



# Bioconcentration of nonylphenol in fathead minnows (*Pimephales promelas*)

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## Abstract

Bioconcentration of *p*-nonylphenol (NP) by fathead minnows was determined under laboratory conditions. Fish were exposed continuously for 42 days to 0.33, 0.93 and 2.36  $\mu\text{g}$  NP/l in a flow-through system. NP was Soxhlet extracted from whole fish homogenates with dichloromethane (DCM). The resulting extract was concentrated and bulk lipids removed by gel permeation and silica-gel chromatography. Compounds were identified and quantified by reverse-phase high-pressure liquid chromatography (RP-HPLC) with fluorescence detection. Mass spectrometry was used for verification of peak assignments. Bioconcentration factors (BCFs) ranged from 245 to 380. © 2001 Elsevier Science Ltd. All rights reserved.

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## 1. Introduction

Alkylphenol polyethoxylates (APE) are non-ionic surfactants that are used in various industrial, institutional, agricultural, and household applications (Talmage, 1994). APE are among the most widely used non-ionic surfactants, with approximately 250 million kg per year used in the United States (US) (Chemical Market Reporter, 18 October, 1999). The molecular structure of APE consists of two moieties, an alkyl-substituted phenol and an ethoxylate oligomer chain. The alkylphenol (AP) portion of the surfactant is fairly non-polar and allows the APEs to dissolve grease and other materials that have small water solubilities. The ethoxylate portion of the surfactant is water-soluble and

aids in the transfer of materials to the aqueous phase. APE are produced commercially by the base-catalyzed ethoxylation of alkylphenols (Cahn and Lynn, 1978). Greater than 90% of the APs used in APE production are of the *para* substituted isomer. Nonylphenol polyethoxylates (NPE) that contain a nine carbon branched isomeric alkyl group ( $\text{C}_9\text{H}_{19}$ ) (Fig. 1) represent approximately 80% of production (Naylor et al., 1992; Talmage, 1994; Lye et al., 1999). The remaining production comprises almost entirely octylphenol polyethoxylates (OPE).

NPE and the less widely used OPE degrade during wastewater treatment or in the environment to form transient intermediates such as alkylphenol ethoxycarboxylates (APEC), shorter-chain oligomers of APE, and alkylphenols (Naylor et al., 1992; Field and Reed, 1996; Bennie et al., 1997; Rudel et al., 1998; Tanghe et al., 1999). The type and efficiency of wastewater treatment affects the total removal rate of NPE and the type and proportion of the transient intermediates. Primary treatment removes only 25–78% of the total NPE

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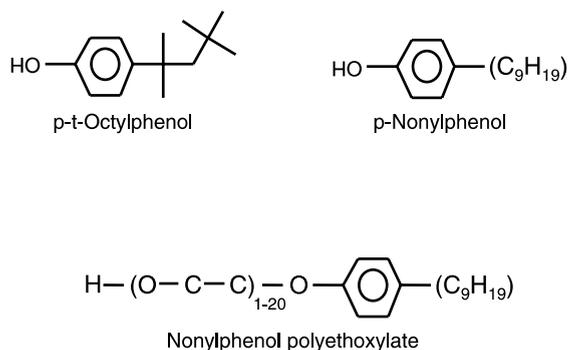


Fig. 1. Structures of target compounds.

entering wastewater treatment plants (WWTP). With primary treatment alone, the hydraulic retention time is short and degradation is minimal. Primary effluents contain approximately 82% NPE<sub>3-20</sub>, 12% NPE<sub>1&2</sub>, 3% nonylphenol monoethoxycarboxylate and nonylphenol diethoxycarboxylate (NPEC<sub>1&2</sub>), and 3% nonylphenol (NP) from the initial NPE loading (Ahel et al., 1994). In the United States (US), WWTP with secondary or tertiary treatment achieve average removal rates in excess of 97% for NPE. Effluents from these types of WWTP contain approximately 28% NPE<sub>3-20</sub>, 22% NPE<sub>1&2</sub>, 46% NPEC<sub>1&2</sub>, and 4% NP from the initial NPE loading (Ahel et al., 1994).

