (Table 1). Based on the consistent water concentrations measured over the course of the study, it would appear that the variable tissue concentrations reflect differences in uptake, biotransformation, and/or elimination rates among individual fish.

Three females exposed to 1,000 μg prometon/L died over the course of the 21-d exposure. Two died on test day 3, while an additional female died on day 5. Following the death on day 5, no other females died, although one female exposed to 20 μg/L was severely battered and in questionable health on test termination on day 21. Fecundity and biochemical data from the four females were excluded from subsequent analyses unless otherwise noted. No males died over the course of the test.

Exposure to prometon did not significantly affect the fecundity of treated fathead minnows relative to that of controls (Fig. 3). This was true whether deceased females were included in or excluded from the analysis. Over the first 7 d of the test, the cumulative eggs spawned per surviving female for the 1,000-μg/L treatment was lower than that observed for the other treatments (Fig. 3). However, over the course of the remaining 14 d, spawning in the 1,000-μg/L treatment group recovered to the point that cumulative fecundity at the end of the test was not significantly different from the controls (Fig.

3). Fertility was not affected by exposure to prometon (data not shown).

Exposure to 20, 200, or 1,000 but not 45 μg prometon/L caused a significant reduction in the fat-pad index (FPI; i.e., wet wt of fat-pad/whole-body wet wt × 100) relative to control males (Fig. 4a). Neither tubercle scores nor tubercle score index (tubercle score divided by whole-body wet wt) in males were significantly affected by prometon exposure (Fig. 4b). Females showed no signs of tubercle development, fat-pad development, or male-like coloration.
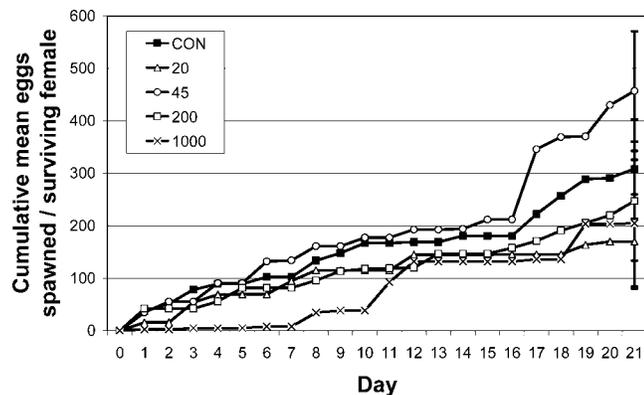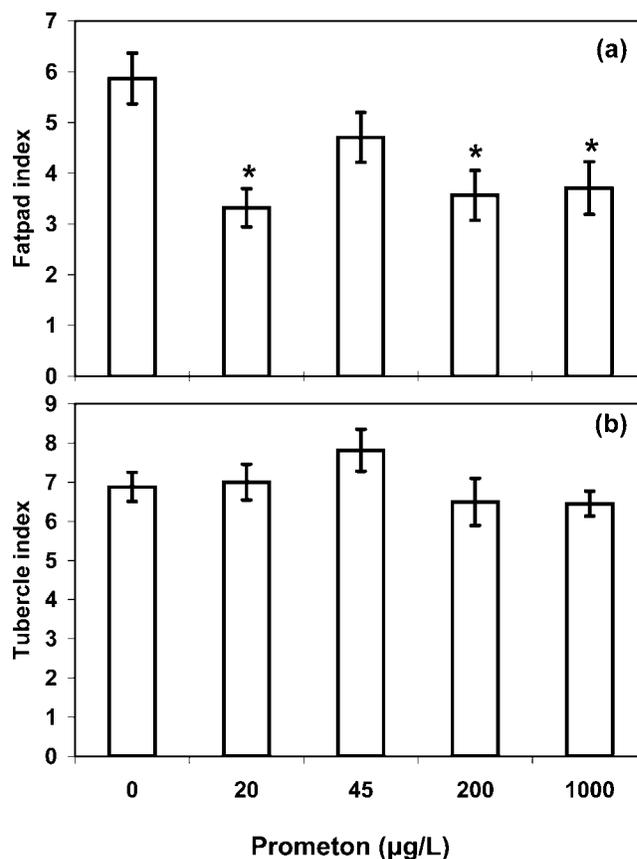


Fig. 3. Effect of exposure to prometon on cumulative fecundity per surviving female during a 21-d fathead minnow reproduction assay. Data are mean values for pairs of control fish (CON; $n = 8$) and animals exposed to prometon ($n = 8$ pairs for all treatments except 1,000 ppm, which had just $n = 5$ pairs with surviving females). Error bars = standard error of the mean.



