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Estrogenic Alkylphenols in Fish Tissues, Sediments, and Waters from the U.K. Tyne and Tees Estuaries

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Nonylphenols and related compounds are common products of biodegradation of a large group of nonionic surfactants, the nonylphenol polyethoxylates. Many of these compounds are known to be environmentally persistent and to elicit estrogenic response in both mammals and fish. In this study, nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), and octylphenol (OP) were found in tissues of mature male flounder, *Platichthys flesus* (5−55 ng/g NP, 190−940 ng/g NP1EO, wet weight), and in tissues of juvenile flounder (30−180 ng/g NP, wet weight). These fish also showed detectable levels of the yolk protein vitellogenin in their plasma, indicative of estrogenic exposure. The compounds were also found in discharges from a major sewage treatment works (3000 ng/L NP, 45 000 ng/L NP1EO) and in sediments from two estuaries in north-east England; the highest levels from the highly industrialized Tees (1600−9050 ng/g NP, 125−3970 ng/g NP1EO, 30−340 ng/g OP, dry weight) and lower levels from the industrialized/urbanised Tyne estuary (30−80 ng/g NP, 160−1400 ng/g NP1EO, 2−20 ng/g OP dry weight). The implications of these findings for fish populations are discussed.

Introduction

Alkylphenol polyethoxylates (AP*n*EOs) comprise one of the largest volume surfactants in production, and, as such, are commonly used in a variety of industrial, agricultural, and household applications (*1*). Over 300 000 tons are produced worldwide annually, with a U.K. production of nonylphenol polyethoxylates (NP*n*EO, $n = 9-10$) of 18 000 tons (*2*). Approximately 50% of the total U.K. production is used in industrial and institutional cleaning products which are disposed of to sewer for subsequent treatment at local sewage treatment works (STWs), and ultimately discharged into the aquatic ecosystems (*2*). During sewage treatment, NP*n*EOs are biodegraded by the hydrolytic removal of ethoxylate groups into short-chain ethoxylates, carboxylic acid derivatives, and finally to alkylphenols themselves (*1*), forming increasingly more lipophilic and persistent metabolites (*3, 4*). These breakdown products are ubiquitous contaminants in the aquatic environment and have been detected not only in sewage effluents but also in surface waters and sediments at concentrations ranging from nanograms to milligrams per liter (*5*).

Recent studies have strongly indicated that several alkylphenolic compounds possess estrogenic activity. Investigations with male rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Salmo salar*), and Atlantic cod (*Gadhus morhua*) have confirmed that alkylphenols are capable of inducing synthesis of the yolk protein vitellogenin (*6*−*8*), which serves as a sensitive biomarker for exposure to "estrogens" (*9*). These compounds have also the ability to inhibit testicular growth in rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), and flounder (*Platichthys flesus*) (*6, 10*; Lye et al., in preparation), and to induce the formation of egg cells in testis of juvenile Japanese medaka (*Oryzias latipes*) (*11*). In light of recent findings suggesting that estrogenic effects in wild fish are more widespread than previously thought (*12*−*14*), the presence of these compounds in the aquatic environment, and their subsequent uptake in aquatic organisms, is of significant concern.

Despite high inputs of NP*n*EOs in the aquatic environment and their potential estrogenic breakdown products, there are no data on the levels of alkylphenols in the sediment column of U.K. estuaries, or in estuarine biota living in close contact with, and feeding on, contaminated sediments. This study focuses on the concentrations of nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), and octylphenol (OP) in sediment, water, and fish tissue (the flounder, *Platichthys flesus*) from two major estuaries in the north-east of England: the industrialized/urbanised Tyne and the highly industrialized Tees estuaries. The concentrations of these compounds were also investigated in the effluent of the major STW in the Tyne at Howdon.

