Abstract—Temporal and dose–response relationships of vitellogenin (VTG) mRNA induction and subsequent plasma VTG accumulation were established for sheepshead minnows (Cyprinodon variegatus) treated with p-nonylphenol (an alkylphenol) and the organochlorine pesticides methoxychlor and endosulfan. Thirty-two adult male fish per treatment were continuously exposed to measured concentrations of 0.64, 5.4, 11.8, 23.3, and 42.7 μg/L p-nonylphenol; 1.1, 2.5, 5.6, 12.1, and 18.4 μg/L methoxychlor; and in two separate tests, 15.9, 36.3, 68.8, 162, 277, 403, 590, and 788 ng/L endosulfan using an intermittent flow-through dosing apparatus. Separate triethylene glycol (50 μL/L) and 17β-estradiol (65.1 ng/L) treatments served as the negative and positive controls, respectively. Four fish were randomly sampled from each test concentration on days 2, 5, 13, 21, 35, and 42 of exposure, and changes in VTG mRNA synthesis and serum VTG levels were measured. Overall, fish exposed to p-nonylphenol or methoxychlor demonstrated a rapid, dose-dependent synthesis of VTG mRNA up to day 5 of exposure, followed by a relatively constant dose-dependent expression through day 42. Both chemicals showed a dose-dependent increase in plasma VTG over the entire time course of exposure, with significantly elevated VTG levels by the fifth day of exposure to p-nonylphenol at concentrations of ≥5.4 μg/L and to methoxychlor at concentrations of ≥2.5 μg/L. Exposure to 0.64 μg/L p-nonylphenol resulted in highly variable plasma VTG levels of less than 6 mg/ml. Exposures with endosulfan failed to induce measurable levels of either hepatic VTG mRNA or serum VTG at the chemical concentrations tested. Our results demonstrate that the sheepshead minnow bioassay is a suitable estuarine/marine teleost model for in vivo screening of potentially estrogenic substances.

Keywords—Fish Vitellogenin p-Nonylphenol Methoxychlor Endosulfan

INTRODUCTION

During the past decade, considerable evidence has been reported describing the estrogenic capability of certain natural and anthropogenic chemicals to modulate normal endocrine processes in oviparous vertebrates. Birds [1,2], amphibians [3], reptiles [4,5], and fish [6–8] display estrogen-like responses to environmental contaminants such as natural and synthetic estrogens [9–11], organochlorine pesticides [12,13], phthalates [9], and nonionic surfactants [14,15]. Concern over the putative relationship between environmental contaminants and alteration of endocrine functions in wildlife populations has prompted U.S. Congressional requirements for development of diagnostic procedures to identify chemicals with endocrine-modulating potential [16,17]. Development of diagnostic procedures specifically targeted to investigate impairment of endocrine processes in whole organisms at multiple cellular and physiological levels should allow development of predictive exposure–effect models in support of environmental risk assessment and management activities.

In vivo assays have been developed with teleost fish using intraperitoneal injections and, more recently, aqueous routes of exposure that incorporate the appearance of vitellogenin (VTG) in the plasma of juvenile or male fish as an indicator of estrogenic activity [7,9–12,18,19]. The egg yolk precursor protein VTG is normally found in the serum of oviparous female fish under the influence of circulating levels of estradiol. Male fish possess the VTG gene, but with little or no circulating estradiol, they do not normally express VTG in the serum. However, if exposed to an exogenous estrogen, male fish are capable of VTG synthesis equivalent to that of mature female fish [20,21].

In a previous study [11], we described an in vivo aqueous exposure system and procedures for measuring hepatic VTG mRNA synthesis and serum VTG levels in male sheepshead minnows (Cyprinodon variegatus), and we established the relative potency of 17β-estradiol and two pharmaceutical estrogens, ethynyl estradiol and diethylstilbestrol, in this estuarine fish species. In the present study, we describe exposures conducted with three xenobiotic compounds—p-nonylphenol, methoxychlor, and endosulfan—that have demonstrated varying degrees of estrogenic potency in assorted in vitro assays. Para-nonylphenol is the primary microbial degradation product of alkylphenol ethoxylates [22]. Alkylphenol ethoxylates are nonionic surfactants used extensively in manufacturing and are components of detergents, emulsifiers, paints, wetting agents, cosmetics, herbicides, and as spermicides in topical contraceptives [7]. Freshwater, estuarine, and marine habitats contaminated with alkylphenols discharged from industrial and sewage treatment plants have been extensively documented in