Fig. 4. Effects of prometon on male fathead minnow secondary sex characteristics as measured by (**a**) fat-pad index (fat-pad wet wt/ whole-body wet wt × 100) and (**b**) tubercle index (tubercle score/ whole-body wet wt). Mean ± standard error. Asterisks indicate statistically significant difference from the control (analysis of variance $p < 0.05$; Dunnett's test at $\alpha = 0.05$).
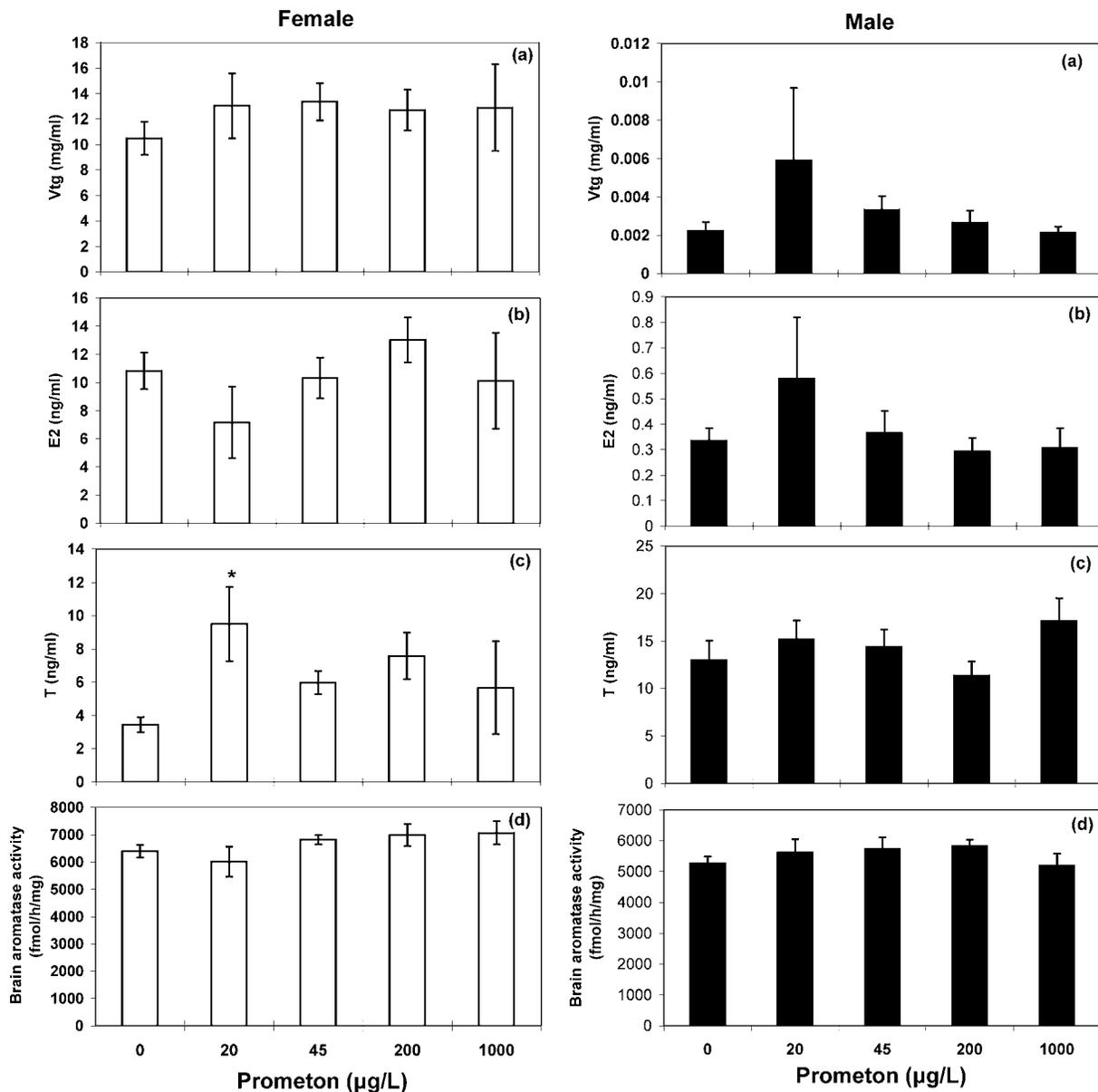
Fig. 5. Effects of exposure to prometon on plasma concentrations of (**a**) vitellogenin (VTG), (**b**) 17β-estradiol (E2), and (**c**) testosterone (T) and on (**d**) brain aromatase activity in female (white bars) and male (black bars) fathead minnows exposed for 21 d. Mean ± standard error shown. Asterisk indicates statistically significant difference from control $p < 0.05$.