Materials and Methods

Chemicals. The standards used in this study were Marlophen NP3 (ethoxylated 4-nonylphenol containing ethoxylated oligomers with 1−7 ethoxy units) (Hüls 95/143343, Marl, Germany), 4-NP (Fluka, U.K.) and 4-OP (Fluka, U.K.; >90%), and 2,4,6-tribromophenol (Aldrich, U.K.; 99%), which was used as the internal standard. Methanol, dichloromethane (DCM), and petroleum ether (light petroleum spirit, boiling point 40−60 °C) were purchased technical grade (Beveridges Ltd., U.K.) and redistilled prior to use. Cyclohexane was purchased as Distol grade solvent (Fisher, U.K.) and used as purchased. Anhydrous sodium sulfate (BDH; AnalaR grade) was heated to 300 °C overnight and stored in a desiccator prior to use. Neutral alumina (BDH) was heated to 300 °C overnight, stored at 120 °C, and deactivated with 15 wt % distilled water immediately prior to use for column chromatography.

The analytical method was adapted principally from the methods of Giger et al. (*15*), Stephanou and Giger (*16*), Ahel and Giger (*17, 18*) and Marcomini and Giger (*19*).

Study Sites and Sample Collection. Analyses were carried out on two suites of samples, one from the Tyne and one from the Tees, sampled in April and June 1997 by R. V. Bernicia (University of Newcastle) and R. V. Water Guardian (U.K. Environment Agency), respectively. Samples consisted of a series of sediment grabs taken at four sites upstream from near the outfall of Howdon STW to Ouseburn (the Tyne) and downstream from near Bamlett Bight to beyond the estuary mouth at Redcar Jetty (the Tees) (for location see Figure 1). Two replicate 0.1 m^2 sediment samples were taken at each

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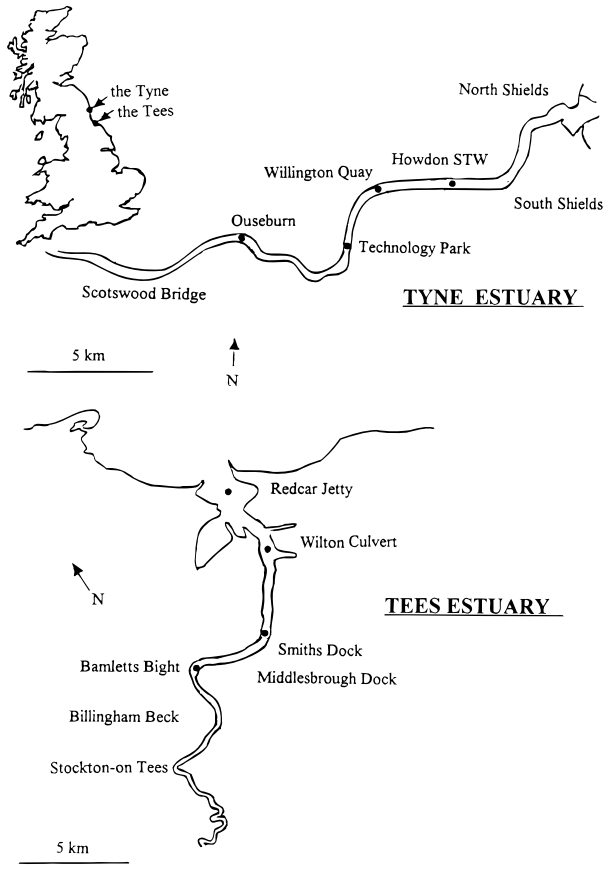

FIGURE 1. Map of sampling locations in the Tyne and Tees estuaries.

site using a van Veen grab; aliquots from the top 10 cm of the sample were collected. Surface water was collected from the immediate vicinity of the discharge from Howdon STW in the Tyne using prerinsed polycarbonate bottles, and 5 L of final effluent was obtained from Howdon STW. Fish were collected from both estuaries by beam trawl, and their tissues were homogenized. Sexually mature male flounder, *Platichthys flesus* ($n = 6$, length >250 mm), collected from the Tyne showing high levels of the female yolk protein vitellogenin (vtg), indicative of estrogenic exposure (see recent papers of Lye et al., e.g., *13*, *14*), were analyzed in this study. Despite trawls over a period of 1 week in the Tees, the catch was very poor ($n = 11$) and was restricted to small fish (<100 mm) being too small to bleed for vitellogenin analysis.

