

Identification and Quantitation of Nonylphenol Ethoxylates and Nonylphenol in Fish Tissues from Michigan

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Nonylphenol (NP) and its lower ethoxylates, nonylphenol monoethoxylate (NPE₁) and nonylphenol diethoxylate (NPE₂), can be present in aquatic environments at total concentrations of more than 10 µg/L. They are metabolites of nonylphenol polyethoxylates (NPE) and have been found to be weakly estrogenic. To evaluate bioaccumulation potential and identify potential risks posed by these chemicals, concentrations of NP, NPE₁, NPE₂, and nonylphenol triethoxylate (NPE₃) were determined in the tissues of fish inhabiting various waters in Michigan. This method involves extraction of samples using exhaustive steam distillation with concurrent liquid extraction. Concentrations of NP among all sites and species ranged from <3.3 to 29.1 ng/g, ww and varied little among sites. NPE₁ was detectable in some samples but at concentrations less than the method detection limit (16.8 ng/g). Concentrations of NPE₂ and NPE₃ in all samples were less than their respective MDLs of 18.2 and 20.6 ng/g.

Introduction

Alkylphenols (APs) and alkylphenol ethoxylates (APEs) have numerous applications, including pesticide formulations, petroleum production, cleaning products, pulp and paper manufacturing, and plastics manufacturing (1). Approximately 80% of the APEs used are nonylphenol ethoxylates (NPEs), while the remaining 20% are almost entirely octylphenol ethoxylates. These compounds enter wastewater treatment plants (WWTPs) where they may undergo degradation. The degradation intermediates NP and its lower oligomer NPEs tend to be more persistent and are often the dominant NPE species in WWTP effluents (2, 3). They are

lipophilic and tend to adsorb to organic surfaces (1). Wastewater treatment in the United States generally removes around 95% of all the NPEs entering the plant (4).

The most comprehensive survey of nonylphenol from the United States reported water and sediment concentrations of NP and NPEs from 30 rivers that are influenced by municipal or industrial wastewater effluents (5). That study found that 60–75% of water samples had no detectable levels of NP, NPE₁, or NPE₂ while 30% of sediments contained detectable levels. However, reports on the concentrations of APs and APEs in fish in U.S. waters are scarce. Recently, NP and NPE have been detected in water of the Las Vegas Bay of Lake Mead, NV (6).

While information on the occurrence of NP in water and sediment is available, few studies have examined the occurrence of NP in fish due to the lack of suitable analytical techniques. Monitoring of NP in fish is important to assess the potential for dietary exposure of humans and wildlife. We have developed a method for the analysis of NP and its ethoxylates in fish (7). In the present study, the method was applied to determine concentrations of NP and NPE_{1–3} in fish from Michigan waters.

The objective of this study was to measure concentrations of NP and NPE_{1–3} in fish from various rivers in Mid-Michigan. Since these fish were not caged, samples from a particular "site" actually represent a segment of water that may range approximately 3 km up or downstream from the sampling location. Thus, analyte concentrations should be associated with a river segment rather than a specific point. This information will prove valuable to guide further studies to measure the environmental occurrence of these compounds.

Experimental Section

Overview of Study Area. Sampling sites were chosen to represent the ambient environmental concentrations of the compounds of interest in fish. Fish were collected from two major regions: the Kalamazoo River Basin, MI, and Lake Michigan near the mouth of the Kalamazoo River (approximately 7 km) (Figure 1). The Kalamazoo River flows through both urban areas and rural areas and receives secondary and tertiary WWTP effluent and industrial discharges including those of paper manufacturing facilities.

Sampling along the Kalamazoo River was conducted up and downstream of WWTPs, whenever possible. Fish were captured within approximately 40 m of the WWTP effluents. The Portage, Battle Creek, Allegan, Marshall, Gun Lake, Augusta, and Albion WWTPs employ activated sludge secondary treatment. The Otsego WWTP employs trickling filter secondary treatment, while the Kalamazoo WWTP has tertiary treatment.

Fish and Sampling. Fish species were selected for analysis based on several considerations. These include availability at sampling sites, size (weight), migratory behavior, and placement in the food chain. Since the fish were meant to represent an area within a river, less migrating species were preferred. Also, fishes that reside primarily in the middle depths of the water column were preferred to best represent the exposure to dissolved analytes and not those bound to sediments. Furthermore, species that are classified as game fish were preferred.