Although NP is a minor degradation component of NPE, significant amounts of this slightly lipophilic, moderately toxic compound enter the environment. NP has a log  $K_{ow}$  of 4.48 (Ahel and Giger, 1993b), water solubility of 5.4 mg/l (Ahel and Giger, 1993a), and a log  $K_{oc}$  of 3.58 (Naylor et al., 1992). This physico-chemical profile indicates that NP may bioaccumulate in aquatic organisms. This has been documented in several species of fish from natural waters and from controlled laboratory exposures (Talmage, 1994). NP bioconcentration factors (BCFs) for the fathead minnow (*Pimephales promelas*) exposed to 5 or 25  $\mu\text{g NP/l}$  for 20 days were determined to be 270 or 350, respectively (Naylor, 1995). Half-times for depuration have been determined to be 1.4 and 1.2 days, respectively, following exposure to 5 and 25  $\mu\text{g NP/l}$  (Naylor, 1995).

Nonylphenol has been shown to be weakly estrogenic in a variety of both in vitro (Mueller and Kim, 1978; Soto et al., 1992; Jobling and Sumpter, 1993; White et al., 1994; Desbrow et al., 1998) and in vivo bioassays (Jobling et al., 1996; Nimrod and Benson, 1996; Giesy, 1998). LC<sub>50</sub> values for acute toxicity to fish range from 0.135 to 0.3 mg NP/l for fathead minnows (*P. promelas*) (Dwyer et al., 1995) and from 0.167 to 0.221 mg NP/l for three species of trout (Naylor, 1995). Reports of APE and their degradation products in the environment in the US are scarce. The most compre-

hensive survey reports concentrations from 30 US rivers that are influenced by municipal or industrial wastewater effluents (Naylor et al., 1992). That study demonstrated that 60–75% of samples had no detectable levels of NP, NPE<sub>1</sub>, and NPE<sub>2</sub>. NP concentrations observed in that study ranged from < 0.11 to 0.64  $\mu\text{g/l}$  (Naylor et al., 1992). Concentrations of NP in effluents and surface waters ranging from less than the detection limit to 37  $\mu\text{g/l}$  also have been reported (Snyder et al., 1999).

The objectives of this study were to develop a suitable method for extraction, clean-up, and quantification of NP in fish tissues and to use this method to determine the BCF for partitioning of NP into fathead minnows during a controlled laboratory exposure.

## 2. Experimental

### 2.1. Standards and reagents

All standards and reagents used were of the highest purity commercially available. High purity standards of *p*-nonylphenol (NP), *p*-tert octylphenol (OP), and *p*-tert butylphenol (BP) were obtained from Schenectady International (Freeport, TX). Pesticide residue grade acetonitrile (ACN), hexane, dichloromethane (DCM) and acetone were obtained from Burdick and Jackson (Muskegon, MI). Reagent water was purified first by reverse osmosis (RO) followed by Nanopure™ (Barnstead, Dubuque, IO) treatment. Glass wool (8  $\mu\text{m}$ , Corning Glass Works, Corning, NY) was Soxhlet extracted overnight with DCM, then dried and stored in glass containers until needed. Glass fiber filters, fine grade (GF/F) with 0.7  $\mu\text{m}$  nominal pore size, were heated at 450°C for 4 h in aluminum foil prior to use. Anhydrous granular sodium sulfate (EM Science, Gibbstown, NJ) was heated at 450°C overnight, then stored at 125°C until day of use.

### 2.2. Fish exposure

Fathead minnows (*P. promelas*) were cultured in the Michigan State University Aquatic Toxicology Laboratory (MSU-ATL) culture facility under a 16:8 light:dark cycle in continuously flowing well water at 17–19°C. Fish were fed a 1:1 (v:v) mixture of Tetramin® (Tetra Werke, Melle Germany) and ground Sterling Silver Cup Trout Chow (2–4 mm pellet size, Murray, UT) once daily at a rate of approximately 0.5% of fresh body weight. The dry food was supplemented with *Artemia nauplii* (brine shrimp). Freeze-dried *Spirulina* sp. algae was fed to fry up to 2.5 cm length. Reproductively mature fish, 12 to 18 months of age, were transferred to randomly assigned 19 l glass aquaria in a circulating water-jacketed bath (Frigid Units Living Stream, To-

ledo, OH). Each tank received 3 l/h treated well water through a solenoid-controlled proportional-flow diluter apparatus (Ace Glass, Vineland, NJ). Each tank received vigorous aeration, and glass lids prevented escape of aerosols from the tanks. The temperature of the water jacket was maintained at 26°C.