Solvent Extraction. Extractions were performed by 3−5 h reflux using a modified Nielsen−Kyger steam distillation apparatus (Ace Glass Inc.; Code 6555; Vineland, U.S.A.) with a 2 L flask and a condenser with a 15 mL solvent holding capacity (*17*). The extraction solvent used was cyclohexane. Water samples and sewage effluents were of 2 and 1 L, respectively, whereas sediment (49−60 g) and fish tissue (2.4−20 g) were extracted as slurry in 1−1.5 L of distilled water. Procedural blanks were run between every four extractions. After extraction, extracts were run down a short anhydrous sodium sulfate-drying column.

Chromatographic Cleanup. Cleanup (*17*) was performed by deactivated neutral alumina column chromatography using 5.6 g of adsorbent slurry packed into a 10 mm i.d. column in petroleum ether. The sequential elution scheme used was 30 mL of petroleum ether, DCM, and methanol, respectively. The second eluate of 30 mL of DCM contained AP and AP*n*EO.

GC-FID and GC-MS Analysis. Analysis was performed by gas chromatography with flame ionization (GC-FID) and mass spectrometry (GC-MS) detection for quantification and confirmation, respectively. The GC-FID was performed by on-column injection (1 μL) of the extract in DCM (1 mL). The GC used was a Carlo Erba 5160 fitted with a 30 m fused silica capillary column of internal diameter 0.25 mm coated with a 0.25 μm film thickness of HP-5 (Hewlett Pachard 5% phenylmethyl silicone). Hydrogen was used as a carrier gas with an approximate 1 mL/min flow rate and 50 kPa inlet pressure. The GC oven temperature was programmed at 50−300 °C at 4 °C/min, were it was held for 10 min. GC-MS was performed by on-column injection (1 μL) of the extract in DCM (1 mL). The GC conditions were identical to those used in the GC-FID analysis except that helium was used as the carrier gas. The mass spectrometer was a Fisons Trio 1000 operated with an ionization energy of 70 eV. Analyses were run either in the full scan mode, scanning from m/z 45 to m/z 500, or in the selected ion monitoring mode (SIM mode). Standards were analyzed to determine fragmentation patterns and principal ions that could be used for selected ion monitoring (SIM) GC-MS analysis. Fragment ions were chosen according to the most abundant ions in each oligomer. These ions were m/z 107, 121, 135 (NP); 107, 179, 193 (NP1EO); 107, 135, 206 (OP); and 141, 222, 332 (TBP).

GC-FID analysis (e.g., Figure 2) was able to resolve OP, NP, and NPE_{1-6}. Response factors relative to the TBP standard were determined for OP, NP, and NP*n*EO standards for quantification. The NP compounds exist as a separate standard; hence, GC-FID response factors, relative to the TBP internal standard, can be determined directly for individual oligomer peak group according to the formula: $RF = A_{is}M_n/A_nM_{is}$ where RF is the response factor for the oligomer (n), A_n and A_{is} are the peak areas for the oligomer peak group and internal standards, respectively, and M_n and M_{is} are the amounts of oligomer and internal standards, respectively. However, as the Marlophen NP3 standard is a mixture of NP*n*EO ($n = 1-7$), direct determination of the GC-FID response for each oligomer peak group is not possible. In this study the GC-FID response to each NPnEO oligomer was assumed to be equal, as individual ethoxylate standards were not available. Hence, levels of NP1EO were calculated from responses and response factors determined from the analysis of the Marlophen NP3 standard and are therefore likely to be a slight overestimate of the true level of NP*n*EO contamination in the samples analyzed. The concentrations in this study were determined by integrating the peak areas of the oligomer in the m/z 107 (NP and NP1EO components), 135 (OP component), and 322 (TBP internal standard) mass chromatograms. The quantification was made from GC-MS analysis by rationing the analyte peak areas against the internal standard peak areas, using a response factor measured from the standard mixture. For quantification using the internal standard, the quantifiable limit of detection was taken as the minimum amount of standard required to give 95% signal reproducibility, or 50 times the base line variation.