Species analyzed include rock bass (*Ambloplites rupestris*), bluegill sunfish (*Lepomis macrochirus*), green sunfish (*Lepomis cyanellus*), smallmouth bass (*Micropterus dolomieu*), white suckers (*Catostomus commersoni*), longnose

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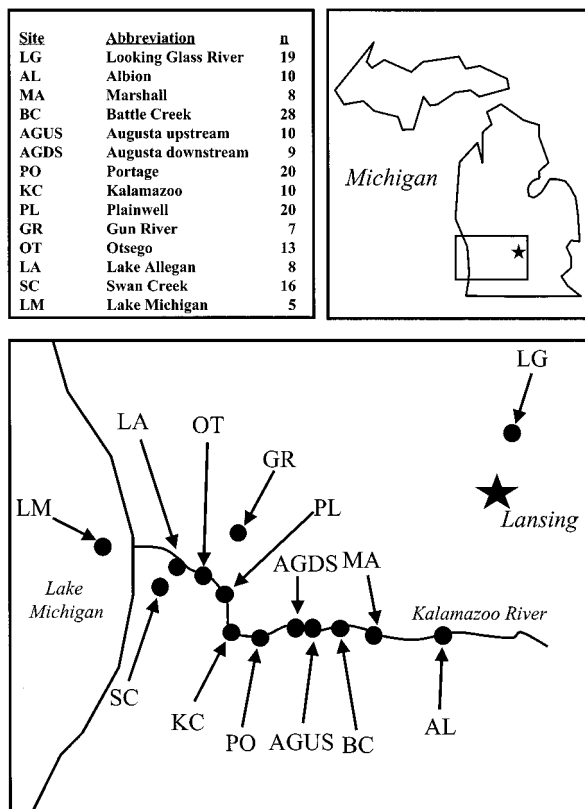


FIGURE 1. Locations of sample collection. The *n* value is the total number of samples for that site.

suckers (*Maxostoma macrolepidotum*), and rainbow smelt (*Osmerus mordax*).

Fish were collected by electroshocking on three occasions between late July and early November 1999 and stored at -20°C until analysis. The area of the fish where NP was likely to accumulate, the digestive/excretory system, was chosen for analysis (8). Fish were cut at the midsection from the pectoral to dorsal fin, and this portion was used for analysis.

Standards and Reagents. High-purity standards ($\geq 96\%$ purity) of *p*-nonylphenol (NP), *p*-cumylphenol, and 4-*tert*-butyl orthocresol were obtained from Schenectady International (Freeport, TX). Standards of NPE_{1-3} were obtained from Huntsman Corporation (Austin, TX). High-purity pesticide residue grade *n*-hexane, dichloromethane (DCM), and isooctane were obtained from Burdick and Jackson (Muskegon, MI). Organic free water was obtained by purification of reverse osmosis treated water followed by Nanopure (Barnstead, Dubuque, IA) treatment. All glassware and stainless steel homogenization equipment were rinsed with organic free water followed by high-purity pesticide residue-grade acetone and *n*-hexane. ACS reagent-grade sodium chloride was obtained from J. T. Baker (Phillipsburg, NJ). Reagent-grade concentrated sulfuric acid was obtained from EM Science (Gibbstown, NJ).

Extraction. Extraction and quantitation methods for nonylphenolics are described in detail elsewhere (7). Briefly, a 20-g cross-section was removed from the sample and homogenized (Blender 700, Waring Corporation, New Hartford, CN). The homogenized sample was poured into a 2-L boiling flask. A total of 20 g of sodium chloride, 3 mL of concentrated sulfuric acid, and 500 ng of the surrogate standard *p*-cumylphenol are added. The sample was extracted using a Nielsen-Kryger improved version steam-distillation column (Ace Glass, Vineland, NJ) for a combined total of 3 h. The resulting extract was concentrated to 1 mL