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Michael J. Hemmer,*‡ Becky L. Hemmer,‡ Chris J. Bowman,§ Kevin J. Kroll,‡ Leroy C. Folmar,‡ Dragoslav Marovich,‡ Marilynn D. Hoglund,‡ and Nancy D. Denslow‡

†U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, Florida 32561
‡Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610, USA

(Received 15 February 2000; Accepted 5 June 2000)

Printed in the USA 0730-7268/01 $9.00 + .00

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both North America and Europe [23–25]. Para-nonylphenol is mildly lipophilic, with reported bioconcentration factors for various fish species ranging from 0.9 to 1.250 [26]. The estrogenic nature of p-nonylphenol has been demonstrated by numerous in vitro studies [27–29], as has its capacity for in vivo induction of VTG in fish [14,30,31]. Methoxychlor and endosulfan, both of which are generally classified as organochlorine pesticides, are used to control a large variety of insect pests on vegetable, fruit, cereal, and forage crops. Methoxychlor, a chlorinated ethane insecticide, is unique, because the parent compound is not estrogenic but must undergo metabolic conversion to active demethylated metabolites before binding to the estrogen receptor [32,33]. Endosulfan, a chlorinated cyclodiene insecticide, is available commercially as a mixture of two endosulfan isomers, α and β, which are typically supplied at the ratio of 65 to 70% α and 30 to 35% β. In vitro assays indicate endosulfan is capable of weakly binding to the mammalian and trout estrogen receptor and can also induce a dose-related proliferation of estrogen-dependent MCF-7 cells [27,34–36]. In this study, we examined the temporal and dose–response relationships associated with hepatic VTG mRNA induction and subsequent expression of VTG in serum of sheepshead minnows continuously exposed to p-nonylphenol, methoxychlor, or endosulfan.

MATERIALS AND METHODS

Fish collection and exposure chemicals

Adult male sheepshead minnows were collected by bag seine from Big Sabine Point, Santa Rosa Sound, Florida, USA, and immediately transported in aerated coolers to the U.S. Environmental Protection Agency’s Gulf Ecology Division in Gulf Breeze, Florida. The fish were acclimated in 60-L tanks receiving a continuous flow of aerated, 20 ± 2 parts per thousand (ppt), filtered seawater at 25 ± 1°C for a minimum of two weeks before being used in exposures. Fish were maintained under a 16:8h light:dark photoperiod and fed Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA) to satiation at least twice daily. Test chemicals were obtained from the following sources: p-nonylphenol (96.4% purity) from Schenectady International (Schenectady, NY, USA), methoxychlor (99.0% purity) and endosulfan (α = 99.5% and β = 98.0% purity) from Chem Service (West Chester, PA, USA), and 17β-estradiol (98.0% purity) from Sigma (St Louis, MO, USA). Chemical stock solutions at the appropriate concentrations were prepared using reagent-grade triethylene glycol (TEG; Spectrum, Gardena, CA, USA) as the solvent.

Exposure conditions

Sheepshead minnows were exposed to p-nonylphenol, methoxychlor, or endosulfan in 135-L glass aquaria using a six-cell dosing apparatus supplying 1 L of test solution to each of five test concentrations, a positive control, and a solvent control at 20 cycles/h. Chemical stock solutions were infused at 50 µL/cycle into the apparatus mixing chambers using three dual-channel Hamilton Microlab 500B dispensers fitted with 100-µL Hamilton syringes (Hamilton Instruments, Reno, NV, USA).

Chemical exposures with p-nonylphenol and methoxychlor were conducted concurrently from August through October 1997 using separate dosing systems at nominal exposure concentrations of 1, 10, 20, 40, and 80 µg/L p-nonylphenol and 1.5, 3, 6, 12, and 24 µg/L methoxychlor. 17β-Estradiol at a nominal concentration of 100 ng/L and TEG at 50 µL/L served as positive and solvent controls, respectively. Two separate exposures were conducted with endosulfan, the first using a low concentration range of 25, 50, 100, and 200 ng/L from May through July 1998 and the second conducted at 300, 600, 900, and 1200 ng/L from November through December 1998. Future references to exposure concentrations refer to the average concentration measured in water samples from the aquaria. For each chemical examined, 208 adult male fish were randomly divided among the treatments, with each chemical exposure aquaria and estradiol positive control receiving 32 fish and the TEG control receiving 16 fish. Sizes for experimental fish were as follows (mean ± standard deviation): for p-nonylphenol, 5.63 ± 0.41 cm and 3.53 ± 0.85 g; for methoxychlor, 5.61 ± 0.41 cm and 3.49 ± 0.86 g; for endosulfan test one, 5.84 ± 0.43 cm and 3.90 ± 0.99 g; and for endosulfan test two, 5.70 ± 0.40 cm and 3.84 ± 0.88 g. Exposure tanks were maintained in a temperature-controlled water bath (25 ± 1°C) with a constant photoperiod of 16:8h light:dark.