Extraction Recoveries and Reproducibility. The recoveries (percent of standard added to sample recovered during extraction) and reproducibility (percent RSD, e.g., relative standard deviation for triplicate analysis) for the extraction step of the method were determined by 3 h steam distillations of distilled water (2 L) and fish tissue (5−10 g) spiked with methanol (50 μL) containing 0.0935 mg of OP, 0.7 mg of NP, and 2.32 mg of Marlophen NP3. The extracts were analyzed by GC-FID without column cleanup. The results of triplicate extraction of distilled water, e.g., NP (104, 2.8), NP1EO (102,1.9), and OP (103, 1.1), and fish tissue, e.g., NP (98, 28.1), NP1EO (98, 4.7), and OP (97, 3.7), show efficient recovery of these compounds.

Cleanup Separation and Recoveries. In the neutral alumina column chromatography cleanup, OP, NP, and Marlophen NP3 standards (0.0935 mg of OP, 0.7 mg of NP,

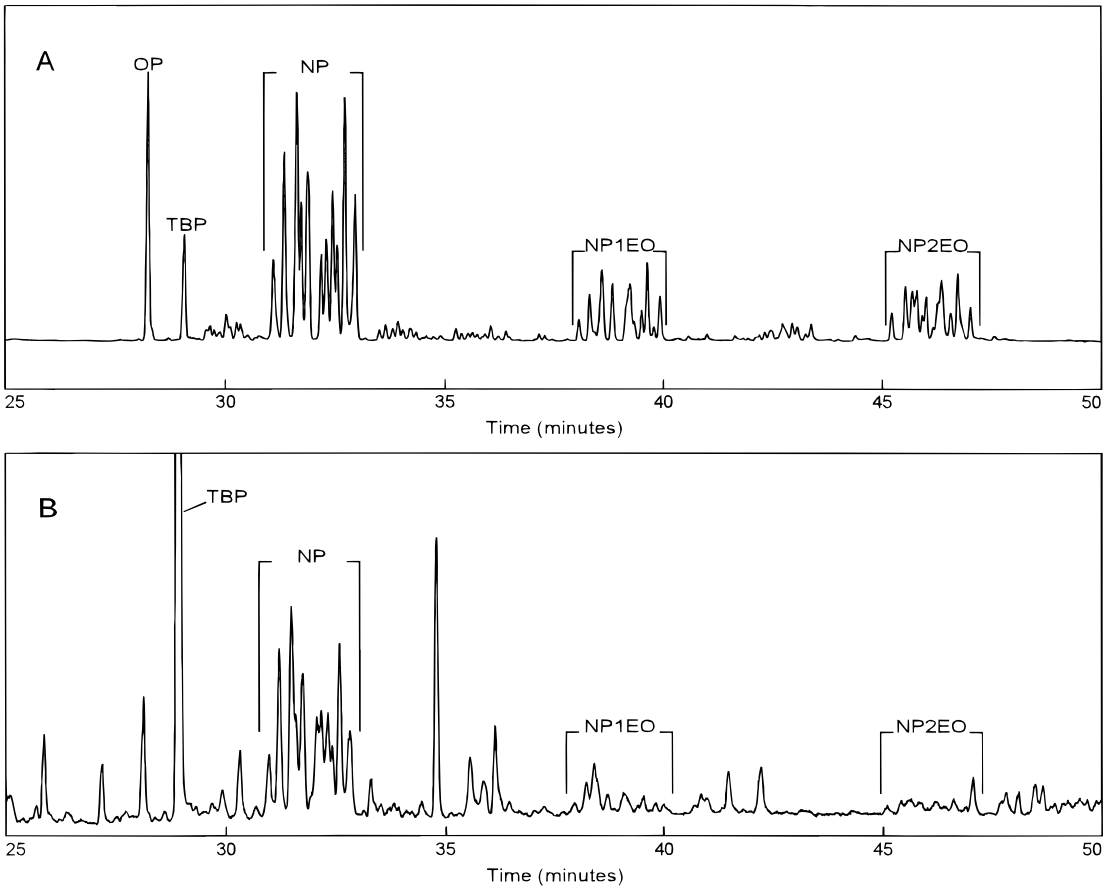

FIGURE 2. Expanded GC-FID chromatogram showing nonylphenol (NP) from (A) water spiked with a nonylphenol standard mixture and (B) from Tees sediment.

TABLE 1. Concentrations[a] of NP, NP1EO, and OP in Fish Tissue, Sediment, Sewage Effluent, and Water Samples from the Tyne

type of sample	no. of samples	NP	NP1EO	OP
homogenized muscle tissue	6	5−55	nd[b]	nd
homogenized liver tissue	6	10−30	190−940	nd
sediment I, Howdon STW	3	60 ± 20	160 ± 65	5 ± 0.6
sediment II, Willington Quay	2	30 ± 1	610 ± 65	2 ± 0.1
sediment III, Technology Park	2	80 ± 20	1400 ± 460	20 ± 1
sediment IV, Ouseburn	2	65 ± 20	910 ± 190	12 ± 4
sewage effluent	2	3000 ± 850	45000 ± 16000	nd
water (adjacent the outfall)	2	130 ± 50	940	nd

[a] Concentrations are expressed as ng/g wet weight for fish tissue, ng/g dry weight for sediments, and ng/L for sewage effluent and water. [b] nd = not detectable.