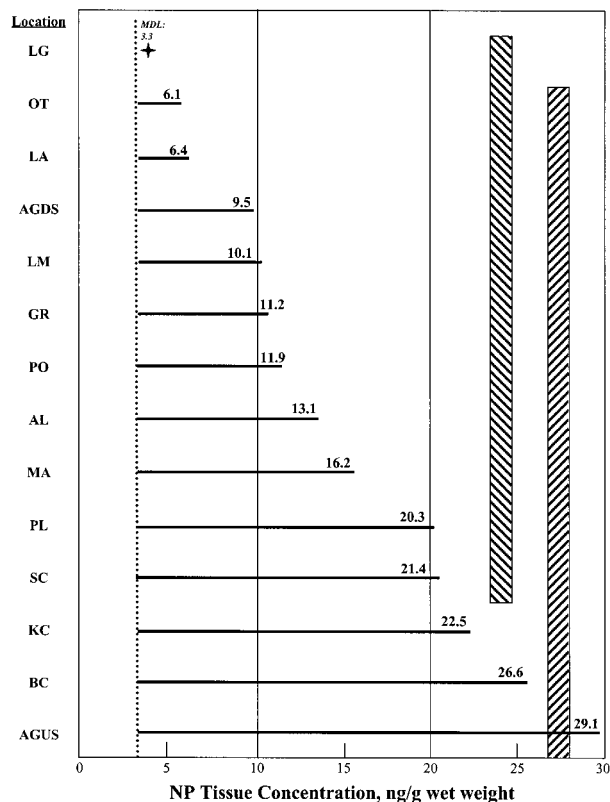


FIGURE 2. Ranges of detectable NP tissue concentrations among sites. Vertical bars represent sampling sites that are not significantly different. (◆) All samples below MDL. Site abbreviations listed in Figure 1.

in isooctane using a nitrogen evaporator (Organomation Associates, Inc., Berlin, MA). To further remove lipids from the sample extract, a Perkin-Elmer (Norwalk, CT) series 200 autosampler and binary pump and a Hewlett-Packard (HP) (Palo Alto, CA) 1046A fluorescence detector were employed to separate lipids from the compounds of interest. A total of 800 μL of the isooctane extract was separated using a Phenomenex Luna 5 μm silica column (250 mm \times 4.6 mm, Torrance, CA) by a 0.65 mL/min isocratic elution using 12% 1:4 MeOH:DCM and 88% hexane. Fluorescence detection was used to determine surrogate recovery during this fractionation. A fraction of HPLC effluent was collected between 7 and 16 min, and 3.0 μg of 4-*tert*-butyl orthocresol was added as an internal standard and concentrated under nitrogen to 100 μL of isooctane. Compounds of interest were identified and quantified using a HP 5890 series II Plus GC and a HP 5972 mass selective detector (MSD). Separation was accomplished using a 30-m DB-17MS capillary column (0.25 mm i.d., 0.15- μm film, J&W Scientific, Folsom, CA). The GC was held at 100 $^{\circ}\text{C}$ for 2 min and ramped to 300 $^{\circ}\text{C}$ at 4 $^{\circ}\text{C}/\text{min}$. The MSD was operated in selected ion monitoring (SIM) mode with three ions monitored for each compound of interest. Internal standard recoveries were greater than 80%. Matrix spike recoveries were greater than 70% for NP and NPE_{1-2} and 17% for NPE_3 . Method detection limits (MDLs) for NP, NPE_1 , NPE_2 , and NPE_3 were 3.3, 16.8, 18.2, and 20.6 ng/g, respectively.

Results and Discussion

All residues in concentrations are reported on a wet weight basis. Concentrations of NP greater than the MDL were found in 75 of 183 (41%) samples across all sites and species with a mean concentration, excluding nondetects, of 12.0 ng of NP/g with a range of 3.3–29.1 ng of NP/g (Figure 2). If nondetects are included, the mean NP concentration across

TABLE 1. Detectable Concentrations of NP in Tissue among Species

species	mean tissue concn (ng/g, ww) ± SD	no. of samples analyzed	no. of samples <MDL
longnose sucker (<i>Maxostoma macrolepidotum</i>)	<3.3	2	2 (100%)
green sunfish (<i>Lepomis cyanellus</i>)	<3.3	4	4 (100%)
bluegill sunfish (<i>Lepomis macrochirus</i>)	5.7 ± 5.2	36	12 (33%)
smallmouth bass (<i>Micropterus dolomieu</i>)	5.8 ± 5.2	27	11 (41%)
white sucker (<i>Catostomus commersoni</i>)	7.2 ± 5.2	60	23 (38%)
rainbow smelt (<i>Osmerus mordax</i>)	7.7 ± 4.0	5	2 (40%)
rock bass (<i>Ambloplites rupestris</i>)	8.1 ± 5.3	49	31 (63%)

all sites and species is 4.0 ng of NP/g. NPE₁ was found in 21 samples but all at concentrations less than the calculated MDL (16.8 ng of NPE₁/g). NPE₂ and NPE₃ were not detected in any of the samples.

Five of the seven species contained detectable concentrations of NP. Rock bass (*A. rupestris*) contained the greatest average detectable NP concentration, 8.1 ng of NP/g, while the rainbow smelt (*O. mordax*) exhibited the second greatest detectable tissue concentration at 7.7 ng of NP/g (Table 1). Longnose sucker (*M. macrolepidotum*) and green sunfish (*L. cyanellus*) contained no detectable concentrations of NP.

There were no significant differences in concentrations of NP among species so the samples were pooled for comparison among sites (ANOVA with a type I error of 0.1 followed by a Tukey's Multiple Range Test). There were significant differences in NP concentrations, however, among sampling sites (Figure 2).