Fish were randomly assigned to 20 groups, each containing two males and two females, and placed into 19 l aquaria. Treatments were in triplicate with the exception of the solvent control group, which due to the restriction of space in the water bath, contained only two aquaria. Fish were allowed to acclimate in control water for 7 d, then exposed to NP for a total of 42 d. The aquaria were assigned randomly to receive treatment concentrations of NP or control solutions delivered by a proportional-flow diluter. Treatments included control, which received only water, and a solvent control, which received the same concentration of solvent (0.0001% ethanol) as the NP treatments. Fathead minnows were exposed to target (nominal) concentrations of 1.0, 3.0 and 10 µg NP/l. The actual, measured concentrations were 0.33, 0.93 and 2.4 µg NP/l (Table 1). Concentrations of NP were determined in 5–7 fishes from each exposure level of NP (Table 1).

### 2.3. Water extraction

Water samples were collected three times during the exposure to determine actual NP concentrations. Analytical methods were adopted from those published previously (Snyder et al., 1999). Samples of 1 l of water were spiked with 1.0 µg BP in methanol as a surrogate standard. The fortified samples were vacuum filtered and extracted using 47 mm GF/F directly above a 47 mm styrenedivinylbenzene (SDB) Empore™ solid-phase extraction (SPE) disk. The SPE disk was frozen until extraction. The SPE disk was solvent extracted with 15 ml of acetone followed by 25 ml DCM. Moisture was removed by passing the extract through 30 g of anhydrous sodium sulfate. The dried extract was concentrated to 1 ml in iso-octane. A 2-g gravimetric silica

column (60–100 mesh, Aldrich Chemical Company, Milwaukee, WI) was washed with 100 ml of 20% DCM in hexane and the extract loaded onto the column. Alkylphenols were eluted with 100 ml of DCM. This fraction was concentrated to 1 ml in acetonitrile (ACN) for reverse-phase high-pressure liquid chromatography (RP-HPLC) analysis.

### 2.4. Fish extraction and clean-up

Individual, whole fathead minnows were weighed, then homogenized with 30 g of anhydrous sodium sulfate. The homogenate was spiked with 0.50 µg of OP as a surrogate standard and Soxhlet extracted for 12 h using 300 ml DCM. The resulting extract was concentrated at 30°C by rotary evaporation followed by a gentle stream of nitrogen to a final volume of 2 ml.

Gel permeation chromatography (GPC) was used to remove bulk lipids from the extracts. The GPC system consisted of a Rheodyne 7725i injector (Cotati, CA) with a 5 ml sample loop, a quaternary high-pressure liquid chromatography (HPLC) pump (Perkin–Elmer, Series 410, Norwalk, CT), and an electronic fraction collector (ISCO Foxy 200, Lincoln, NE). Two Phenomenex (Torrance, CA) GPC columns were connected in tandem: a Phenogel™ 5 µm 500 Å (300 × 21.20 mm) followed by an Envirosep-ABC (350 × 21.20 mm). Both GPC columns were preceded by an Envirosep-ABC (60 × 21.20 mm) guard column (Phenomenex, Torrance, CA). The pump was operated isocratically at 3 ml/min with DCM as the mobile-phase solvent. A fraction containing OP and NP was collected from 20–35 min. This fraction was concentrated and cleaned-up in the same manner as the water extracts.

### 2.5. Quantitation of compounds

Extracts were analyzed by RP-HPLC as described previously (Snyder et al., 1999). The reverse-phase HPLC system included a Perkin–Elmer (PE) (Norwalk, CT) series 200 autosampler, a PE series 200 binary

Table 1  
Concentrations of NP in water and fish tissues and bioconcentration factors

Nominal concentration in water (µg NP/L)	1.0	3.0	10.0
Mean measured concentration in water (µg NP/L)	0.33	0.93	2.36
Standard error ( <i>n</i> )	0.0164 (2)	0.032 (3)	0.19 (3)
Mean measured concentration in fish (mg NP/kg, ww) <sup>a</sup>	81.2	403	912
Standard error ( <i>n</i> )	2.2 (5)	6.08 (6)	31.54 (7)
95% Confidence interval	6.0	15.6	77.11
Mean bioconcentration factor (BCF)	245.9	434	380.2
Standard error ( <i>n</i> )	6.5 (5)	6.5 (7)	13.13 (7)
95% Confidence interval	18.1	16.77	32.13 (7)

<sup>a</sup> Method detection limit (MDL) = (67 µg/kg).