and 2.32 mg of Marlophen NP3 as used in extraction tests) elute primarily in the DCM fraction. Mean recoveries of alkylphenols from spiked distilled water were in the range of 93−99%.

Results

GC-MS total ion current (TIC) chromatograms of the different extracts from the Tyne and the Tees revealed peaks in positions corresponding directly with NP, NP1EO, and OP standards. TIC traces were then correlated with the ion chromatograms of the principal ions associated with each compound, which also revealed peaks at the same positions as the standard and provided confirmation of peak identify.

Fish. NP (5−60 ng/g, wet weight) was detected in muscle and liver tissue of flounder from the Tyne in 3 out of 6 extracts and NP1EO (190−940 ng/g wet weight) in liver tissue in 5 out of 6 extracts examined (Table 1). Due to poor catches in the Tees, homogenized fish tissue in the Tees included both muscle and liver tissue. NP was present (30−180 ng/g wet weight) in 6 out of 11 fish samples (Table 2).

Sediment. All three compounds measured were found in the sediment samples analyzed from the Tyne, NP, and NPE1O at levels similar to those in fish (e.g., 30−80 and 160−1400 ng/g dry weight) and OP ranging between 2 and 20 ng/g dry weight (Table 1). No clear pattern could be detected in sediment concentrations although the levels of NP1EO and OP were elevated at the Technology Park site in the Tyne compared to other sites (Figure 3). Concentrations of NP in sediment samples from the Tees were generally 20−300 times higher than the sediment samples collected in the Tyne (Table 2). The highest NP concentration recorded was 9050 ng/g dry weight at Bamletts Bight and decreased seaward, so that the lowest NP concentration, 1600 ng/g dry weight, was recorded at Redcar Jetty at the mouth of the estuary. A similar pattern was found in the concentrations of NP1EO (3970 and 120 ng/g dry weight) and OP (340 and 30 ng/g dry weight)