Sample collection

Four fish were randomly sampled from each exposure concentration at 0, 2, 5, 13, 21, 35, and 42 d of exposure, or until all fish in that group were sacrificed. Four TEG control fish were sampled on days 0, 2, 21, and the last exposure day. Total length and wet weight (blotted) were recorded for each fish sampled and for mortalities occurring during the exposures. Blood was sampled from each fish by severing the tail at the caudal peduncle with a razor blade and collecting the blood in a heparinized capillary tube. The tubes were centrifuged for 3 min at 13,700 g, and the plasma was aspirated and transferred to a 1.5-ml Eppendorf microfuge tube. The tubes were then quickly frozen in a −70°C freezer. After exsanguination, each fish was split ventrally, and the liver was removed and placed in a 2-ml cryovial. The vials were flash frozen in liquid nitrogen, then transferred to a −70°C freezer for storage. At the end of each exposure, the plasma and liver samples were shipped on dry ice to the University of Florida at Gainesville for VTG analysis.

Chemical analysis

All chemical stock solutions were analyzed before the start of exposures. Water samples were collected from the exposure tanks 96 h after the start of chemical flow, 24 h after introduction of the fish, and once weekly thereafter to determine the exposure concentrations. One-liter water samples were siphoned from the mid-water column of each exposure aquaria, and 500 ml were taken from the TEG and estradiol control aquaria. Water samples collected from the TEG aquaria were spiked with 25 µl of the chemical stock solutions diluted to equal the exposure concentrations. Hewlett-Packard (HP) Chemstation software (Palo Alto, CA, USA) was used to control gas chromatograph (GC) parameters and to collect and process the data.

p-Nonylphenol. Seawater samples containing p-nonylphenol were acidified to a pH of approximately 3 with 3 ml of 1.8 N H₂SO₄ and extracted with 25 ml of hexane on a magnetic stir plate for 45 min. Depending on the exposure concentration, 5 to 15 ml of the hexane extract was concentrated to 1.0 ml under a gentle stream of nitrogen. Analyses were performed using a HP model 5890 series II GC equipped with a fused-silica nonpolar capillary column (HP-5, 25 × 0.32 mm i.d., 0.17-µm film thickness) and flame-ionization detector. Helium
was used as the carrier gas. The temperature program for the GC was 250°C for the injection port, 300°C for the detector, and 50°C for 1 min for the oven, then increasing at 8°C/min to 220°C and then at 20°C/min to 275°C for 4 min. The mean percentage recovery and standard deviation of eight TEG control samples spiked at 2.1 μg/L p-nonyphenol was 89 ± 11.7%.

**Methoxychlor.** Sample volumes used to determine methoxychlor residues in seawater samples were 1 L for the lowest nominal exposure concentration of 1.5 μg/L and 100 ml for the remaining exposure concentrations. Samples were extracted twice with 25 ml of hexane, and the two extracts were combined and concentrated under a gentle stream of nitrogen to a final volume of 10 ml. A HP model 6890 GC containing a methyl siloxane capillary column (HP-1, 30 × 0.25 mm i.d., 0.25-μm film thickness) equipped with an electron-capture detector was used to measure exposure concentrations. Helium was used as the carrier gas. The temperature program for the GC was 290°C for the injection port, 310°C for the detector (with P-10 gas at an anode flow of 40 ml/min), and 100°C for 1 min for the oven, then increasing at 15°C/min to 280°C for 2 min. The mean percentage recovery and standard deviation of eight TEG control samples spiked at 2.6 μg/L methoxychlor was 103 ± 6.3%.

**Endosulfan.** Seawater samples containing endosulfan were adjusted in volume to 475 ml and extracted with 25 ml of hexane on a stir plate for 45 min. Hexane extracts were not concentrated before analysis. A HP model 6890 dual-column GC (front column: HP-1 [methyl siloxane], 30 × 0.25 mm i.d., 0.25-μm film thickness; back column: HP-1701 [14% cyanopropyl phenyl methyl], 30 × 0.25 mm i.d., 0.25-μm film thickness) equipped with an electron-capture detector was used to measure exposure concentrations. Helium was used as the carrier gas. The temperature program for the GC was 290°C for the injection port, 310°C for the detector (with P-10 gas at an anode flow of 40 ml/min), and 50°C for 1 min for the oven, increasing at 25°C/min to 150°C and then at 2.5°C/min to 280°C for 3 min. The mean percentage recovery and standard deviation of six TEG control samples spiked at 9.5 μg/L endosulfan was 117 ± 6%.