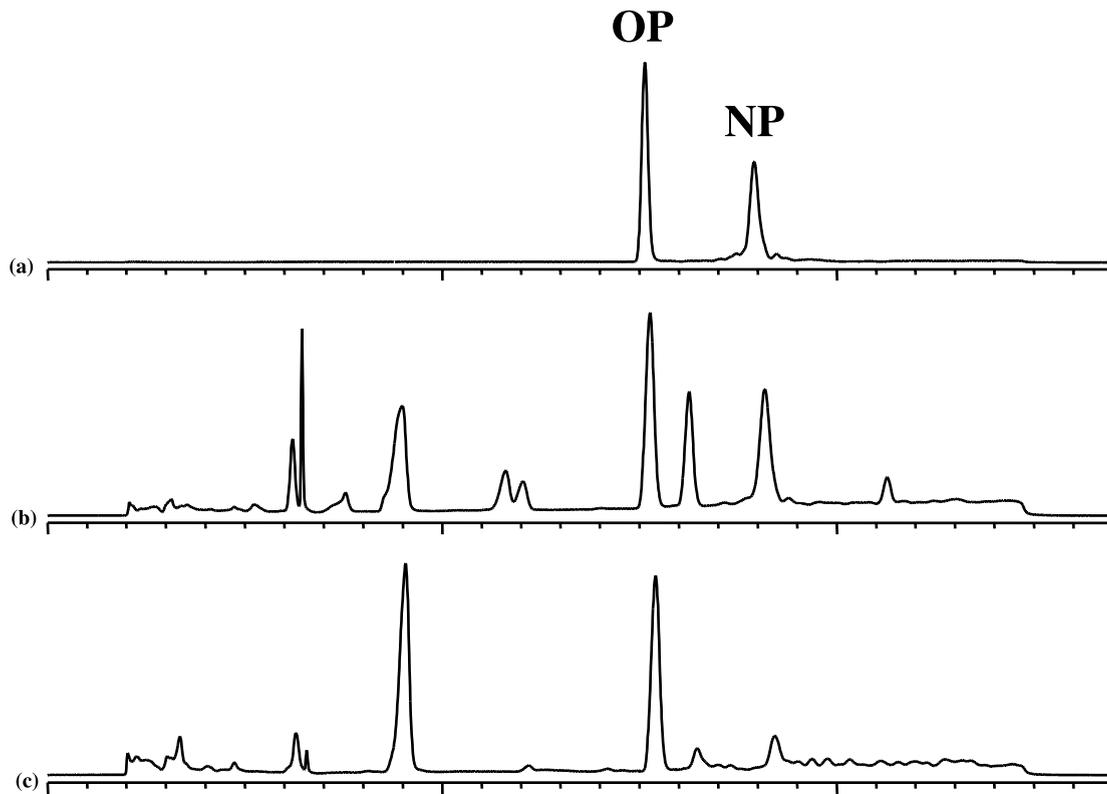


Fig. 2. Chromatograms of target compounds in fish tissues: (a) standards; (b) fish tissue extract from 2.36  $\mu\text{g}$  NP/l exposure; (c) fish tissue extract from 0.33  $\mu\text{g}$  NP/l exposure.

pump, a Hewlett Packard (HP) 1046A fluorescence detector, and PE TurboChrome 4.0 data software package. A Phenomenex Prodigy™ 5  $\mu\text{m}$  octadecylsilica (ODS) 100 A° (250  $\times$  4.6 mm, Torrance, CA) column preceded by a 30  $\times$  4.6 mm guard column of the same packing material was used. For all compounds of interest, the fluorescence detector settings were: 229 nm excitation, 310 nm emission, PMT gain of 12, lamp time of -1, response time of 2 s, stop time at 27 min, gate and delay at 0.