TABLE 2. Concentrations[a] of NP, NP1EO, and OP in Fish Tissue and Sediment Samples from the Tees

type of sample	no. of samples	NP	NPE1O	OP
homogenized fish tissue	11	30 ± 180	nd[b]	17[c]
sediment I, *Redcar Jetty*	2	1600 ± 320	120 ± 40	30 ± 10
sediment II, *Wilton*	2	3360 ± 110	220 ± 60	110 ± 30
sediment III, *Smiths Dock*	2	4830 ± 5	890 ± 250	230 ± 10
sediment IV, *Bamletts Bight*	2	9050 ± 1140	3970 ± 1990	340 ± 60

[a] Concentrations are expressed as ng/g wet weight for fish tissue and ng/g dry weight for sediments. [b] nd = not detectable. [c] One specimen.

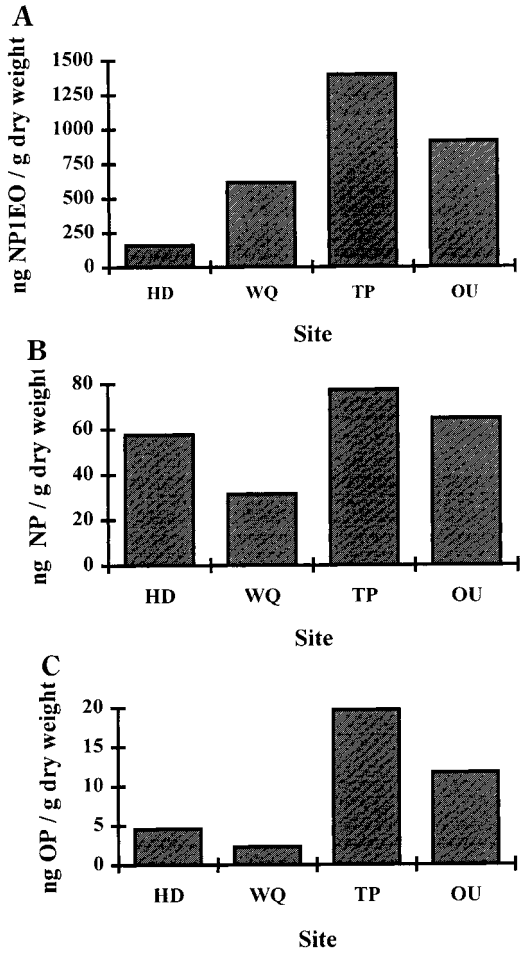

FIGURE 3. Concentrations of (A) nonylphenol monoethoxylate (NP1EO), (B) nonylphenol (NP), and (C) octylphenol (OP) (ng/g dry weight) in sediment samples from the mouth of Tyne Estuary and further upstream (HD = Howdon, WQ = Willington Quay, TP = Technology Park, Ou = Ouseburn).