**17β-Estradiol.** Seawater samples containing estradiol were filtered through E-18 solid-phase extraction tubes (Supelco, Bellefonte, PA, USA) and eluted from the column with 5.0 ml of methanol (MeOH). Sample volume was reduced under nitrogen to 0.5 ml in a N-Vap (Organomation, Northborough, MA, USA) at 55 to 60°C and evaporated to dryness using a Centri-Vap (Savant Instruments, Farmingdale, NY, USA). After evaporation, samples were stored at -20°C until analyzed by radioimmunoassay. Samples and standards were performed in triplicate and conducted in a single run for each series of exposures. Standard curves were prepared using radioimmunoassay estradiol in assay buffer (60 mM H3BO3, and 1% bovine serum albumin) at concentrations of 6.25, 12.5, 25, 50, 100, and 200 pg/tube. Labeled estradiol was reconstituted using 1 ml of borate buffer (65 mM H3BO3, [pH 8.0]) and 1% MeOH solution. Assay tubes containing 200 μl of estradiol antisera (E26-47; Endocrine Sciences, Calabasas, CA, USA), 100 μl of tritiated estradiol at 12,000 cpm/ml (TRK587; Amersham International, Buckinghamshire, England), 100 μl of borate buffer, 100 μl of assay buffer, and 100 μl of reconstituted sample or estradiol standard were vortexed and then incubated for 24 h at 4°C. Separation of bound and free estradiol was achieved by adding 500 μl of 5% charcoal/0.5% dextran in phosphate-buffered saline (PBS; 37 mM NaH2PO4, 94 mM Na2HPO4, 150 mM NaCl, and 0.025% NaN3 [pH 7.5]), fol-

**Analysis of hepatic VTG mRNA**

**Total RNA isolation and in vitro synthesis of standards.** Analysis of VTG mRNA was accomplished using the methods described by Bowman and Denslow [38]. Individual liver tissues were processed for RNA using RNeasy mini-spin columns (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendations, then measured spectrophotometrically at 260 and 280 nm. The 260-nm reading was used to estimate the total RNA recovered from the isolation. The 260:280-nm ratio, as well as a 1% agarose-formaldehyde gel stained with ethidium bromide, was used to verify the quality of the RNA. To quantitate VTG mRNA, it was necessary to transcribe complimentary sheepshead minnow VTG RNA (cRNA) from the same cloned sheepshead minnow VTG cDNA fragment used to make the cDNA probe for hybridization (see below). The in vitro transcription reactions were performed with the cloned sheepshead minnow VTG fragment as the template, T7 RNA polymerase, and Ambion’s Megascript kit (Austin, TX, USA) according to the manufacturer’s directions. The VTG cRNA was analyzed for purity by electrophoresis and quantitated by spectrophotometry.

**Slot blot.** The synthesized cRNAs and 12 μg of sample RNA were denatured in slot blot denaturing solution (50% formamide, 7% formaldehyde, 0.15 M NaCl, and 0.015 M Na2C6H5O7 [pH 7.0]) for 15 min at 68°C and then loaded onto a Biodyne B nylon membrane (Life Technologies, Rockville, MD, USA) using a slot blot apparatus (Schleicher and Schuell, Keene, NH, USA). Sheepshead minnow VTG cRNA standards ranging from 0.01 to 100 ng were applied to all blots. Once the samples and standards were loaded, the RNA was ultraviolet-crosslinked in a Stratalinker 1800 (Stratagene, La Jolla, CA, USA), then stained with methylene blue to ensure even loading of the nucleic acid [39].

**cDNA probes.** The cDNA probes for VTG and β-actin were made from the plasmids (pSHMVTG2a) and (pSHMβactA1), respectively. The insert corresponding to the fragment of sheepshead minnow VTG or β-actin was cut from the plasmid vector pGEM-T Easy (Promega, Madison, WI, USA) using the EcoRI restriction enzyme. The fragment was purified by gel electrophoresis and served as a template for synthesis of the cRNA standards and [α-35]P-labeled cDNA probes. The cDNA probes were made using Ambion’s StripEZ kit according to the manufacturer’s instructions. The probes were then purified using TE-Midi Select-D, G50 columns (5 Prime-3 Prime, Boulder, CO, USA).