Gonadal somatic index (wet wt of gonad/whole-body wet wt × 100) for both males and females was unaffected by prometon. Exposure to 200 or 1000 μg/L of prometon had little effect on gonad histology. Among males, no differences in testicular stage (all fish at stage 5, mature with plentiful sperm [34]) or in numbers or morphology of Sertoli and Leydig cells were observed. Males exposed to prometon appeared to have greater quantities of luminal sperm, but the histological sampling design was not adequate for a quantitative assessment. Most of the ovaries examined were at stage 4 (17/21; late vitellogenic). The ovaries of the remaining four females (two controls and one each from the 200- and 1,000-μg/L exposures) were at stage 3 (early vitellogenic) or 5 (mature oocytes; see Leino et al. [34] for details on ovarian staging). Three of the prometon-treated females (two exposed to 200 μg/L; one exposed to 1,000 μg/L; all at stage 4) appeared to have unusually high numbers of preovulatory atretic follicles.

Another five fish from the prometon-treated groups but none from the controls had ovaries in which no postovulatory follicles were observed.

Exposure to prometon had no significant effect on plasma VTG or steroid concentrations or on brain aromatase activity in males (Fig. 5a to d). The seemingly elevated VTG mean for the 20-μg/L treatment was due to a single male with a plasma VTG concentration of 0.0322 mg/ml, which was approximately 10-fold greater than that observed for most males but still 300- to 500-fold less than concentrations observed for females. A similar profile was observed for plasma E2, with an elevated mean for the 20-μg/L treatment group caused by a single male with a plasma E2 concentration of 2.15 ng/ml, approximately fivefold greater than that observed for most males. The male with elevated E2 was not the same as that with elevated VTG.

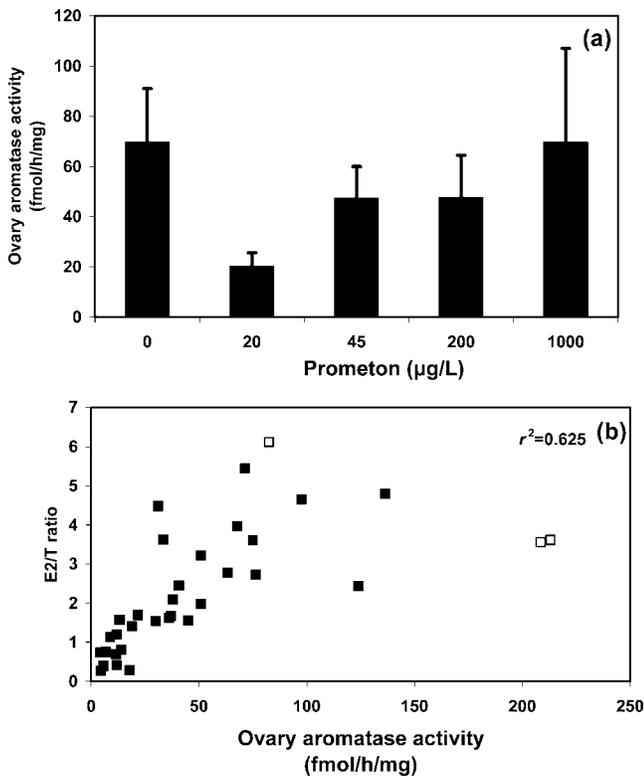As in males, exposure to prometon had no significant effect

Fig. 6. (**a**) Effects of exposure to prometon on ovary aromatase activity in female fathead minnows and (**b**) correlation between ovary aromatase activity and the ratio of 17β-estradiol/testosterone (E2/T) measured in plasma from female fathead minnows (all treatments included). $r^2$ = Pearson correlation coefficient ($p < 0.0001$). White boxes indicate values outside 95% confidence limits.



