Journal of Chromatography A, 1217 (2010) 112–118

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Gas–liquid chromatography–tandem mass spectrometry methodology for the quantitation of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents and from wild populations

Kate A. Fenlon [a], Andrew C. Johnson [b], Charles R. Tyler [c], Elizabeth M. Hill [a,*]

[a] School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK
[b] Centre for Ecology and Hydrology, CEH Wallingford, Wallingford OX10 8BB, UK
[c] School of Biosciences, University of Exeter, Exeter EX4 4PS, UK

A R T I C L E I N F O

Article history:
Received 6 July 2009
Received in revised form 1 October 2009
Accepted 23 October 2009
Available online 30 October 2009

Keywords:
Bile
Fish
Estrogen
GC–MS/MS
Alkylphenol
Bisphenol

A B S T R A C T

Fish can be exposed to a complex mixture of chemical contaminants arising from the exposure to wastewater treatment works (WwTWs) effluents. Some of these contaminants are estrogenic and have been associated with feminisation of male fish and the presence of populations containing intersex individuals. However the detection of trace levels (ng/L) of estrogenic chemicals surface waters can be difficult and does not give information on the exposure of aquatic organisms to these contaminants. In this study we assessed whether the analysis of estrogenic substances that bioconcentrate in fish bile can be used to detect the exposure of fish to feminising contaminants in receiving waters and effluents, and thus facilitate their monitoring of these substances in aquatic environments. Estrogenic metabolites in bile were deconjugated using enzymatic hydrolysis and partially purified by solid phase extraction. Steroidal and xenoestrogens were derivatized to their trimethylsilyl ethers and quantified by gas–liquid chromatography–mass spectrometry (GC–MS/MS) using multiple reaction monitoring. The method was validated using spiked bile samples from immature female rainbow trout (*Oncorhynchus mykiss*) as well as bile from sexually mature roach (*Rutilus rutilus*) that had been exposed to either tap water or an undiluted estrogenic effluent for 10 days or captured from a river site downstream of a WwTWs effluent discharge. The mean recovery of target analytes from spiked bile was between 86 and 99% and the limit of detection was between 0.1 and 0.7 ng/mL bile for bisphenol A (BPA), 17β-estradiol (E2), estrone (E1) and 17α-ethinylestradiol (EE2), and 11, 60 and 327 ng/mL bile for branched nonyl chain isomeric mixtures of 4-nonylphenolethoxylate (NP1EO), 4-nonylphenol (NP) and 4-nonylphenoldiethoxylate (NP2EO), respectively. All target analytes were detected in bile from roach exposed directly to a WwTWs effluent, with concentrations between 6–13 μg/mL bile for NP, 18–21 μg/mL for NP1EO, 75–135 μg/mL for NP2EO, 0.7–2.5 μg/mL for BPA, E2 and E1 and 17–29 ng/mL for EE2. With the exception of NP2EO, all analytes were detected in at least 2 out of the 5 fish sampled from the River Thames. BPA and NP1EO were detected in all three reference fish held in tap water indicating possible contamination from laboratory plastics. The work shows that analysis of 20–100 μL quantities of bile could be a useful approach in detecting exposure to mixtures of estrogenic contaminants taken up by fish from WwTW effluents and has the potential for monitoring the efficacy of remediation strategies that may be adopted for reduction of these endocrine disrupting chemicals in the aquatic environment.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The detection of estrogenic responses in fish exposed to wastewater treatment works (WwTWs) effluents was first reported in the UK in peer-reviewed literature in 1994 [1]. Since then, sexual development has been shown to be affected in wild fish populations exposed to WwTWs effluent discharges in a number of countries, and in both freshwater and estuarine environments [2]. One of the most widely reported feminised phenotypes is the condition of intersex, where both male and female sex tissues are contained within the same gonad, and this has been shown to be as a result of exposure to endocrine disrupting compounds which are introduced into the aquatic environment predominantly from WwTWs effluents and industrial outputs [3]. Fish captured or held below WwTWs discharges biosynthesize high levels of plasma vitellogenin, a biomarker of estrogen exposure, and bioconcentrate estrogenic substances [3,4] which include the naturally

* Corresponding author at: School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK. Tel.: +44 1273 678 382; fax: +44 1273 678 511.
E-mail address: E.M.Hill@sussex.ac.uk (E.M. Hill).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.10.063

produced estrogens estrone (E1) and 17β-estradiol (E2), the pharmaceutical estrogens 17α-ethinylestradiol (EE2), equilenin and 17β-dihydroequilenin, and the industrial compounds nonylphenol (NP), nonylphenol ethoxylates (NPEOs) and bisphenol A (BPA) [5–7]. The most potent of these compounds are the steroidal estrogens, and in comparison, the estrogenic activity of non-steroidal phenolics such as NP, NPEOs and BPA are relatively weak [8]. Exposure to EE2, at environmentally relevant concentrations has been shown to induce intersex in roach, a species indigenous to UK, and to cause complete gonadal sex reversal at an exposure to 4 ngEE2/L (a concentration sometimes recorded in the most polluting effluents) [9]. However, there is evidence that mixtures of chemicals with similar modes of action can act in an additive manner indicating the importance of determining the exposure to all the estrogenic contaminants that could bioconcentrate in effluent-exposed fish [10,11].

The estrogens E1 and E2, as well as the birth control pharmaceutical EE2, are excreted by humans as conjugated metabolites. However, during transport and processing at the WwTWs, these metabolites are hydrolysed back to the parent compounds resulting in concentrations in UK final WwTWs effluents ranging from 0.1 to 90 ng/L for E2, 1 to 80 ng/L for E1 and <0.1 to 4.3 ng/L for EE2 [12–16]. NP and NPEOs were widely used surfactants which have now been included on the EU's list of priority pollutants and have restricted use; however, their concentrations in WwTWs effluents been reported at between 0.2 and 230 μg/L [13–15]. BPA is mainly used in the synthesis of polycarbonate plastics and epoxy resins and its concentrations in German WwTWs effluents have been reported to be between 0.01 and 1 μg/L [17,18]. The concentrations of estrogenic contaminants in river water are expected to be orders of magnitude lower, and a recent survey of EU rivers detected E1 in 16% of samples with a mean concentration of 4 ng/L, BPA in 34% of samples with a mean of 25 ng/L, and NP in 29% of samples with a mean of 134 ng/L, however, E2 and EE2 were not detected in any samples [19]. In other studies, E1 has been detected in river water at concentrations of <0.4–12.2 ng/L, E2 at 0.4–4.3 ng/L and EE2 at 0.4–3.4 ng/L [20,21].