The greatest average concentrations were found in fish from the section of the river near Kalamazoo and Battle Creek. While these cities employ advanced wastewater treatment technologies, industrial discharges, either through the municipal WWTP or direct effluent discharge may have contributed to greater concentrations of nonylphenolic compounds near these cities.

Estimated Water Concentrations and Bioconcentration Factors. On the basis of the concentrations of NP measured in fish and an assumed bioconcentration factor (BCF), concentrations of biologically available NP were estimated. BCFs for NP have been reported earlier (9–12). A BCF value of 300 was used as an approximate estimate for various species. The BCF for bluegill sunfish and fathead minnow (*Pimephales promelas*) have been estimated as 220 (11) and 271 (12), respectively. To validate this BCF value, fathead minnows were exposed to a nominal 10 ng of NP/mL for 29 days. Further details of exposure and analysis of fish are given elsewhere (13). These fish were prepared and extracted as described above. The resulting cross-section tissue NP concentration was 2.93 µg/g (n = 4), which corresponds to a BCF of 293.

By applying a BCF of 300 and the least and greatest observed tissue concentrations of 3.3 and 29.1 ng/g, respectively, the estimated water concentrations ranged from 11 to 97 ng of NP/L, which is comparable to the values reported by others (5, 14).

Ranges of Possible Concentrations. Since concentrations of NP in some fish were less than the MDL, there were uncertainties associated with presenting actual concentration ranges. Proxy values were substituted for samples for which the actual concentrations were nondetectable. These proxy values are chosen such that the greatest possible ranges of concentration were calculated. Samples containing nondetectable concentrations were first assigned the least possible concentration of zero, which returned the most conservative estimate. These same samples were then assigned the greatest possible nondetectable concentration, the MDL for that analyte, which returned the least conservative estimate. Samples that had detectable concentrations were not altered.

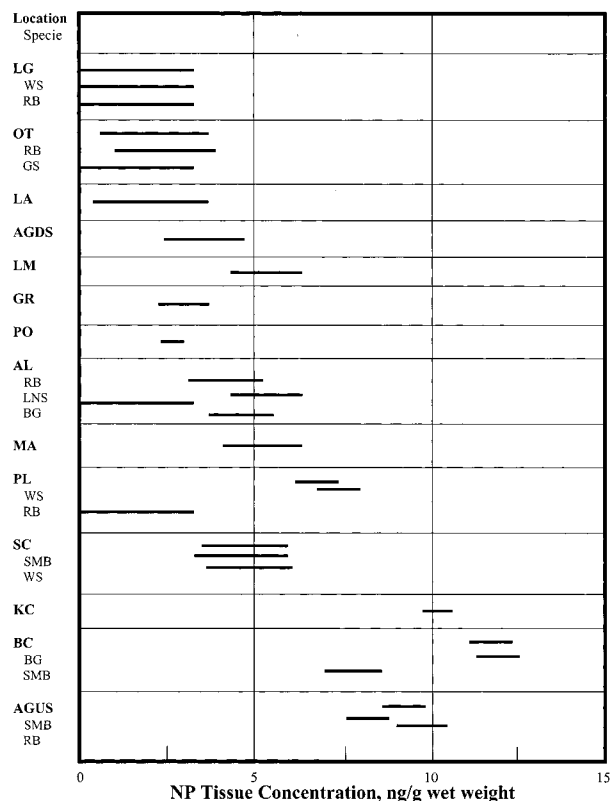


FIGURE 3. Proxy value ranges of NP tissue concentrations among sites and species. Species: WS, white sucker; RB, rock bass; GS, green sunfish; LNS, longnose sucker; BG, bluegill sunfish; SMB, smallmouth bass.

A nonparametric Wilcoxin's Rank Test was performed on each site and species to determine a range of possible concentrations (Figure 3). When using this proxy value substitution, tissue concentrations among all species and sites ranged from 4.4 to 6.4 ng of NP/g. Concentrations of NPE_{1–3} were all less than their respective MDLs. The estimated ranges were 0.0–16.8, 0.0–18.2, and 0.0–20.6 ng/g, respectively, for NPE₁, NPE₂ and NPE₃.

The results of this study suggest the presence of nonylphenolics in fish but at relatively small concentrations. NP is the predominant compound, with concentrations of NPEs less than those of NP. Fishes collected near WWTP effluent discharge sites contain relatively greater concentrations than those collected from more remote areas. Actual measured concentrations of NP in the water and sediments would allow better interpretation of the fish tissue data by providing a direct correlation with environmental exposure.

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