Bioconcentration and metabolism of 4-*tert*-octylphenol in roach (*Rutilus rutilus*) fry

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Early stages of fish have been reported to be particularly sensitive to the effects of xenoestrogens which may cause irreversible changes in sexual differentiation and development. In this study we report the uptake and metabolism of an oestrogenic alkylphenol, 4-*tert*-octylphenol (*t*-OP) in roach (*Rutilus rutilus*) fry. Roach fry, aged 7 days post hatch (DPH), were exposed to a concentration 5.8 ± 0.8 µg/L of radiolabelled [^{14}C] *t*-OP in a semi-static system and were sampled 5, 12 and 19 days after exposure. After 5 days of exposure the bioconcentration factor (BCF) for *t*-OP residues was 346, whereas after 12 and 19 days of exposure steady state conditions in the fish were reached with BCFs of 1061 and 1134 respectively. Radio-HPLC analysis of 7 DPH fry exposed for 5 days to *t*-OP revealed that the majority of residues were present as the parent compound. However when 26 DPH fry were exposed to *t*-OP for 5 days, *t*-OP only made up 22 % of the total radioactivity, the remainder of which were a mixture of metabolites. The major metabolite was identified as the glucuronide conjugate of *t*-OP. This study suggests that alkylphenols can bioconcentrate to high levels in larval fish but that roach fry can rapidly conjugate them to more polar metabolites.

Introduction

A recent report has demonstrated a high incidence of intersexuality in wild populations of the freshwater fish, roach, in UK rivers [1]. Intersex fish were characterised by the appearance of female characteristics, such as an ovarian cavity or primary or secondary oocytes, in male tissue. The incidence of feminised fish was particularly high downstream of sewage treatment works (STWs). Some of the oestrogenic chemicals in STW effluent have been identified as steroidal oestrogens originating from human waste [2]. However other oestrogenic chemicals originating from industrial waste may be present such as bisphenol A and 4-alkylphenols [3]. In some rivers alkylphenolics are thought to be the main oestrogenic pollutants responsible for the high incidence of intersex fish at some sites [4]. 4-Alkylphenols are the degradation products of alkylphenol ethoxylates (APEOs) which are nonionic surfactants commonly used in the production of paints, cleaning agents, plastics, pesticides and therefore are major constituents of waste waters of some chemical industries. Bacterial degradation of these compounds into hydrophobic alkylphenols (AP), such as 4-nonylphenol (NP) and 4-octylphenol (OP), occurs in sewage treatment works. Their release to the aquatic environment is mainly from discharges of waste water into rivers and sea and from sewage sludge. 4-*tert*.- alkylphenols have been reported to be the most oestrogenic structures and 4-*tert*.octylphenol (*t*-OP) is the most oestrogenic of the alkylphenolics tested to date [5].

Laboratory studies have shown that feminisation can be permanent if exposure occurs over long periods of time or at critical stages early in the development of the organism. For instance, in fish, sex determination occurs during the egg/fry stage, which is a period when they appear to be more sensitive to hormones and hormone mimicking compounds than during adulthood [6]. Exposure to the endocrine-regulating chemicals during a narrow window in early life stages can cause irreversible developmental effects. This "window" normally coincides with the period when differentiation of the gonad and reproductive tract are occurring [7]. Studies with natural and synthetic oestrogens, used in hormonal manipulation of sex in fish, have demonstrated that treatment during early stages of development with sex steroids for periods as short as 2 hours resulted in sterilisation or reversal of sexual differentiation [6,8]. Exposure of sexually undifferentiated male carp (*Cyprinus carpio*) to the xenoestrogen 4-tert-pentylphenol resulted in formation of an oviduct and ovo-testis [9]. Similarly exposure of male Japanese medaka (*Oryzias latipes*) to *t*-OP during gonadal differentiation resulted in the intersex condition [10].

There is little information concerning the bioconcentration and metabolic fate of alkylphenols in fish when exposed at very early stages of development. In this study we report on the uptake and biotansformation of the xenoestrogen *t*-OP in roach fry exposed to an environmentally relevant concentration of *t*-OP (5.8 ± 0.8 µg/L) which has been shown to produce an oestrogenic response in fish [11]. This concentration is well below the reported 14 day NOEC level for *t*-OP in fish [12].

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Materials and methods

Chemicals

Radiolabelled 4-(1,1,3,3-tetramethylbutyl)[U-^{14}C]phenol (*t*-OP, specific activity 3.23 MBq/mg, radiochemical and chemical purity > 99 %) was purchased from BlyChem Ltd., Billingham, UK. EcoLite and CytoScint liquid scintillation cocktails and methylbenzothonium hydroxide tissue solubiliser were purchased from ICN Radiochemicals, Irvine, CA, USA. 4-(1,1,3,3-tetramethylbutyl)phenol, 3-aminobenzoic acid ethyl ester (MS222), β-glucuronidase Type VII-A enzyme from *Escherichia coli* and all other chemicals were purchased from Sigma-Aldrich Company, Poole, Dorset, UK. All solvents were HPLC grade purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland.