The low levels of estrogenic chemicals in river water make measurement and monitoring of these contaminants in the environment challenging. It has, however, been demonstrated that much higher concentrations of estrogenic compounds may accumulate in fish bile, and in laboratory studies the bioconcentration of waterborne alkylphenols in bile was between 20,000- and 70,000-fold [22–26]. In fish exposed to WwTW effluents, the concentration factors in bile were 4000–6000-fold for EE2 and 10,000–13,000-fold for E2 and E1 [5,6]. The dominant metabolites of these phenolic contaminants in fish bile are phase II metabolites which are mainly conjugates of glucuronic acid, and hydrolysis of bile results in release of the parent compounds which can be readily quantified [27,28]. Analysis of fish bile has also been used for the identification and quantitation of polycyclic aromatic hydrocarbons [29,30] chlorinated phenols [31], and resin and fatty acids from pulp and paper mill effluents [32] and represents a snap shot of the recent exposure of the animal to mixtures of bioavailable contaminants in the environment.

The aim of this study was to determine whether analysis of microlitre amounts of fish bile can be used to detect exposure to mixtures of estrogenic compounds originating from WwTWs effluents, and thus enables a new and more sensitive method for the determination of exposure to these environmental contaminants. We refined an analytical method [33], previously validated for the quantitation of estrogenic chemicals in wastewater samples, and used GS–MS/MS instead of GC–MS to increase the selectivity and sensitivity of the analysis. Estrogenic contaminants in bile samples were deconjugated and extracted on an OASIS HLB solid phase extraction (SPE) cartridge. Following derivatisation, samples were analysed by GC–MS/MS using multiple reaction monitoring mode (MRM). The analytical method was tested on bile samples from roach (*Rutilus rutilus*) held in cages downstream of a WwTW effluent and from fish captured from the Temple Lock reach of the River Thames.

2. Experimental

2.1. Chemicals

E1, E2, EE2, technical nonylphenol (NP), BPA, 2,2′-dihydroxybiphenyl, bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), pyridine, 4-nitrophenol, potassium 4-nitrophenyl sulfate, 4-nitrophenol β-D-glucuronide, β-glucuronidase (type VII-A extracted from *Escherichia coli*), sulfatase (VI from *Aerobacter aerogenes*) and all other chemicals were purchased from Sigma–Aldrich (Poole, UK). [2,4,16,16-$^{4}H_2$]Estrone (E1-d_4), [2,4,16,16-$^{4}H_2$]17β-estradiol (E2-d_4), [2,4,16,16-$^{4}H_2$]17α-ethinylestradiol (EE2-d_4) (isotope purity 96%, chemical purity > 98%), [U-$^{13}C_6$-ring] n-nonylphenol (^{13}C-NP) (isotope purity 99%), [U-$^{13}C_6$-ring] bisphenol A (^{13}C-BPA) (isotope purity 99%), [U-$^{13}C_6$-ring] n-nonylphenol monoethoxylate (^{13}C-NP1EO) (isotope purity 99%, chemical purity ≥ 98%), nonylphenol monoethoxylate (NP1EO) (mixture of alkyl isomers, chemical purity ≥ 98%) and nonylphenol diethoxylate (NP2EO) (mixture of alkyl isomers, chemical purity ≥ 98%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All solvents were of HPLC-grade purchased from Rathburn Chemicals (Walkerburn, Scotland, UK).

2.2. Fish exposure and sample collection

Wild roach (*R. rutilus*) of mixed sex were captured by electrofishing from the Temple Lock stretch of the River Thames on 3rd September 2007. The fish were collected as part of the annual fish monitoring survey which is carried out by the Environment Agency in all the major rivers of England and Wales. Temple reach comprises a 0.5 km stretch between grid references SU8380584364 and SU8518286132, and is 6.2 km downstream of Henley WwTWs which has a population equivalent of 11,620. Another population of maturing roach of mixed sex and 3+ years old were purchased from a fishery (Framlingham Fisheries, Suffolk) and subsequently exposed downstream of the discharge from a WwTWs (Co. Durham, UK) for 10 days in heavy-duty galvanised steel-mesh cages. The wastewater influent had a population equivalent of 47,569 with some industrial inputs, with the influent treated by primary sedimentation and secondary trickling filter. Fish were not fed for the duration of the exposure, though they were able to take any natural food available. Additionally, some roach were kept as reference controls in standard stock tanks with a flow-through system of dechlorinated tap water at 15 °C. At harvest, fish were anaesthetised with ethyl 4-aminobenzoate, stunned by a blow to the head and the spinal cord severed. For caged and laboratory-held control fish, bile was obtained by puncturing the gall bladder with a needle and drawing the fluid into a syringe. For wild fish, gall bladders were removed and frozen, and the bile extracted prior to sample preparation. Bile was stored in vials and frozen on dry ice for transport before storage at −70 °C.