Fig. 7. Effects of exposure to prometon on female fathead minnow follicle-stimulating hormone–like (FSHβ; white bars) and lutenizing hormone–like β-subunit (LHβ; black bars) mRNA expression in pituitary tissue. Mean ± standard error.

## DISCUSSION

### Prometon in the environment

Prometon has commonly been detected in both regional and nationwide studies of pesticide contamination in groundwater and surface water [4–8]. For example, a National Water Quality Assessment (NAWQA) program study of the occurrence of pesticides in shallow groundwater of the United States found prometon to be the third most frequently detected pesticide [6]. Initial results from the NAWQA study of pesticides in streams from the United States found prometon to be one of the pesticides most commonly detected at urban sites, with a mean annual detection frequency greater than 80% [7]. Despite the fact that prometon has no registered agricultural use, it still ranked as the fourth most frequently detected herbicide in agricultural areas, suggesting extensive use for noncrop applications [1,39]. Prometon concentrations are generally within an order of magnitude of those reported for the chlorotriazines atrazine and simazine, with prometon tending to be somewhat more prominent in urban areas and less common in agricultural regions and areas of low population density [3–5,6–8]. Overall, concentrations of prometon detected in groundwater and surface waters typically range from approximately 1 ng/L (near analytical detection limit) to 10 μg/L, with concentrations as great as 80 μg/L reported in some California, USA, groundwaters [3–5,6–8] (http://infoventures.com/e-hlth/pestcide/prometon.html). As a whole, both surface water and groundwater studies suggest that some degree of human and wildlife exposure to prometon is likely in many regions of the continental United States.

The in vitro and in vivo prometon concentrations tested in this study covered a wide range. The 20- and 45-μg/L in vivo exposure concentrations represent the relatively high end (i.e., low likelihood of occurrence) of the range observed in surface and groundwater. The two greatest in vivo exposure concentrations, 200 and 1000 μg/L, would be expected to be rare in the environment, perhaps only observed as pulse events following direct application adjacent to surface waters or through accidental discharge/spills and/or occupational exposures in humans. Similarly, the in vitro concentrations tested spanned the range from environmentally relevant, with a reasonable possibility of occurrence (e.g., as low as 5.64 ng/L), through concentrations not likely to occur in the environment (e.g., 1 mg/L).

on plasma VTG or E2 concentrations in females (Fig. 5a and b). However, a significant difference among treatments was observed for plasma testosterone in females (Fig. 5c). Mean testosterone concentrations were greater in females exposed to 20 μg/L prometon than in the controls (Fig. 5c). As in males, brain aromatase activity was not significantly affected (Fig. 5d), but females exposed to 20 μg prometon/L had mean ovary aromatase activity that was more than fourfold less than that in control fish (Fig. 6a). While this was not statistically significant (Kruskal–Wallis $p = 0.0827$), changes in other endpoints in the females exposed to 20 μg/L were consistent with inhibition of ovary aromatase activity. For example, exposure to 20 μg/L of prometon caused not only a significant increase in plasma testosterone but also a 33% (albeit nonsignificant) decrease in plasma E2 (Fig. 5b and c). Across all treatments, a significant negative correlation was observed between ovary aromatase activity and both testosterone concentration and GSI as well as a significant positive correlation between ovary aromatase activity and both E2 concentration and E2/testosterone ratio (as shown in Fig. 6b).