Experimental conditions

Roach fry, aged 7 days post-hatching (DPH), were supplied by the Environment Agency National Coarse Fish Farm, Calverton, UK. Fish were held in constantly aerated tap water which had been previously filtered (20 µm), and passed through an activated charcoal filter. Fish were exposed to a photoperiod of 12 hours light and 12 hours dark and the water temperature was kept between 13-14 °C. The pH of the water was 7.6-7.7, conductivity 350-380 µS/cm and the dissolved oxygen levels varied from 10-11 mg/L. Throughout the experiment, fish were fed once daily with a commercial fry food (BOCM, Renfrew, UK).

Roach fry, age 7 DPH, were exposed for up to 19 days to [U-^{14}C] *t*-OP in a 5 litre semi-static system. The octylphenol was dissolved in methanol and the final concentration of methanol in the dosing water did not exceed 0.1 % v/v. The water was changed daily and the concentration of *t*-OP in the tank water monitored daily by collecting three replicates of water and the radioactivity determined by scintillation counting. The mean measured concentration of *t*-OP throughout the time course of the experiment was 5.8 ± 0.8 (s.e. n = 3) µg/L of [^{14}C]*t*-OP (9.5 kBq/L) (the nominal concentration was 6.0 µg/L). This exposure concentration was well below the NOEC level of 84 µg/L reported for a 14 days exposure of rainbow trout to *t*-OP [12].

Approximately 50, 25 and 20 fry were sacrificed by terminal anaesthesia with MS222 at days 5, 12 and 19 of exposure, equivalent to 12, 19 and 26 DPH, respectively. The fry were briefly rinsed with deionised water (10 mL, 2 sec), all traces of water removed and the fry weighed before extraction with methanol. Analysis of the rinsing water showed that there no significant losses of radioactivity from the fry tissue. In order to investigate metabolism of *t*-OP in older fry, 26 DPH fish were dosed for 5 days with [^{14}C]*t*-OP in conditions as described above.

Extraction of t-OP residues

Soluble radioactive residues were extracted from fry tissue by 3 × 0.5 mL methanol (MeOH) and centrifugation (3000 rpm for 10 min). The supernatants were combined and three aliquots taken for determination of radioactivity by liquid scintillation counting. Residual radioactivity in the tissue pellets was determined after solubilisation with 1M methylbenzothonium hydroxide in methanol and counted by liquid scintillation counting. Levels of methanol-insoluble radioactivity present after digestion of the tissue pellets were always lower than 3 % of the total radioactive material extracted with methanol.

Solid phase extraction

t-OP residues were purified using 3 mL solid phase polymer-based extraction cartridge (OASIS HLB, Waters, MA, USA). The cartridge was preconditioned with 1 mL of MeOH followed by 1 mL of 5 % MeOH in 17.5 mM ammonium acetate buffer, pH 4.0. The sample was diluted to 5 % MeOH with the same pH 4.0 buffer before being loaded into the cartridge, and was drawn through the cartridge on a vacuum chamber at a flow rate of 1 mL/min. The cartridge was washed with 1 mL of 5 % MeOH in 17.5 mM ammonium acetate buffer, pH 4.0, and finally eluted with 2 mL of MeOH which was reduced to 100-250 µL for HPLC analysis. The recovery of radiolabelled residues by the solid extraction procedure was determined for each sample and was typically > 90 %.

Radio-HPLC analysis

The metabolite profiles were obtained by reversed-phase radio-HPLC analysis of the SPE extract using a Waters 600 HPLC instrument with a 4.6 × 150 mm (5 µm) C_{18} HPLC column and a C_{18} guard column (Nova-Pak, Waters Millipore Corporation, MA, USA). Metabolites were detected using a photodiode array UV spectrophotometer (Waters 996) connected in series with a radioactivity detector (Berthold LB 506 C-1, Wildbad, Germany). A HPLC step gradient program was employed using methanol:water:acetic acid (30:70:0.1 v/v, adjusted to pH 4.0 with NH_4OH) for 10 min, increasing to 40 % MeOH (20 minutes), 50 % MeOH (35 minutes), and finally to 100 % MeOH over 15 minutes in a linear gradient. The flow rate was 1 mL/min over the entire run. The radioactive fractions were collected and the HPLC eluent evaporated under vacuum at room temperature in order to isolate metabolites for further structural analysis.

β-glucuronidase hydrolysis

Aliquots of metabolites fractionated by HPLC were incubated with β-glucuronidase (80 units/mL in 100 mM phosphate buffer, pH 6.75) for 12 hours at 37 °C. After acidification to pH 4 with 1M hydrochloric acid, the metabolites were extracted with ethyl acetate (3 × 1 mL) and the solvent was evaporated to dryness under nitrogen and samples were redissolved in 50:50 water:MeOH (pH 4.0) prior to re-analysis by radio-HPLC.