2.3. Hydrolysis of estrogenic contaminants in bile samples

Bile (20–100 μL) was added to 200 μL of β-glucuronidase type VII from *E. coli* (1000 units/mL), 200 μL sulfatase type VI from *A. aerogenes* (2 units/mL) and 500 μL 0.1 M phosphate buffer (0.2 M) sodium dihydrogen orthophosphate:0.2 M disodium hydrogen orthophosphate:water (4:1:5, v/v/v) at pH 6.0. The samples

were gently mixed and incubated at 32 °C, and after 18 h hydrolysis was stopped by the addition of 90 μL of glacial acetic acid. Simultaneously, the activity and specificity of 20 μL of β-glucuronidase and sulfatase was monitored by incubation with standard substrates (10 μL of 10 mg/mL) of 4-nitrophenol β-D-glucuronide and potassium 4-nitrophenyl sulfate, respectively, in 50 μL 0.1 M phosphate buffer (pH 6.0) and 20 μL water. Incubation with the enzymes produced a yellow colour as the nitrophenol was hydrolysed from the glucuronide or sulfate, group and enzyme activity was checked by monitoring nitrophenol formation which was determined by spectroscopy at 405 nm with comparison to a standard curve of 4-nitrophenol in 0.1 M phosphate buffer. Using this methodology, the efficiency of hydrolysis of the nitrophenol substrates was between 90 and 100% for both enzymes and was similar to that in previously reported studies [6,28].

2.4. Solid phase extraction and method validation

The recoveries of a mixture of the target estrogenic chemicals were determined using a modified solid phase extraction (SPE) method from Gibson et al. [33]. In initial work, the recoveries of a solution of NP, NP1EO, NP2EO (500 ng/mL) and BPA, E1, E2 and EE2 (5 ng/mL) in water were compared using either ethyl acetate or acetone as elution solvents. OASIS HLB SPE cartridges (60 mg, Waters, Milford, MA) were conditioned with 2× 5 mL of either acetone or ethyl acetate followed by 5 mL 0.1% acetic acid. The mixture of estrogenic chemicals (in 1 mL water containing 0.1% acetic acid) was loaded onto the cartridge under vacuum. The cartridge was washed with 2× 5 mL water and 5 mL acetone:0.1 M sodium hydrogencarbonate buffer (40:60, v/v, adjusted to pH 10 using 1.0 M sodium hydroxide solution) and 1 mL acetone:water (25:75, v/v) and dried under vacuum for 30 min before elution of estrogenic compounds with 4 mL of either ethyl acetate or acetone. Following evaporation of the elution solvent under vacuum, 100 μL of the stable isotope internal standard mixture (containing 50 ng ^{13}C-NP and ^{13}C-BPA, 25 ng E1-d4 and E2-d4, 75 ng EE2-d4, and 125 ng ^{13}C-NP1EO) was added to monitor recoveries of the analytes and the solvent removed under a gentle stream of N_2. Prior to GC–MS/MS analysis, samples were derivatized with 25 μL BSTFA (1% TMCS) and 25 μL pyridine heated to 60 °C for 30 min. Following cooling to room temperature, samples were transferred to GC vials. Recoveries were calculated after subtraction of concentrations of any target analytes that were detected above the limit of quantitation (LOQ) in the work up of non-spiked water (100 μL) samples. The recoveries of target analytes spiked into bile from immature female rainbow trout were determined in a similar manner. However in all the following work acetone was used as the elution solvent from SPE cartridges. Bile (30 μL) was spiked with two masses of analytes (either 50 ng or 500 ng for NP, NP1EO and NP2EO and either 0.5 or 5 ng for BPA, E1, E2 and EE2 for low and high concentrations, respectively). Estrogenic contaminants in spiked and non-spiked bile samples were hydrolysed to release conjugated metabolites. Samples in the hydrolysis buffer (1 mL) were extracted by SPE as described above. Following evaporation of the acetone elution solvent, internal standards were added to monitor recoveries of the target analytes and the samples derivitized for GC–MS/MS analysis. Recoveries were calculated after subtraction of concentrations of any target analytes detected in non-spiked bile samples.

Estrogenic metabolites present in bile samples (20–100 μL) obtained from reference, effluent-exposed and wild roach were hydrolysed and extracted by SPE as described above. However prior to SPE loading of samples of either hydrolysed bile (total volume 1 mL) or the blank work up (100 μL water in 0.9 mL hydrolysis buffer), the stable isotope internal standards were added at this point to account for any losses of the target analytes. After SPE, an additional standard of 10 μL of 20 μg/mL 2,2′-dihydroxybiphenyl was added to monitor recoveries of the stable isotope internal standards. Samples were then analysed by GC–MS/MS.

2.5. GC–MS/MS analysis

GC–MS/MS analysis was carried out using a Waters Micromass Quattro micro tandem mass spectrometer, with an integrated Agilent 6890 GC fitted with a 30 m HP5-MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was helium at a constant flow of 1.0 mL min^{-1}, injection port temperature was 280 °C, GC interface temperature was 280 °C, source temperature was 250 °C with electron energy of 70 eV. The collision gas was argon at a pressure of 1×10^{-4} mbar, and the collision energy was 12 V for all ion transitions. The MS detector was used in MRM mode with a dwell time of 0.08 s. The sample (1 μL) was introduced using splitless injection and the oven temperature programme was 90 °C for 1 min, 15 °C/min to 150 °C, 150 °C for 11 min, 10 °C/min to 200 °C, 15 °C/min to 280 °C, 280 °C for 6 min. A minimum of two transition ions were monitored for each compound,

Table 1
MRM ions used for the GC–MS/MS analysis of estrogenic contaminants and deuterated or ^{13}C labelled internal standards.