**Slot blot hybridization with VTG and β-actin cDNA probes.** Nylon membranes containing the slot blotted samples were prehybridized in a glass cylinder using a Techne Hybridiser oven (Techne, Princeton, NJ, USA) with ExpressHyb buffer (Clontech, Palo Alto, CA, USA) for 30 min at 68°C. The membrane was then incubated at 68°C for 1 h in fresh hybridization buffer containing 3 × 105 dpm VTG probe/ml.
Vitellogenin induction in sheepshead minnow

After incubation, the nylon membranes were washed twice with 2× standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) for 20 min at 25°C, then twice with 0.1× SSC and 0.1% SDS for 30 min at 50°C. After washing, the nylon membranes were wrapped in Saran Wrap and exposed to BioMax MR x-ray film (Eastman Kodak, Rochester, NY, USA). The exposed data were collected on a phosphor screen and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). The VTG cDNA data were used to generate a standard curve for each blot to determine the amount of VTG mRNA (ng/μg total mRNA per sample). The nylon membranes were then stripped using Ambion’s Strip E2 kit according to manufacturer’s instructions. The stripped blots were reprobed using 3 × 10^5 dpm of β-actin probe/ml under the same conditions described above for VTG hybridization. The β-actin mRNA data were collected and analyzed by phosphor imaging. The VTG mRNA levels for each individual sample were then normalized using the derived β-actin levels. An autoradiogram of a typical slot blot membrane used to quantitate VTG mRNA is presented in Figure 1.

Analysis of plasma VTG

Vitellogenin standard preparation. Vitellogenin was purified from the serum of estradiol-injected male fish by anion-exchange chromatography on a POROS 20HQ column (Perseptive Biosystems, Framingham, MA, USA) equilibrated with 20 mM bis-Tris propane and 150 mM NaCl (pH 9.0) [40,41]. Vitellogenin was eluted using a linear salt gradient (150–1,000 mM). The VTG fractions were pooled and adjusted to pH 7.0 with 500 mM bis-Tris propane (pH 6.5), then treated with aprotinin (10 kallikrein inhibitor units (KIU)/ml) and sodium azide (0.02%). Purified samples were aliquoted, mixed with an equal volume of glycerol, and stored at −80°C. Samples were then thawed, used once as standards in the Western blot analyses or enzyme-linked immunosorbent assays (ELISA), and discarded.

Quantitation of VTG by ELISA. Plasma VTG was quantitated by direct ELISA using an avidin-biotin complex kit (Pierce, Rockford, IL, USA). Sheepshead minnow VTG purified by anion exchange was used as a standard [11]. Samples, blanks, and standards (0, 0.01, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml) were diluted with PBS and sodium azide (PBSZ; 10 mM NaH2PO4, 150 mM NaCl, and 0.02% NaN3 [pH 7.2] containing 10 KIU/ml aprotinin). A significant plasma protein effect depresses the absorbance at lower plasma dilutions (M. Chow and N. Denslow, unpublished data). To minimize this effect, VTG-free male plasma was added back to the standards at the same concentration as the sample dilutions. The samples were loaded into a 96-well microtiter plate (50 μl/well) and incubated overnight in a humidified chamber at 4°C. The next day, the plate was washed four times with Tris-buffered saline with Tween (10 mM Tris, 150 mM NaCl, and 0.05% Tween) and blocked with 360 μl/well of 1.0% bovine serum albumin in Tris-buffered saline with Tween for 2 h at room temperature. The plates were rewashed with PBSZ and 0.05% Tween (PBST), then incubated overnight at 4°C with 50 μl/well of primary antibody diluted to 0.1 μg/ml with blocking buffer. The primary antibody used was HL 1330 (SC9-4A8) raised against sheepshead minnow VTG. The plate was then washed with PBST, followed by the addition of 50 μl of the secondary antibody supplied with the avidin-biotin complex kit (biotinylated horse, anti-mouse IgG) to each well and incubated at room temperature for 30 min. During the incubation period, a fresh solution of avidin and biotinylated alkaline phosphatase included with the avidin-biotin complex kit was prepared. After 30 min, the plate was washed with PBST, and the alkaline phosphatase solution was added at 50 μl/well. Again, the plate was incubated at room temperature for 30 min. After incubation, the plate was washed with PBST, and 100 μl of the substrate solution (p-nitrophenol in carbonate buffer with 2 mM MgCl2 [pH 9.6]) was added to each well. After 30 min of incubation at room temperature, the intensity of the yellow color was determined at 405 nm in an ELISA plate reader. All samples and standards were run in triplicate. Samples were diluted in the range of 1:200 to 1:1,000,000 in PBSZ with aprotinin for the values to fall into the range of the standard curve. The coefficients of variation and correlation coefficients for this assay were 10% or less and 0.95 or greater, respectively.