The elution profile was a 20 min gradient (curve = -2) from 50% water in ACN to 2% water in ACN followed by a 10 min ACN purge. Each sequence began with one blank and one standard. The injection volume of standards and samples was 25  $\mu\text{l}$ . Peak areas were determined by electronic integration, and sample concentrations were determined using Microsoft Excel version 7.0. Chromatography and retention times for the compounds of interest are given (Fig. 2). All calibration curves were linear ( $r^2 > 0.99$ ). An HP 5890 series II plus GC with a 30 m DB5-MS capillary column (J and W Scientific, Folsom, CA) and an HP 5972 series mass selective detector (MSD) were used for confirmation of peak assignments.

### 2.6. Recovery and precision

Instrumental detection limits (IDLs) and limits of quantitation (LOQs) were determined by analyzing dilute standards (near the estimated IDL) and calculating the signal-to-noise ratio (peak height (mV) divided by baseline noise) for each standard concentration. Linear regression of the signal to noise ratios against concentrations was then used to determine the IDL (signal to noise ratio = 3) and LOQ (signal to noise ratio = 10). The method detection limit (MDL) was determined by spiking seven 1.5 g homogenized fathead minnow tissue samples with dilute concentrations of NP and OP (at the estimated MDL) and analyzing as described previously. The MDL was calculated by multiplying the standard deviation (SD) of the resulting concentrations by a *t*-test value of 3.1 (for  $n = 7$ ) (Taylor, 1987).

### 3. Results

Recoveries for each of the compounds of interest were in excess of 70%. For both water and tissue analyses, data were adjusted for surrogate recovery. The

IDLs, LOQs, and MDLs for these compounds in water and tissue are shown (Table 1). The clean-up steps for this method did not remove all lipids present in the fish tissue extracts, resulting in an increased baseline and elevated operating pressures. Therefore, after approximately five injections of fish tissue extracts, the analytical column was purged with successively less polar solvents to remove residual lipids.

With the exception of the controls, NP was detected in the water in each of the exposure tanks (Table 1). Nonylphenol was not detectable in water or fish tissues from the control or solvent control exposure. Therefore, bioconcentration factors (BCF) were calculated only for the 0.33, 0.93 and 2.4  $\mu\text{g NP/l}$  (measured) exposure concentrations (Table 1). The coefficient of variation (CV) for replicate water samples was 6.9%, 6.1% and 14% for the 0.33, 0.93 and 2.4  $\mu\text{g NP/l}$  exposure concentrations, respectively. The CV for replicate measurements of NP in fish tissue was 5.9%, 3.7% and 9.1%, for the 0.33, 0.93 and 2.4  $\mu\text{g NP/l}$ , respectively. BCF, calculated by dividing the average tissue concentration by the average measured exposure concentration ranged from approximately 246 in the least exposure to 434 in the intermediate exposure (Table 1) with an average of 353.

#### 4. Discussion

The BCFs determined here are similar to previously reported values ranging from 100–300 (Naylor, 1995; Staples et al., 1998; Liber et al., 1999). The analytical method described here works well for tissue samples with a mass of less than 1.5 g. For environmentally exposed fish, it would be necessary to assure that other residues that can fluoresce (for example, polycyclic aromatic hydrocarbons) present in the tissue do not interfere with the HPLC analysis. Concentrations of NP in surface waters are often less than 0.16  $\mu\text{g NP/l}$  (Naylor et al., 1992). Fish exposed to 0.16  $\mu\text{g NP/l}$  would, assuming a BCF of 353, contain a concentration of 56.5  $\mu\text{g NP/kg}$ , which is only slightly less than the MDL of 67  $\mu\text{g NP/kg}$ . Thus to determine the concentration of NP in fish from surface waters containing small concentrations of NP, larger masses of tissue could be needed to meet the required detection limits.