Compound	Retention time	Molecular mass	Mass after derivatisation[a]	Quantitation ion transition	Confirmation ion transition	Ratio quantitation/confirmation transition (mean ± SD)[b]
NP	15.8–18.4	220	292	292 > 235, 221, 193, 179		
	16.2			292 > 221	292 > 193	1.3 ± 0.1
^{13}C-n-NP	21.0	226	298	298 > 185		
NP1EO	22.8–23.6	264	336	336 > 307, 265, 251, 193		
	23.00			336 > 251	336 > 193	2.5 ± 0.2
NP2EO	25.2–25.7	308	380	380 > 309, 295, 161		
	25.30			380 > 309	380 > 161	0.4 ± 0.1
^{13}C-n-NP1EO	24.5	270	342	342 > 185		
BPA	24.4	228	372	372 > 357	372 > 191	46.0 ± 3.8
^{13}C-BPA	24.4	240	384	369 > 197		
E1	27.3	270	342	342 > 257	342 > 327	40.9 ± 2.7
E1-d4	27.3	274	346	346 > 261		
E2	27.6	272	416	416 > 285	416 > 326	8.3 ± 1.3
E2-d4	27.6	276	420	420 > 287		
EE2	28.6	296	440	425 > 193	440 > 425	10.9 ± 1.9
EE2-d4	28.6	300	444	429 > 195		

[a] Prior to analysis samples were derivatized to their trimethylsilyl ethers by reaction with BSTFA.
[b] Ratios calculated for concentrations of 1–100 ng/μL injection volume for NP and NPEOs, 0.01–10 ng/μL for E1, E2 and EE2 and 0.05–10 ng/μL for BPA.

with the most abundant used for quantitation and the second for confirmation (Table 1). The identification of analytes was performed by comparison of retention times, spectra and the ratio of the quantitation to the confirmation transition in the samples with those observed for authentic standards. The peak areas of the multiple ion transitions used for the quantitation of NP, NP1EO and NP2EO were summed to give a total area for the analyte. Quantitation was carried out by determining the response factor of the target to its respective internal standard and comparing this to a standard curve. Deuterated or ^{13}C analogues were used as internal standards for their respective target analytes, with the exception of NP2EO which was quantified by ^{13}C-NP1EO. Concentrations of analytes were corrected for any amounts detected in the blank (100 μL water) work up samples prior to the determination of the final concentration in the bile samples. The Student's *t*-test was used to investigate statistical differences between datasets.

3. Results and discussion

3.1. Selection of GC–MS/MS transitions

The selection of transition ions for the GC–tandem MS analysis of the trimethylsilyl derivatives of E1, E2, EE2 and BPA is well established e.g. [34,35] (Table 1). However, the measurement of technical NP is complicated by its composition of at least 22 branched chain alkyl isomers which are only separated using lengthy GC run times [36], and under the GC conditions used in this study, 14 isomers of NP were completely resolved. For quantitation of NP, four ion transition pairs (292 > 235, 221, 193, 179) were identified which comprised 12 of the most abundant alkyl isomers of the technical mixture. The ion transition pairs were formed from fragmentation of the alkyl chain, and the formation of the trimethylsiloxy tropylium ion at *m*/*z* 179 [36]. The confirmation ratio for NP in fish samples was based on the transition ion pair ratio of 292 > 221 to 292 > 193 at 16.34 min as this was the most abundant and frequently detected isomer in bile samples.

The NP1EO was quantified using four transition ion pairs (336 > 307, 265, 251, 193) which accounted for 10 branched chain alkyl isomers that were resolved on GC–MS/MS. The fragments *m*/*z* 307, 265 and 251 were formed from increasing losses from the alkyl chain, and *m*/*z* 193 from loss of C_7H_{15} as well as OC_2H_4 and subsequent rearrangement of the silyl group. Confirmation of NP1EO was determined by analysis of the ratio of the transition ion pair of 336 > 251 to 336 > 193 at a retention time of 23.00 min.

Two transition ions of NP2EO (380 > 309, 295) accounted for 8 of the 10 isomers separated on GC–MS/MS and one confirmation transition ion pair (380 > 161) was common to all isomers. The fragments *m*/*z* 309 and 295 were likely to arise from losses of alkyl groups from the nonyl chain, and *m*/*z* 161 from a combination of fragmentation of the alkyl and ethoxylate chains.

The presence of the target analytes in the bile samples was confirmed by the ratio of the quantitation to the confirmation transition ion pair at the specified retention time (Table 1), of the calibration standards. The ratio of the quantitation to the confirmation ion for each analyte was considered acceptable providing they were within ±25% of ratio of the standard. For all analytes, the ratios fell within the acceptable criterion at concentrations down to $\geq 2 \times$ the LOQ value.

3.2. Sample preparation, recoveries and limits of detection

The calibration curve for GC–MS/MS quantitation of the target analytes was linear over the range of concentrations measured of 1–100 ng/μL for NP and NPEOs, and 0.01–10 ng/μL BPA, E1, E2 and EE2, with R^2 values of 0.9822–0.9994 (Table 2).

The recovery of the compounds from SPE extraction of spiked water was compared using two different elution solvents. Ethyl acetate has often been used for the elution of the steroidal estrogens from SPE, however acetone has been reported to be a suitable solvent for the elution of both alkylphenols and steroidal estrogens [33]. Recoveries of the steroidal estrogens from spiked water were similar for the two elution solvents, however the recoveries of BPA, NP and both NPEOs were significantly higher, and less variable using acetone as the SPE elution solvent (Table 2). Subsequently, the recovery of target compounds from bile samples spiked at two concentrations of the analytes was determined using acetone as the SPE elution solvent. The recoveries of all analytes spiked at the high concentration were between 86 and 99%. The recoveries of NP1EO, BPA, E1 or E2 from bile were independent of concentration ($P > 0.05$). However, the recoveries of NP, NP2EO and EE2 were significantly less ($P < 0.05$) at the lower concentration, whilst all recoveries remained >60%. It was possible that losses of low amounts of these analytes occurred in the wash or elution step which was only apparent at the lower spiking concentration. The internal standard added during sample preparation indicated little loss during processing, with mean recoveries (±SD) of 109.5 ± 18.7% for ^{13}C-NP, 111.5 ± 37.4% for ^{13}C-BPA, 128.9 ± 22.6% for ^{13}C-NP1EO, 123.7 ± 14.6% for E1-d_4, 96.5 ± 13.3% for E2-d_4 and 129.5 ± 19.7% for EE2-d_4.