Finally, transcript levels of pituitary FSHβ for females exposed to 45 and 1,000 μg/L of prometon were approximately 2.3-fold and 1.6-fold greater, respectively, than mean control FSHβ transcript levels. Because of the considerable variability among individuals, these differences were not significant (Fig. 7). Mean LHβ transcript levels were somewhat less variable than those of FSHβ within treatments and, similarly, were not significantly affected by prometon exposure (Fig. 7).
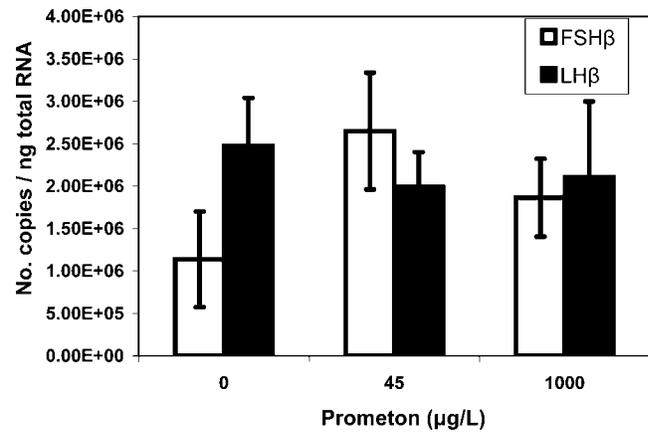
*Prometon as a potential EDC*

Prometon is structurally similar to the widely used agricultural herbicide atrazine, which has recently come under scrutiny for its potential to adversely affect development and reproduction in vertebrates through effects on endocrine function. Exposure to comparatively high doses (100–300 mg/kg/d) of atrazine has been linked to a wide variety of endocrine-related responses in rodents, including early onset of mammary tumors [11,12], premature aging of the reproductive system [11,12], suppression of estrous cycling [13], suppressed secretion of gonadotropins [40], disrupted ovarian function (≥75 mg/kg/d) [15], and delayed puberty in male Wistar rats (≥12.5 mg/kg/d) [16]. In wildlife, changes in aromatase activity [14], alterations in response to pheromones [17], histological and morphological changes [18–20], and altered levels of plasma steroids [19] have all been associated with exposure to atrazine. Although consensus has not been reached regarding potential toxic mechanisms of action, studies with the herbicide are suggestive enough to warrant concern that other triazines, such as prometon, may have unanticipated endocrine-related effects on humans and/or wildlife.

In vitro, atrazine has no appreciable affinity for the ER and has not been shown to consistently induce ER-mediated responses [41–43]. Atrazine has, however, been shown to cause concentration-dependent increases in aromatase activity in H295R adrenocortical carcinoma cells [29,43]. In JEG-3 human choriocarcinoma cells, atrazine did not induce aromatase activity, but it did increase cytochrome P4501A activity, suggesting that the herbicide might affect the metabolism of steroid hormones in vivo and/or interact with AhR-mediated toxicity pathways [44]. In this study, prometon as well as secbumeton, terbumeton, and atraton were evaluated using a variety of cell-based in vitro bioassays. The methoxytriazines did not interact with the AhR, ER, or AR/GR to induce reporter gene expression in H4IIE-luc cells, MVLN cells, or MDA-kb2 cells, respectively (Fig. 1). In contrast to atrazine, the methoxytriazines did not induce or inhibit aromatase activity in H295R cells (Fig. 2a). Similarly, the methoxytriazines did not inhibit aromatase activity in fathead minnow ovary postmitochondrial supernatants following in vitro exposure (Fig. 2b). Overall, the mechanism-specific in vitro screening assays used in this study provided no evidence to suggest that methoxytriazines were likely to act as EDCs through direct interactions with nuclear steroid hormone receptors, the AhR, or aromatase.

In vivo, it was indicated that high concentrations of prometon (1,000 μg/L) adversely affected fish, at least early in the assay. During the first 7 d of the test, three females exposed to 1,000 μg prometon/L died, and cumulative mean eggs spawned per surviving female was less than that for the other groups (Fig. 3). The reproduction assay is not designed for detecting treatment-related effects on survival, so it was uncertain whether the female mortalities observed were related to the prometon treatment as opposed to some other, more random, secondary factor, such as male aggression. Nonetheless, the mortalities and poor fecundity suggest a treatment-related effect. Further, the large variability observed in tissue concentrations of prometon, particularly for females (Table 1), suggests varying capacities to accumulate and/or eliminate the herbicide, which may partially explain the differential susceptibility of individuals. In particular, differences in the spawning activity of various pairs may have contributed to the variability in tissue concentrations and would readily explain why the

tissue concentrations in females were more variable. With that said, a clear relationship between overall fecundity and tissue concentration was not detected. Whatever the case, during the last two weeks of the test, surviving animals appeared to have compensated for prometon-related stress and were spawning in a manner that was not significantly different from that of controls (Fig. 3). Overall, prometon concentrations of 200 μg/L or less appeared to have no effect on survival or fecundity, and even a concentration approaching (or causing) direct toxicity (i.e., 1,000 μg/L) appeared to have little adverse impact on long-term reproductive output of surviving fish. Correspondingly, atrazine concentrations as great as 224 μg/L did not significantly affect reproductive endpoints, including fecundity, eggs per female per day, number of spawns, or eggs per spawn, in fathead minnows exposed for 21 d [45,46].