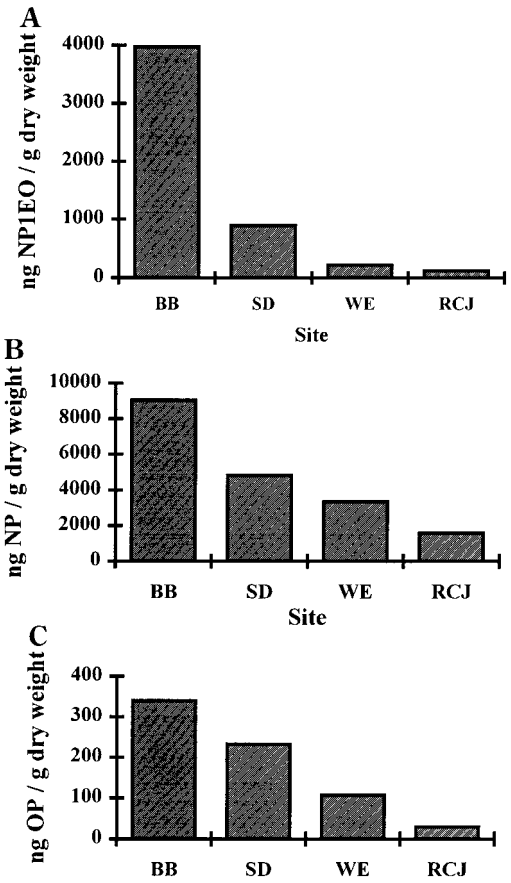

FIGURE 4. Concentrations of (A) nonylphenol monoethoxylate (NP1EO), (B) nonylphenol (NP), and (C) octylphenol (OP) (ng/g dry weight) in sediment samples from the Bamlett Bight of the Tees Estuary and further downstream. (RCJ = Redcar Jetty, WE = Wilton, SD = Smiths Dock, BB = Bamlett Bight).

in sediment samples. The distribution of the measured NP, NP1EO, and OP concentrations on the estuary profile showed a clear trend from high concentrations at Bamletts Bight to low concentrations at Redcar Jetty (Figure 4).

Sewage Effluent and Water Samples. The NP and NP1EO concentrations in the treated sewage effluent samples from Howdon STW, Tyne estuary, were 3000 and 45 000 ng/L, respectively (Table 1). Equivalent figures for water samples from the Tyne adjacent to the outfall were 130 and 940 ng/L. OP was not detectable in these samples (Table 1).

Discussion

In this study, concentrations of nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), and octylphenol (OP) have been measured in sediment samples from two estuaries in the north-east of England, the Tyne and the Tees, with the highest concentrations consistently being found in the Tees. The concentrations recorded in the Tees sediment varied in the range of 1600−9050 ng/g dry weight for NP, 120−3970 ng/g dry weight for NPE1O, and 30−340 ng/g dry weight for OP. These concentrations are consistent with those of Blackburn and Waldock (*20*), who measured levels of NP (5.2 μg/L) and OP (10 μg/L) in Tees water. Although the concentrations in the sediments were high, they are within the same range (190−13 100 ng/g d.m. NP) as the levels found in sediment samples from contaminated areas close to municipal STWs and industries in Europe and the United States (*21*, *22*), and still lower compared to the levels (up to 37 μg/g NP; 23 μg/g OP) reported in Great Lakes sediment (*23*).

The elevated concentrations in the Tees sediment are likely to be attributed to a number of sources, only some of them identified. Blackburn and Waldock (*20*) recently reported that effluent from three randomly selected outfalls in the Tees, including a STWs outfall, contained levels between 0.3 and 27 μg/L NP. Other sources may result from inputs from

surfactant manufacture in the Tees (*2*) as the major U.K. production works for nonylphenol is sited at Billingham, and nonylphenol is ethoxylated to NP1EO at Wilton (*2*). Washout of the reactors effluent has been reported to contain on average 0.5% of the NP1EO products (*2*). The high concentrations recorded for NP, NP1EO, and OP at Billingham diminished markedly with distance downstream (Figure 4), indicating that activities at this site could be a significant contributor to the inputs of alkylphenols in the Tees. The relatively lower abundance of octylphenol compared to NP and NP1EO at all sites probably reflects the lower commercial use of octylphenol polyethoxylate (20%) compared to NP*n*EO (80%) (*5*).

The NP, NP1EO, and OP concentrations measured in the Tyne sediments (30−80 ng/g dry weight of NP, 160−1400 ng/g dry weight of NP1EO, and 2−20 ng/g dry weight of OP) were lower than those measured in the Tees and are likely to be at least partly attributed to the presence of NP (3 μg/L) and NP1EO (45 μg/L) in treated effluent extracts from the major STW on the Tyne. These levels are comparable to concentrations (<0.2−330 μg/L NP) reported in effluents discharged into other U.K. rivers (*21*) and consistent with results from previous studies in which sewage effluents from Europe and the United States have been analyzed (*21*, *24*, *25*).