In this study, the limit of detection (LOD) of the target analytes in spiked bile was considerably lower than that reported previously using GC–MS with selected ion monitoring methodology In two studies using GC–MS, the LODs of EE2 have been reported to be <6.4 ng/mL and <0.1 μg/g, and E2 to be 15.5 ng/mL and <0.04 μg/g

Table 2

Recoveries (mean% ± SD), GC–MS/MS calibration and limits of detection of estrogenic analytes. The recoveries of estrogenic analytes from spiked water samples were determined after SPE and elution of samples with either acetone or ethyl acetate. The recoveries were determined of estrogenic analytes spiked into bile at two concentrations, extracted by SPE and eluted with acetone. All determinations are a mean of 3 replicates.

Compound	Recovery water		Recovery bile[A]		R^2	LOD[B] (ng/mL bile)
	Acetone	Ethyl acetate	High	Low		
NP	85.8 ± 2.5	76.2 ± 3.7	86.4 ± 1.4	62.5 ± 3.0	0.9822	60.2
NP1EO	103 ± 2.2	63.0 ± 39.2	90.0 ± 8.8[a]	79.1 ± 9.5[a]	0.9942	11.0
NP2EO	113 ± 2.8	70.4 ± 42.1	97.4 ± 9.7	72.7 ± 10.0	0.9961	327.0
BPA	99.2 ± 3.0	90.5 ± 0.8	99.2 ± 2.7[b]	99.0 ± 4.5[b]	0.9893	0.1
E1	98.9 ± 1.3	97.1 ± 2.4	93.4 ± 1.9[c]	103.7 ± 7.1[c]	0.9990	0.7
E2	93.9 ± 7.6	93.6 ± 1.7	86.7 ± 6.8[d]	80.7 ± 4.7[d]	0.9994	0.4
EE2	93.9 ± 5.0	94.4 ± 10.4	91.4 ± 2.3	79.7 ± 1.2	0.9991	0.4

Recovery values followed by the same letter are not significantly different between the two spike concentrations ($P > 0.05$). Calibration covered 1–100 ng/μL for NP and NPEOs, and 0.01–10 ng/μL BPA, E1, E2 and EE2.

[A] 30 μL bile was spiked at 2 levels; 500 ng NP, NP1EO and NP2EO, and 5 ng BPA, E1, E2 and EE2 (high); 50 ng NP, NP1EO and NP2EO, and 0.5 ng L^{-1} BPA, E1, E2 and EE2 (low).

[B] LOD based on 100 μL bile sample, and LOD and LOQ values were determined from 3 to 9 times the signal to noise values of the spiked standards.

Table 3
The concentrations of estrogenic compounds (ng/mL) detected in bile from individual roach held in tap water (control), exposed to WwTW effluent, or caught in River Thames. Values are blank corrected.

Sample	Sex	Bile volume	NP	NP1EO	NP2EO	BPA	E1	E2	EE2
Blank workup	–	–	428 ± 149	49.6 ± 15.5	<LOD	6.1 ± 0.6	(3.67 ± 0.3)[a]	<LOD	<LOQ
Control	Male	20	<Blank	266	<LOQ	257	2.8	26.7	<LOQ
Control	Male	20	<Blank	452	<LOQ	157	9.1	24.9	<LOQ
Control	Female	20	951	1,656	<LOQ	195	15.3	76.5	<LOQ
Effluent-exposed	Male	20	6576	20,756	128179	763	565	158	16.9
Effluent-exposed	Male	20	5531	18,826	135489	1551	717	332	16.9
Effluent-exposed	Female	20	12678	18,080	94074	1141	1426	2503	22.4
Effluent-exposed	Female	40	9180	20,840	75064	1951	684	549	29.1
River Thames	Female	20	2453	21.5	<LOD	<Blank	11.3	42.8	5.6
River Thames	Female	40	<Blank	16.9	<LOD	2.2	3.2	10.4	<LOD
River Thames	Male	100	195	110.8	<LOD	18.2	7.0	15.3	<LOD
River Thames	Male	100	<Blank	24.4	<LOD	10.6	2.5	7.0	1.7
River Thames	Male	20	1418	83.3	<LOD	68.0	96.7	401	<LOQ

Wild fish from Temple reach 1 collected 3 September 2007. Blank concentrations are the mean of 11 replicates and are based on a bile volume of 100 μL.
[a] An E1 type signal was detected, however there was no confirmation ion present.

bile [5,38]. In our work, the LOD of EE2 and E2 were <1 ng/mL which may reflect the higher signal to noise and increased sensitivity of the method using GC–MS/MS in multiple reaction monitoring mode.