In the amphibian *Xenopus laevis*, exposure to relatively low concentrations of atrazine (<25 μg/L) have been linked to a reduction in larynx size [19]. This has been interpreted as a demasculinizing effect since larynx growth in *X. laevis* is androgen dependent [19,47]. In fathead minnows, fat-pad and breeding tubercle development have been shown to be androgen dependent [48]. Exposure to the androgenic growth promoter 17β-trenbolone, for example, has been shown to induce tubercle and fat-pad development in female fathead minnows [49]. Three of the four prometon concentrations tested caused a reduction in FPI in males (Fig. 4a), suggesting a demasculinizing effect. However, no significant effects were observed on tubercle index. Bringolf et al. [45] found that atrazine exposure had no effect on numbers of breeding tubercles, but they did not measure fat-pad weights in their study. Thorpe et al. observed a significant reduction in fat-pad weight following exposure to the antiandrogen linuron (Karen L. Thorpe, University of Exeter, Exeter, UK, personal communication). To date, data are not sufficient to ascertain whether effects on FPI might be a more sensitive response to EDCs than effects on tubercles. Regardless, the effect of FPI observed in this study is suggestive of a potential demasculinizing effect of prometon, although clear concentration dependence of the response was not evident.

In addition to effects on the larynx, exposure to low concentrations of atrazine (<25 μg/L) has been linked to a variety of morphological and histological abnormalities in developing gonads of frogs [18–20]. Atrazine exposure has not been reported to cause similar gonadal abnormalities in reproductively mature fathead minnows [45,46]. In this study as well, no evidence was observed for mixed sex or intersex gonad morphology or histology, as has been reported for developing amphibians. However, such effects would be unlikely in mature fish that had already completed sexual differentiation. The limited histological analyses conducted as part of this study suggested that prometon may have had some subtle effects on gonad histology in the adult fish. However, no compelling evidence suggested that 21 d of exposure to prometon resulted in abnormalities in gonad morphology or histology severe enough to cause adverse effects on reproduction (i.e., reduced fertility) over a relatively short term.

Exposure to prometon had no apparent effect on plasma concentrations of VTG, E2, or testosterone in male fathead minnows. Thus, the biochemical endpoints revealed no clear correlation with the observed effect on FPI. Additionally, in vitro bioassay results provided no evidence to suggest that direct interactions between prometon and steroid hormone receptors and/or aromatase would explain the FPI effect, al-

though they do not rule out the possibility that a metabolite of prometon may have interacted with those biomolecules. Without greater understanding of the molecular biology and endocrinology controlling fathead minnow dorsal fat-pad and tubercle development and/or the potential effects of prometon metabolites, it is difficult to assess the mechanistic basis and/or significance of prometon's apparent demasculinizing effect.

Atrazine exposure has been linked to abnormal aromatase activity in juvenile alligators [14], and Hayes et al. [19] proposed induction of aromatase activity as a potential mechanism of action for effects observed in *X. laevis*. Furthermore, a range of environmental contaminants, including different fungicides, insecticides, chlorinated organics, and polycyclic aromatic hydrocarbons, have been shown to inhibit aromatase activity, at least in vitro [29,30,50]. In previous studies with the fathead minnow, fadrozole, prochloraz, and perfluorooctanesulfonate have all been shown to affect brain aromatase activity [31,33,37]. Prometon exposure had no significant effect on brain aromatase activity in the species (Fig. 5d). Elevated plasma testosterone concentrations in females exposed to 20 µg prometon/L would be consistent with a potential effect on ovary aromatase activity. Additionally, E2/testosterone ratios were significantly correlated with ovary aromatase activity within individual females, across all treatments (Fig. 6b). However, no corresponding, statistically significant effects were observed on either ovary aromatase activity or plasma E2 concentrations to corroborate a potential effect on aromatase.