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#### References

- Ahel, M., Giger, W., 1993a. Aqueous solubility of alkylphenols and alkylphenol polyethoxylates. *Chemosphere* 26, 1461–1470.
- Ahel, M., Giger, W., 1993b. Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic solvents. *Chemosphere* 26, 1471–1478.
- Ahel, M., Giger, W., Koch, M., 1994. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment – II. Occurrence and transformation in sewage treatment. *Wat. Res.* 28, 1131–1142.
- Bennie, D.T., Sullivan, C.A., Lee, H.B., Peart, T.E., Maguire, R.J., 1997. Occurrence of alkylphenols and alkylphenol mono- and diethoxylates in natural waters of the Laurentian Great Lakes basin and the upper St. Lawrence River. *Sci. Total Environ.* 193, 263–275.
- Cahn, A., Lynn Jr., J.L., 1978. Surfactants and detergent systems. In: Kirk-Othmer Encyclopedia of Chemical Technology 22, Wiley, New York, pp. 365.
- Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of estrogenic chemicals in STW effluent. 1. chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* 32, 1549–1558.
- Dwyer, F.J., Sappington, L.C., Buckler, D.R., Jones, S.B., 1995. Use of surrogate species in assessing contaminant risk to endangered and threatened species, National Technical Information Service, Springfield, VA, pp. 71.
- Field, J.A., Reed, R.L., 1996. Nonylphenol polyethoxy carboxylate metabolites of nonionic surfactants in U.S. paper mill effluents, municipal sewage treatment plant effluents, and river waters. *Environ. Sci. Technol.* 30, 3544–3550.
- Giesy, J.P., 1998. Environmental Estrogens. *Wat. Reports* 97, 90–98.
- Jobling, S., Sumpter, J.P., 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: an in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat. Toxicol.* 27, 361–372.
- Jobling, S., Sheahan, D., Osborne, J.A., Matthiessen, P., Sumpter, J.P., 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ. Toxicol. Chem.* 15, 194–202.
- Liber, K., Gangl, J.A., Corry, T.D., Heinis, L.J., Stay, F.S., 1999. Lethality and bioaccumulation of 4-nonylphenol in bluegill sunfish in littoral enclosures. *Environ. Toxicol. Chem.* 18, 394–400.
- Lye, C.M., Frid, C.L.J., Gill, M.E., Cooper, D.W., Jones, D.M., 1999. Estrogenic alkylphenols in fish tissues, sediments, and waters from the U.K. Tyne and Tees estuaries. *Environ. Sci. Technol.* 33, 1009–1014.
- Mueller, G.C., Kim, U.H., 1978. Displacement of estradiol from estrogen receptors by simple alkylphenols. *Endocrinol.* 102, 1429–1435.
- Naylor, C.G., 1995. Environmental fate and safety of nonylphenol ethoxylates. *Text. Chem. Color.* 27, 29–33.
- Naylor, C.G., Mieure, J.P., Adams, W.J., Weeks, J.A., Castaldi, F.J., Ogle, L.D., Romano, R.R., 1992. Alkylphenol ethoxylates in the environment. *JAOCS* 69, 695–703.

- Nimrod, A.C., Benson, W.H., 1996. Environmental estrogenic effects of alkylphenol ethoxylates. *Crit. Rev. Toxicol.* 26, 335–364.
- Rudel, R.A., Melly, S.J., Geno, P.W., Sun, G., Brody, J.G., 1998. Identification of alkylphenols and other estrogenic phenolic compounds in wastewater, septage, and groundwater on Cape Cod, Massachusetts. *Environ. Sci. Technol.* 32, 861–869.
- Snyder, S.A., Keith, T.L., Verbrugge, D.A., Snyder, E.M., Gross, T.S., Kannan, K., Giesy, J.P., 1999. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ. Sci. Technol.* 33, 2814–2820.
- Soto, A.M., Tien-Min, L., Honorato, J., Silvia, R.M., Sonnenschein, C., 1992. An “in culture” bioassay to assess the estrogenicity of xenobiotics (E-screen). In: M. A. Mehlman (Ed.), *Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection*. Vol. 21, Princeton Scientific Publishing Co., Inc, Princeton, NJ.
- Staples, C.A., Weeks, J., Hall, J., Naylor, C., 1998. Evaluation of aquatic toxicity and bioaccumulation of C8- and C9-alkylphenol ethoxylates. *Environ. Toxicol. Chem.* 17, 2470–2480.
- Talmage, S.S., 1994. *Environmental and Human Safety of Major Surfactants: Alcohol Ethoxylates and Alkylphenol Ethoxylates*. Lewis Publishers, Boca Raton.
- Tanghe, T., Dhooge, W., Verstraete, W., 1999. Isolation of a bacterial strain able to degrade branched nonylphenol. *Appl. Environ. Microbiol.* 65, 746–751.
- Taylor, J.K., 1987. *Quality Assurance of Chemical Measurements*. Lewis Publishers, Inc. Chelsea, MI, USA, 328.
- White, R., Jobling, S., Hoare, S.A., Sumpter, J.P., Parker, M.G., 1994. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinol.* 135, 175–182.

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