3.3. *Measurement of estrogenic compounds in bile*

The analytical method developed in this work was used to quantify estrogenic contaminants in the bile of roach in a preliminary study of fish which had been caged downstream of a WwTWs or captured in the River Thames. A total ion chromatogram of the MRM transitions used for quantitation of the target analytes is given in Fig. 1A. An analysis of the work up samples, containing ultrapure water instead of bile, revealed contamination with NP and NP1EOs and to a lesser extent BPA, and it is likely that the alkylphenolic contaminants may have originated from polypropylene plastics used in the SPE, the SPE packing material, or the solvents (Table 3). The bile from sexually mature control roach held in tap water contained E1 and E2 (2–76 ng/mL) which were likely to be endogenous, originating from circulating sex steroids present naturally in the fish (Table 3 and Fig. 1C). However, these control fish also contained significant signals for NP1EO, BPA, and in one sample NP (Fig. 1C which is shown without correction for blank workup values). After subtraction of the work up values, the concentrations of NP1EO were between 266 and 1656 ng/mL and BPA between 157 and 257 ng/mL bile, indicating some contamination of the roach held in tap water under laboratory conditions. All the target analytes were identified in fish exposed to WwTWs effluent (Table 3 and Fig. 1D), and their identity was confirmed by the presence of the confirmation ion transition pairs, and the ratio of the transitions of the quantitation to confirmation ions which were all within ±25% of the standard values. The detection of target analytes in samples from wild fish caught from the Temple Lock stretch of the River Thames was sample dependent and the concentrations of analytes were generally lower than those found in the effluent-exposed fish. However, within the sample set, all target compounds, except NP2EO, were identified in wild fish (Table 3 and Fig. 1B). Blank samples were prepared with each batch of bile samples, and there was no statistical difference (t-test, $P > 0.2$) in the levels of nonylphenolics or BPA between the different batches of blank samples. Although NP, NP1EO and BPA were detected in bile from some of the wild fish captured in the River Thames, and there was no statistical difference between the levels of nonylphenolics and BPA in wild fish and in the blanks ($P > 0.3$). However the concentrations of these analytes were very variable in the wild fish and it possible that levels of nonylphenolics and BPA were present above blank levels in some individuals. The concentrations of estrogenic contaminants reported here from wild and effluent-exposed fish are in broad agreement with other published data of these compounds in fish bile which used selected ion monitoring GC–MS techniques to quantify estrogenic contaminants arising from exposure to WwTWs effluents [5,37,38]. Our methodology differs, in that it allows for the additional analysis of short chain NPEOs alongside other estrogenic contaminants and uses tandem MS which is a highly selective technique which allows for a simple one step clean up of the sample. In our study, the concentrations of the synthetic estrogen EE2 in roach captured in the River Thames ranged between <0.4 and 5.6 ng/mL bile, which was similar to reported concentrations (<6.4 ng/mL) in other studies of EE2 in bile from cyprinid fish captured downstream from WwTWs effluents [38]. The situation of assessing the exposure and effects on pharmaceuticals on wild fish is more challenging than that with the traditional lipophilic persistent organic pollutants since they will be metabolised and excreted in fish, just as in humans. Thus, it may be possible to witness recent exposure, but not that which may have occurred weeks earlier. Concentrations of EE2 were much fold higher (17–29 ng/mL) in bile of roach caged below the WwTWs effluent, indicating that potential for exposure of fish to much higher concentrations of EE2 when they are located near to discharges of WwTWs effluent. However, in other studies, where fish have been exposed to either more estrogenic or undiluted WwTWs effluents, EE2 concentrations in bile have been even higher at 240–380 ng/mL [5,39]. The concentrations of the naturally produced estrogens, E1 and E2, were generally similar between the mixed sex samples of control roach and the wild fish captured from the River Thames (E1: 3–15 and 3–97; E2: 25–77 and 7–401 ng/mL for control and wild roach, respectively). It should be noted that the lower main stem of the Thames, where these fish came from, is predicted (based on sewage input and dilution) to have a low combined steroid estrogen content of just over 1 ng/L E2 equivalents [21]. However, concentrations of estrogens in bile of effluent-exposed fish were markedly elevated, with E1 between 565–1426 ng/mL and E2 158–2503 ng/mL, indicating additional accumulation in the bile from the WwTWs effluent. Previous work has also reported similar high levels of estrogens in bile of fish captured downstream of an effluent discharge [5] however, not all the of E1 and E2 is necessarily derived from exposure to WwTWs and concentrations of endogenous E1 and E2 can vary markedly in fish due to differences not only in sex, but in maturity, season and condition. The levels of these environmental estrogens within the WwTWs exposed fish