Neither the effect on testosterone concentrations in females nor the effect on FPI in males exhibited classic monotonic concentration dependence. However, a lack of monotonic concentration–response relationships is both theoretically plausible in autoregulatory biological systems [51,52] and has been previously reported in vivo in fathead minnow studies with potent endocrine disruptors. For example, exposure to fadrozole, a potent competitive inhibitor of aromatase, for 7 d resulted in an inverted U–shaped concentration response for ovary aromatase activity characterized by an increase in activity at lower concentrations of fadrozole followed by a decrease back to control levels at greater concentrations [37]. In that study, the contrast between in vivo induction of ovary aromatase and fadrozole's concentration-dependent inhibition of ovary aromatase activity in vitro appeared to be explained by a significant increase in aromatase gene expression in vivo, as indicated by significant increases in fathead minnow CYP19a (the ovary isoform of aromatase) transcript levels in ovary [37]. In another study with the fathead minnow, 17β-trenbolone was shown to produce U-shaped in vivo concentration responses for a number of endpoints in females, including T, E2, and VTG [49]. Thus, deviation from strict dose dependence is not unprecedented and may result from compensatory endocrine and/or physiological responses to a stressor.

In vitro assays conducted as part of this study provided no evidence for direct interaction between prometon and the AR or aromatase. This supports the idea that the non–concentration-dependent effects of prometon on male FPI and female testosterone were probably indirect. It also raises the possibility that metabolites of prometon may be responsible for some of the effects observed in vivo. Cooper et al. [15] reported that exposure to atrazine could suppress gonadotropin release from the pituitary of rodents. This suggested at least one potential means by which prometon might indirectly influence testosterone concentrations and/or secondary sex characteristics. Although methods to measure fathead minnow go-

nadotropins in plasma were not available for this study, we recently developed quantitative polymerase chain reaction assays to examine the expression of fathead minnow LHβ and FSHβ transcript levels in fathead minnow pituitary tissues [38]. Prometon did not significantly affect LHβ or FSHβ pituitary transcript levels in female fathead minnows exposed to 45 or 1,000 µg/L, although the FSHβ expression was quite variable among fish (Fig. 7). Overall, the range of LHβ and FSHβ expression levels observed in this study was similar to "baseline" values observed for reproductively active, nonexposed female fathead minnows [38]. Thus, based on transcript levels, no indication was seen that prometon affected pituitary gonadotropin release in female fathead minnow exposed to 45 or 1,000 µg prometon/L. Unfortunately, pituitary RNA from fish exposed to 20 µg/L was unavailable for analysis of LHβ and FSHβ transcript levels.

## CONCLUSION

The weight of evidence from this study indicates that prometon is not a potent EDC, at least in fish. In a series of in vitro bioassays relevant to common receptor-mediated mechanisms of action as well as a key step in steroidogenesis (aromatase), prometon produced no direct effects. In the fathead minnow reproduction assay, prometon had only a modest effect on fecundity early in the 21-d exposure and only at the greatest test concentration evaluated, which approaches concentrations previously reported to be acutely toxic to aquatic organisms. Furthermore, most of the diagnostic supplementary in vivo endpoints measured in this study provided little direct evidence for prometon effects on endocrine-mediated toxicity pathways in the fish.

While the results of this study do not indicate that prometon acts directly through some of the most commonly examined mechanisms of EDC action (i.e., steroid receptor agonism or antagonism or direct effects on aromatase) or through effects on gonadotropin β-subunit mRNA expression, evidence suggested a demasculinizing effect in males as well as increased testosterone production in females. It seems likely that these responses were due to indirect effects of prometon mediated through a currently undefined toxicant mechanism of action, a compensatory response, and/or the actions of one of more of prometon's biotransformation products. However, over the course of the 21-d test, the apparent effects of prometon on male secondary sex characteristics and female testosterone concentrations did not translate into a significant reduction in reproductive success. A greater understanding of fathead minnow endocrine physiology and the molecular, biochemical, and physiological function of the fathead minnow brain–pituitary–gonadal axis as an integrated and dynamic organ system is needed to fully understand the potential relevance of such effects for hazard assessment.

products or trade names does not indicate endorsement by the federal government. Conclusions drawn in this study neither constitute nor necessarily reflect U.S. EPA regulatory policy concerning the test chemicals.

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