Statistical analysis. For each chemical tested, mean plasma VTG concentrations were graphed to compare response curves over time at each exposure concentration. Analyses for differential rates of VTG accumulation among exposure concentrations were conducted by comparing the slopes of the response curves using the SAS General Linear Models procedures [42]. Family confidence intervals for the analysis of each chemical were set at α = 0.05. Unequal error variances were stabilized using a square-root transformation before analysis [37].

RESULTS

17β-Estradiol positive controls

Measured estradiol exposure concentrations in the test aquaria were an average of 71% of the expected nominal concentrations (Tables 1 and 2). Estradiol control exposures running concurrently with each xenobiotic exposure demonstrated a combined mortality rate of approximately 2%.

Synthesis of hepatic VTG mRNA increased 28-fold by the fifth day of exposure, then stabilized at a mean concentration of 1,256 pg of VTG per 1 μg of total mRNA (range = 1,220–1,520 pg/μg) for the remainder of the 42-d exposure period.
Fig. 2). Detectable amounts of plasma VTG were observed in male fish within 5 d of the initiation of exposure and increased linearly at a calculated accumulation rate of approximately 1.8 mg/ml per day (Fig. 2).

**Triethylene glycol solvent controls**

Triethylene glycol solvent control exposures running concurrently with each chemical exposure demonstrated a combined mortality rate of less than 4%. Because of the low number of TEG samples, hepatic VTG mRNA analyses of the solvent control fish from the p-nonylphenol/methoxychlor exposures were conducted and presented with the results for methoxychlor in Figure 3A. The data show a constant, low-level expression of VTG mRNA in fish sampled from the TEG negative control tanks on days 0, 2, and 21 in both the p-nonylphenol and methoxychlor exposures (Fig. 3A). A similar basal level of VTG mRNA was observed in the TEG controls from both tests with endosulfan (data not shown). No VTG was detected in the plasma of any TEG control fish during the course of the exposures.

**p-Nonylphenol**

Measured p-nonylphenol concentrations remained steady-state throughout the exposure period at approximately 58% of the expected nominal concentrations (Table 1). Mortality rates associated with exposure to p-nonylphenol were 9% or less for treatments at or below 11.8 μg/L. Concentrations of 23.3 and 42.7 μg/L resulted in 28 and 19% mortality, respectively, during the course of the experiment.

Fish exposed to 0.64 μg/L demonstrated a constant, low-level synthesis of VTG mRNA throughout the exposure period (Fig. 4A). The remaining concentrations of 5.4 μg/L or greater showed a rapid rate of VTG mRNA induction by the second day of exposure, which was followed by relatively constant, dose-dependent synthesis from day 5 through the remainder of the test.

Para-nonylphenol precipitated a dose-dependent increase in plasma levels of VTG during the time course of the exposure (Fig. 4B). Fish exposed to 0.64 μg/L exhibited variable VTG levels of less than 6 mg/ml and a significantly lower rate of VTG accumulation compared to all other exposure concentrations tested (α = 0.05). Significantly elevated levels of VTG were detected by the fifth day of exposure at nominal concentrations of 5.4 μg/L or greater. No significant difference in the rates of VTG accumulation was observed in fish treated at 5.4, 11.8, 23.3, and 42.7 μg/L.

**Methoxychlor**

Measured exposure concentrations of methoxychlor were stable throughout the exposure period at approximately 85% of the nominal concentrations (Table 1). Exposure to 18.4 μg/L was acutely toxic to 75% of the fish within 6 d, and exposure to 12.1 μg/L resulted in a 38% mortality rate during the entire test period.

Fish exposed to methoxychlor demonstrated a clear, dose-dependent upregulation of VTG mRNA. A low, relatively constant level of VTG mRNA synthesis was observed in liver samples from the 1.1-μg/L treatment group through the end of the exposure period (Fig. 3A). Fish exposed to 18.4 μg/L displayed the highest rate and level of VTG mRNA synthesis from the second to the fifth day of exposure; however, mortality associated with the acute toxicity of methoxychlor at this treatment level precluded further sampling.