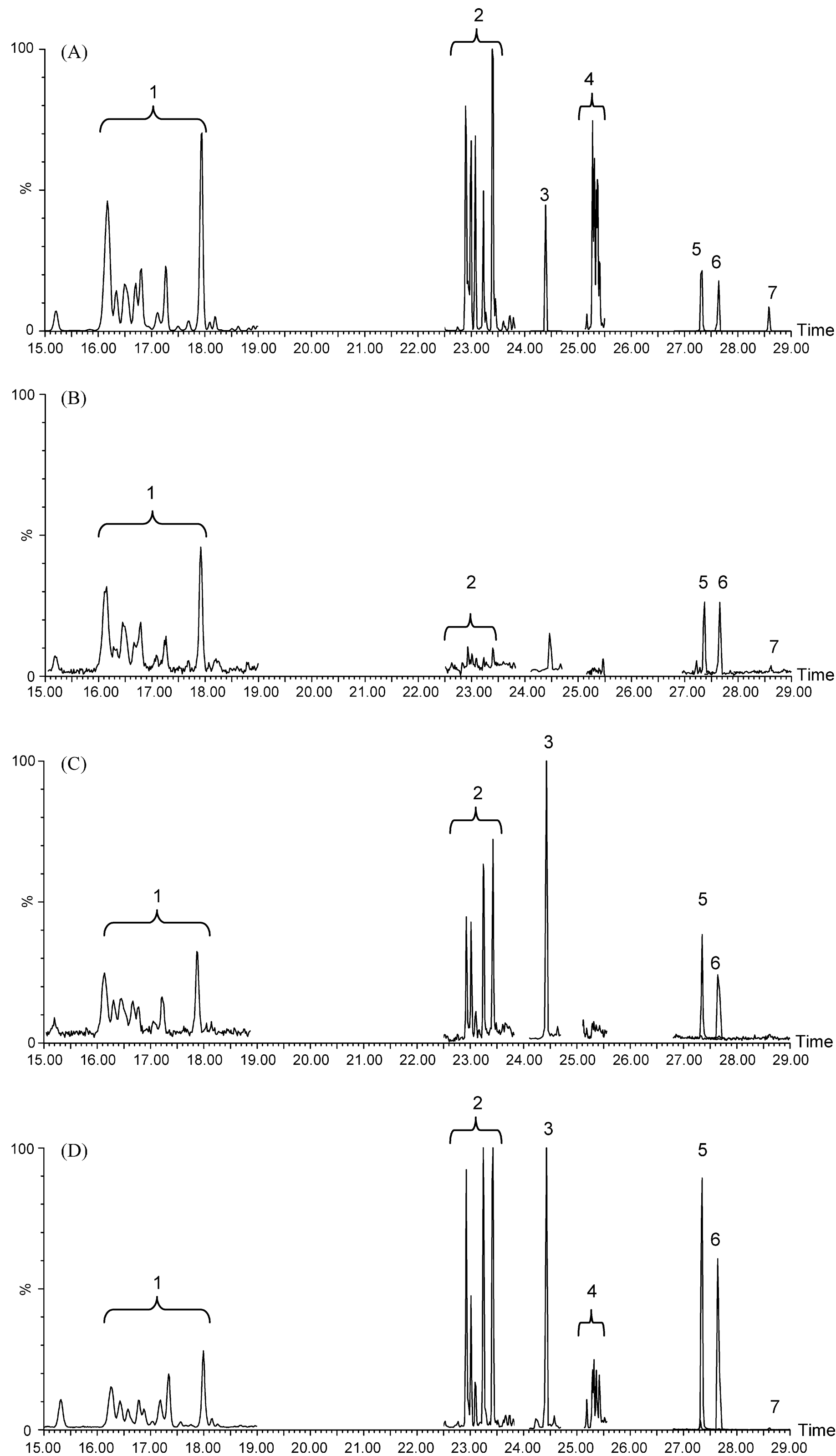

Fig. 1. GC–MS/MS analysis of trimethylsilyl derivatives of estrogenic compounds; representative total ion chromatograms of the MRM transitions used for quantitation of target analytes. (A) Analysis of standards, 100 ng μL^{-1} of NP, NP1EO and NP2EO, and 10 ng μL^{-1} of BPA E1, E2, and EE2, (B) analysis of 20 μL of bile from a female roach captured in River Thames, (C) analysis of 20 μL of bile from a female roach held in laboratory tap water, and (D) analysis of 20 μL bile from a female roach caged in a WwTWs effluent. (1) Nonylphenol isomers; (2) nonylphenol monoethoxylate isomers; (3) bisphenol A; (4) nonylphenol diethoxylate isomers; (5) estrone; (6) estradiol; (7) ethinylestradiol. Where labelled, analytes were >LOD. All analytes are on the same scale within each chromatogram.

emphasises the importance of considering their ability to concentrate in fish when extrapolating for possible biological effects from estrogen contaminant concentrations in the aquatic environment (a very common practice).

The concentrations of BPA and NP1EO detected in the wild roach from the River Thames were on average 10–15-fold lower than that found in bile from the control fish which indicated that fish held in the laboratory were exposed to low concentrations of these contaminants possibly from plastics used in piping supplying water to the tanks or from estrogenic contaminants leaching from the containment tanks. This finding highlights the importance for careful consideration of the housing and water supply systems used in the laboratory for studies where biological effects of estrogenic chemicals (or other EDCs) are being investigated. Significant concentrations of all the industrially derived target analytes were detected in fish caged below to WwTW effluent, with concentrations of ranging between 5.5–12.6 μg/mL bile for NP, 18.0–20.8 μg/mL for NP1EO, 75–135 μg/mL for NP2EO and 0.7–1.9 μg/mL for BPA. The concentrations of NP and BPA were similar to the range of concentrations reported in bile from fish exposed to other European WwTWs effluents where NP concentrations ranged between 1 and 30 μg/mL and BPA from 2 to 25 μg/mL [5,39]. In our study, the high concentrations of NP and NPEOs in the bile suggest that these contaminants are still prevalent in some WwTWs, despite their restricted use in the UK. Possible sources of NPEOs could be due to their continued use in car washes and in service industries [40].

4. Conclusions

The GC–MS/MS methodology developed in this study included MRM analysis of both quantitation and confirmation ion pairs which ensured selective discrimination of target compounds in a complex matrix such as bile which could contain a number of potentially interfering compounds. MRM analysis was successfully applied for determination of nonyl isomers of NP, NP1EO and NP2EO contaminants by selecting transition ion pairs for each of the most abundant alkyl isomers for quantitation of the analytes. The methods were successfully applied to the quantitation of steroidal and phenolic estrogenic contaminants in fish exposed to WwTWs effluents and to fish captured from what might be considered only mildly contaminated river water. The analysis of estrogenic contaminants in fish bile maybe advantageous to that of effluent or receiving water because of the high reported bioconcentration of these chemicals and so could be used to monitor recent exposure to, and importantly, uptake of, trace concentrations of these chemicals in river reaches.

Acknowledgements

We are grateful for the assistance of Dr. A. Abdul-Sada, University of Sussex, with GC–MS/MS analysis, the members of the Fish Team at the University of Exeter and the participating water company for their support in conducting the effluent exposure studies and to the Environment Agency for cooperation with fish collection from the Thames. The work was funded from NERC grants (NE/E017363/1 and NE/E016634/1) to EMH and CRT and the authors are also grateful for support from the Centre for Ecology and Hydrology.