The presence of elevated concentrations of NP and NP1EO in sediment samples from both the Tyne and Tees estuaries compared to their concentrations in water samples from the same area (*20*) is not surprising, since NP and NP1EO are hydrophobic [logarithm of octanol/water partition coefficient (log K_{ow}) of 4.0−4.6 (*26*)] and have a tendency to become associated with particulate matter and ultimately sediment (*5*). This property, coupled with the fact that ultimate biodegradation of NP and NP1EO to CO_2 and H_2O proceeds slowly because of the slow breakage of the phenol ring (*1*), may result in the sediment acting as a sink for these compounds. This means that flounder could be exposed to estrogenic alkylphenols both directly from the sediments in which they live, as observed with other sediment-associated contaminants (e.g., organochlorines, *27*), and indirectly via the sediment-dwelling invertebrates on which they feed (*28*).

In this study, low but detectable levels of NP (5−180 ng/g) and NP1EO (190−940 ng/g) were found in fish from the Tyne and Tees estuaries, demonstrating an uptake either via gills, diet, or both. Furthermore, since the concentrations measured in some of the sediments in this study (e.g., up to 9 μg/g NP) are within the same magnitude as the concentrations (1−10 μg/L NP) required to produce biological effects in rainbow trout (*Oncorhynchus mykiss*) in vivo (*6*, *29*), it is possible that at least in the Tees sediments, concentrations of NP could be high enough to approach chronic effect levels to wild fish. Therefore, although factors such as bioavailability and the possibility of biomagnification of these compounds by sediment-dwelling invertebrates, and a major difference in species sensitivity to estrogenic alkylphenols (*8*, *12*, *29*) have not been taken into account in this study, the concentrations measured in some of the sediment samples could raise concern about the health of exposed flounders for several reasons. First, detectable levels of NP were found in the tissue of juvenile flounder. It is well-known that developmental stages, including embryonic, larval, and juvenile stages, are more sensitive to chronic estrogenic exposure than adults (*30*), and that NP exposure in early life stages can result in lethality prior to estrogenic responses (*31*). Although the present study was unable to confirm estrogenic responses in juvenile flounder from the Tees, extremely high levels of vitellogenin (>100 000 ng/mL) and the presence of ovarian tissue in testes of male flounders from the Tees have recently been demonstrated by other investigators (*12*). Second, concentrations of NP and NP1EO, known to elicit estrogenic responses, were recorded in tissue of male fish from the Tyne that also showed strong vitellogenin induction (*13*, *14*), thus suggesting exposure to estrogenic xenobiotics.

However, recent studies in the U.K. have revealed that the natural (estrone and 17β-estradiol) and synthetic (ethinyl estradiol from contraceptive pills) estrogens excreted by humans are present in domestic sewage effluent (*31*) and that the concentrations found are high enough to induce vitellogenin (*29*, *33*). Many endocrine-disrupting substances are also strongly lipophilic and adsorptive, and therefore preferentially accumulate in tissue (*34*, *35*) and sediment (*36*, *37*). This increases the probability that estuarine fish are simultaneously exposed to a very complex mixture of estrogenic compounds, which sometimes may have even greater than additive effects (*38*). The estrogenic responses demonstrated in the Tyne and the Tees (*12*−*14*) are therefore unlikely to be accounted for solely by the presence of NP and NP1EO as measured in this study. Nevertheless, although levels of NP, NP1EO, and OP measured in the Tyne and in some Tees sediments may be at individually harmless concentrations, the possibility remains that these compounds could exert some joint action with other estrogenic substances, thereby contributing to the overall effect of environmental estrogens in the aquatic environment.

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