Fish exposed to 1.1 and 2.5 μg/L demonstrated a time-dependent, low-level induction of VTG, reaching detectable levels by day 13 of exposure (Fig. 3B). Plasma VTG was rapidly induced during the first 21 d in a dose-dependent manner at levels of 5.6 μg/L and higher. After 21 d of exposure, plasma VTG levels decreased in fish exposed to 5.6 μg/L, whereas fish exposed to 12.1 μg/L demonstrated a steady-state, linear increase in plasma VTG levels. Fish exposed to 1.1 and 2.5 μg/L showed significantly lower rates of VTG accumulation compared with those exposed to 5.6 and 12.1 μg/L (α

| Table 1. Nominal and average measured concentrations (± standard deviation) of p-nonylphenol, methoxychlor, and 17β-estradiol (shared positive control) in weekly water samples during flow-through exposures of male sheepshead minnows (Cyprinodon variegatus) |
|-----------------|-----------------|-----------------|-----------------|
| **Nominal concentration** (μg/L) | **Measured water concentration** (μg/L) | **Nominal concentration** (μg/L) | **Measured water concentration** (μg/L) |
| 1.0             | 0.64 ± 0.06     | 1.5             | 1.08 ± 0.25     |
| 10.0            | 5.38 ± 0.45     | 3.0             | 2.49 ± 0.57     |
| 20.0            | 11.81 ± 1.09    | 6.0             | 5.59 ± 0.63     |
| 40.0            | 23.27 ± 3.61    | 12.0            | 12.08 ± 1.95    |
| 80.0            | 42.67 ± 5.10    | 24.0            | 18.39 ± 2.25    |
| Estradiol control (100 ng/L) | 65.14 ± 15.80 ng/L | Estradiol control (100 ng/L) | 65.14 ± 15.80 ng/L |

| Table 2. Nominal and average measured concentrations (± standard deviation) of total endosulfan and 17β-estradiol (positive control) in weekly water samples during flow-through exposures of male sheepshead minnows (Cyprinodon variegatus) |
|-----------------|-----------------|-----------------|-----------------|
| **Nominal concentration** (ng/L) | **Measured water concentration** (ng/L) | **Nominal concentration** (ng/L) | **Measured water concentration** (ng/L) |
| 25              | 15.90 ± 2.70    | 300             | 277.17 ± 74.79  |
| 50              | 36.26 ± 5.79    | 600             | 403.50 ± 113.43 |
| 100             | 68.80 ± 14.16   | 900             | 590.50 ± 150.4  |
| 200             | 162.27 ± 26.22  | 1200            | 788.33 ± 212.60 |
| Estradiol control (100 ng/L) | 71.92 ± 21.03   | Estradiol control (100 ng/L) | 75.73 ± 13.64   |
Vitellogenin induction in sheepshead minnow

Fig. 2. Vitellogenin (VTG) mRNA (pg/μg total mRNA) induction and plasma VTG accumulation in sheepshead minnows (Cyprinodon variegatus) continuously exposed to 65 ng/L 17β-estradiol (positive control) for 42 d. Each data point/bar represents the mean ± standard error of the mean of four fish.

Fig. 4. Dose response and time course of (A) hepatic vitellogenin (VTG) mRNA induction (pg/μg total mRNA) and (B) plasma VTG accumulation (mg/ml) in sheepshead minnows (Cyprinodon variegatus) exposed to p-nonylphenol. Each point/bar represents the mean ± standard error of the mean of four fish.

Endosulfan

Measured endosulfan exposure concentrations were an average of 72% of the expected nominal concentrations (Table 2). The combined mortality for all treatments was 9% or less in the first series of low-range exposures (15.9–162.3 ng/L). The second series of exposures at higher concentrations (277–788 ng/L) resulted in 41% fish mortality at 590 ng/L and 59% mortality within 96 h at 788 ng/L.

Exposure to 15.9, 36.3, 68.8, 162.3, 277, and 403 ng/L for up to 40 d failed to induce hepatic VTG mRNA levels over background or detectable levels of plasma VTG in adult male sheepshead minnows. The surviving fish exposed to 590 ng/L for up to 20 d and to 788 ng/L for 4 d also showed no detectable mRNA induction or accumulation of VTG in the plasma.

DISCUSSION

Selection of the test concentrations for p-nonylphenol and the lower range of exposures with endosulfan were based on concentrations previously measured in benthic sediments, sewage effluent, and natural receiving waters [23–25,43]. Methoxychlor exposure concentrations were determined by selecting a series of 50% dilutions, starting with the approximate upper value of the maximum acceptable toxicant concentration at 23 μg/L derived in a partial-life-cycle toxicity test with sheepshead minnows [44].