References

[1] C.E. Purdom, P.A. Hardiman, V.V.J. Bye, N.C. Eno, C.R. Tyler, J.P. Sumpter, Chem. Ecol. 8 (1994) 275.
[2] C.R. Tyler, S. Jobling, J.P. Sumpter, Crit. Rev. Toxicol. 28 (1998) 319.
[3] S. Jobling, M. Nolan, C.R. Tyler, G. Brighty, J.P. Sumpter, Environ. Sci. Technol. 32 (1998) 2498.
[4] C.R. Tyler, C. Spary, R. Gibson, E.M. Santos, J. Shears, E.M. Hill, Environ. Sci. Technol. 39 (2005) 2599.
[5] D.G.J. Larsson, M. Adolfsson-Erici, J. Parkkonen, M. Pettersson, A.H. Berg, P.E. Olsson, L. Forlin, Aquat. Toxicol. 45 (1999) 91.
[6] R. Gibson, M.D. Smith, C.J. Spary, C.R. Tyler, E.M. Hill, Environ. Sci. Technol. 39 (2005) 2461.
[7] C.R. Tyler, A.L. Filby, L.K. Bickley, R.I. Cumming, R. Gibson, P. Labadie, Y. Katsu, K.E. Liney, J.A. Shears, V. Silva-Castro, H. Urushitani, A. Lange, M.J. Winter, T. Iguchi, E.M. Hill, Environ. Sci. Technol. 43 (2009) 3897.
[8] H.R. Andersen, A.M. Andersson, S.F. Arnold, H. Autrup, M. Barfoed, N.A. Beresford, P. Bjerregaard, L.B. Christiansen, B. Gissel, R. Hummel, E.B. Jorgensen, B. Korsgaard, R. Le Guevel, H. Leffers, J. McLachlan, A. Moller, J.B. Nielsen, N. Olea, A. Oles-Karasko, F. Pakdel, K.L. Pedersen, P. Perez, N.E. Skakkeboek, C. Sonnenschein, A.M. Soto, J.P. Sumpter, S.M. Thorpe, P. Grandjean, Environ. Health Perspect. 107 (1999) 89.
[9] A. Lange, G.C. Paull, T.S. Coe, Y. Katsu, H. Urushitani, T. Iguchi, C.R. Tyler, Environ. Sci. Technol. 43 (2009) 1219.
[10] K.L. Thorpe, T.H. Hutchinson, M.J. Hetheridge, M. Scholze, J.P. Sumpter, C.R. Tyler, Environ. Sci. Technol. 35 (2001) 2476.
[11] J.V. Brian, C.A. Harris, M. Scholze, A. Kortenkamp, P. Booy, M. Lamoree, G. Pojana, N. Jonkers, A. Marcomini, J.P. Sumpter, Environ. Sci. Technol. 41 (2007) 337.
[12] C. Desbrow, E.J. Routledge, G.C. Brighty, J.P. Sumpter, M. Waldock, Environ. Sci. Technol. 32 (1998) 1549.
[13] A.C. Johnson, J.P. Sumpter, Environ. Sci. Technol. 35 (2001) 4697.
[14] T.P. Rodgers-Gray, S. Jobling, S. Morris, C. Kelly, S. Kirby, A. Janbakhsh, J.E. Harries, M.J. Waldock, J.P. Sumpter, C.R. Tyler, Environ. Sci. Technol. 34 (2000) 1521.
[15] D.A. Sheahan, G.C. Brighty, M. Daniel, S.J. Kirby, M.R. Hurst, J. Kennedy, S. Morris, E.J. Routledge, J.P. Sumpter, M.J. Waldock, Environ. Toxicol. Chem. 21 (2002) 507.
[16] A.C. Johnson, R.J. Williams, P. Simpson, R. Kanda, Environ. Pollut. 147 (2007) 194.
[17] P. Spengler, W. Korner, J.W. Metzger, Environ. Toxicol. Chem. 20 (2001) 2133.
[18] C. Hohne, W. Puttmann, Environ. Sci. Pollut. Res. 15 (2008) 405.
[19] R. Loos, B.M. Gawlik, G. Locoro, E. Rimaviciute, S. Contini, G. Bidoglio, Environ. Pollut. 157 (2009) 561.
[20] R.J. Williams, A.C. Johnson, J.J.L. Smith, R. Kanda, Environ. Sci. Technol. 37 (2003) 1744.
[21] R.J. Williams, V.D.J. Keller, A.C. Johnson, A.R. Young, M.G.R. Holmes, C. Wells, M. Gross-Sorokin, R. Benstead, Environ. Toxicol. Chem. 28 (2009) 220.
[22] R.T. Pedersen, E.M. Hill, Environ. Sci. Technol. 36 (2002) 3275.
[23] A.M.R. Ferreira-Leach, E.M. Hill, Mar. Environ. Res. 51 (2001) 75.
[24] J. Legler, A. Jonas, J. Lahr, A.D. Vethaak, A. Brouwer, A.J. Murk, Environ. Toxicol. Chem. 21 (2002) 473.
[25] M.D. Smith, E.M. Hill, Aquat. Toxicol. 69 (2004) 359.
[26] M.D. Smith, E.M. Hill, Sci. Total Environ. 356 (2006) 100.
[27] G. Jonsson, A. Cavcic, T.U. Stokke, J. Beyer, R.C. Sundt, C. Brede, J. Chromatogr. A 1183 (2008) 6.
[28] R. Gibson, C.R. Tyler, E.M. Hill, J. Chromatogr. A 1066 (2005) 33.
[29] P.J. Ruddock, D.J. Bird, J. McEvoy, L.D. Peters, Sci. Total Environ. 301 (2003) 105.
[30] B. Johnson-Restrepo, J. Olivero-Verbel, S.J. Lu, J. Guette-Fernandez, R. Baldiris-Avila, I. O'Byrne-Hoyos, K.M. Aldous, R. Addink, K. Kannan, Environ. Pollut. 151 (2008) 452.
[31] C.M. Brumley, V.S. Haritos, J.T. Ahokas, D.A. Holdway, Ecotoxicol. Environ. Safe. 39 (1998) 147.
[32] H. Leppänen, S. Marttinen, A. Oikari, Chemosphere 36 (1998) 2621.
[33] R. Gibson, E. Becerril-Bravo, V. Silva-Castro, B. Jimenez, J. Chromatogr. A 1169 (2007) 31.
[34] A. Flores, E.M. Hill, Chemosphere 73 (2008) 1115.
[35] S. Kinani, S. Bouchonnet, S. Bourcier, N. Creusot, J.M. Porcher, S. Ait-Aissa, Rapid Commun. Mass Spectrom. 22 (2008) 3651.
[36] T.F. Wheeler, J.R. Heim, M.R. LaTorre, A.B. Janes, J. Chromatogr. Sci. 35 (1997) 19.
[37] M. Pettersson, E. Hahlbeck, L. Katsiadaki, L. Asplund, B.E. Bengtsson, Mar. Pollut. Bull. 54 (2007) 1868.
[38] L. Vigano, A. Mandich, E. Benfenati, R. Bertolotti, S. Bottero, E. Porazzi, E. Agradi, Arch. Environ. Contam. Toxicol. 51 (2006) 641.
[39] K.L. Rule, S.D.W. Comber, D. Ross, A. Thornton, C.K. Makropoulos, R. Rautiu, Chemosphere 63 (2006) 581.
[40] M. Pettersson, M. Adolfsson-Erici, J. Parkkonen, L. Forlin, L. Asplund, Sci. Total Environ. 366 (2006) 174.