The mortalities observed at the higher p-nonylphenol concentrations of 23.3 and 42.7 μg/L were consistent with reported values for sheepshead minnow and other fish species. In studies where measured concentrations, static renewal, or flow-through exposures were used, LC50 values for p-nonylphenol...
were reported at 319 µg/L for sheepshead minnow, 138 to 300 µg/L for fathead minnow (Pimephales promelas), and 221 to 270 µg/L for rainbow trout (Oncorhynchus mykiss) [26]. Mortalities associated with exposure to methoxychlor and endosulfan in this study were also consistent with LC50 values of 49 µg/L methoxychlor for sheepshead minnows [44] and 0.86 µg/L endosulfan for fathead minnows [45].

The estrogenic responses of p-nonylphenol and methoxychlor exhibited in this assay show excellent correlation with findings from previously described in vitro investigations (Table 3). In general, sheepshead minnows exposed to estradiol, p-nonylphenol, or methoxychlor demonstrated rapid, dose-dependent induction of hepatic VTG mRNA for the first 2 to 5 d (Figs. 2, 3A, and 4A). In the case of estradiol and p-nonylphenol, relatively constant VTG mRNA levels were maintained at all exposure concentrations after the fifth day of exposure. Although fish exposed to p-nonylphenol concentrations of 5.4 µg/L or greater demonstrated a clear, dose-related increase in VTG mRNA independent of exposure duration, their plasma VTG levels followed both a temporal and dose-dependent accumulation throughout the 42-d test period (Fig. 4B). At the lowest p-nonylphenol concentration tested (0.64 µg/L), VTG mRNA levels were only slightly above the threshold levels observed for the TEG controls. However, even at this consistently low mRNA induction level, VTG was detectable in the plasma by the 21st day of exposure (Fig. 4B).

Lech et al. [46] found similar results with juvenile rainbow trout exposed to 4-t-nonylphenol, which demonstrated VTG mRNA induction within 72 h at the lowest concentration tested (10 µg/L).

In contrast, fish exposed to methoxychlor demonstrated both a temporal and dose-related mRNA induction pattern, with a 2- and a 13-d delay in appreciable increases of VTG mRNA levels at 5.6 and 2.5 µg/L methoxychlor, respectively (Fig. 3A). The delay in VTG mRNA induction may have been a function of the conversion of methoxychlor by P450 enzymes to estrogenic mono- or bis-demethylated derivatives [32,33] and their subsequent accumulation to levels capable of eliciting a measurable estrogenic response. The delay in VTG induction is clearly reflected in plasma expression, as shown by the approximate 5-, 13-, and 35-d lags in measurable plasma levels of VTG at methoxychlor concentrations of 5.6, 2.5, and 1.1 µg/L, respectively (Fig. 3B). Temporal delays observed in VTG mRNA induction and expression profiles illustrate the importance of adequate exposure duration where bioactivation may be necessary for estrogenic activity.

In our first test series with endosulfan, concentrations of 162 µg/L or less failed to elicit a vitellogenic response in male fish during the course of 42 d. For this reason, a second series of endosulfan exposures were conducted using nominal concentrations spanning the maximum acceptable toxicant concentration of greater than 0.58 to less than 1.2 µg/L reported for sheepshead minnow [47]. Again, no vitellogenic response was observed for any endosulfan concentration tested, including those causing acute lethality. Our in vivo assay results with endosulfan agree with the negative results reported by Shelby et al. [28], including an in vivo mouse uterotrophic assay, but they contrast with those of several previous in vitro investigations (Table 3). Inconsistent results between in vitro and in vivo assays suggest that although endosulfan may weakly bind to the estrogen receptor at extremely high concentrations, in vivo pharmacokinetic considerations such as uptake, metabolism, and ultimate tissue distribution and concentration may negate endosulfan’s weak estrogenic potential.

In conclusion, results observed with p-nonylphenol and methoxychlor in our sheepshead minnow VTG production assay correlate well with results reported for a variety of other in vitro and in vivo estrogenic assays. Negative results with endosulfan in our assay contrast with positive results reported for several in vitro assays, demonstrating the importance of conducting whole-animal in vivo assays for compounds with suspected endocrine-disrupting potential. Results of this study demonstrate the sheepshead minnow is well suited for use in an in vivo screening assay for estrogenic substances and, possibly, as a sentinel species in estuarine field-monitoring programs.

Acknowledgement—This study was supported, in part, by the U.S. Environmental Protection Agency Cooperative Agreement CR826357-01 to N.D. Denslow. The authors would like to acknowledge the valuable technical assistance of Calvin Walker and Stephanie Friedman.

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