Results and discussion

Bioconcentration of t-*OP residues*

t-OP residues (parent compound plus metabolites, measured as *t*-OP equivalents) accumulated in roach fry after 5 days exposure and reached steady state concentrations after 12 days exposure (Tab. I). At this time the bioconcentration factor (BCF) of *t*-OP residues in the fry was between 1061 and 1134 for 12 and 19 days exposure respectively. The BCF in roach fry was similar to the BCF of 1300 reported for radioactive NP residues in adult stickleback [13] but lower than the BCF of 280 calculated for NP in juvenile salmon [14].

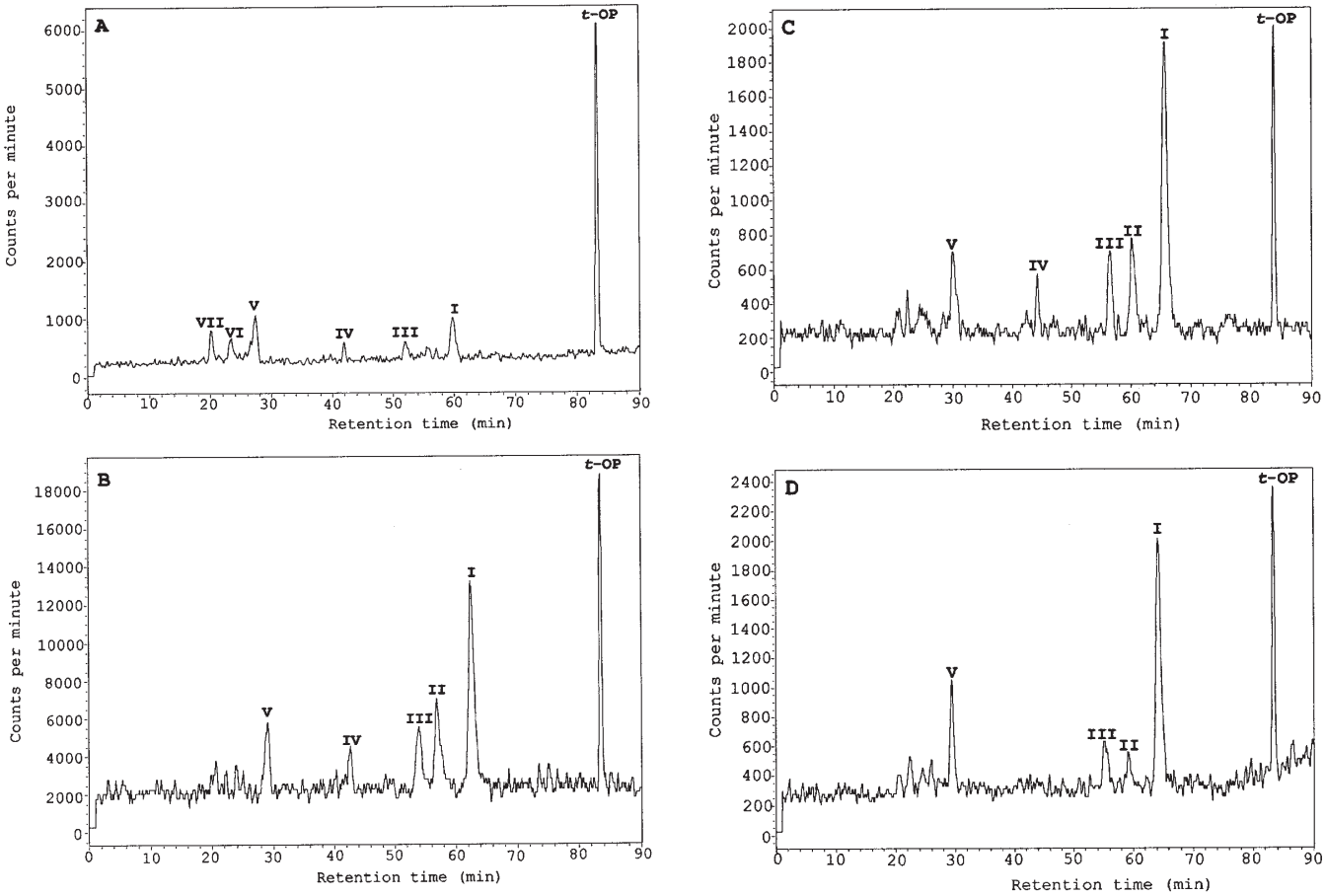

Figure 1. Reversed phase radio-HPLC profiles of methanol extracts of roach fry exposed to 5.8 µg/L [^{14}C] *t*-OP. A = 7 DPH fry exposed for 5 days. B = 7 DPH fry exposed for 12 days. C = 7 DPH fry exposed for 19 days. D = 26 DPH fry exposed for 5 days.

Table I. Accumulation of *t*-OP residues in roach fry.

Age of fry DPH	*Days of exposure*	*Concentration of t-OP residues (ng/g wet weight fish)*	*BCF of t-OP residues*	*Concentration of t-OP (% of total radioactivity)**
7-12	5	2006	346	57
7-19	12	6156	1061	24
7-26	19	6579	1134	21
26-31	5	4034	696	26

*= calculated from integration of radio-HPLC peaks.

Metabolite profile

Reversed-phase radio-HPLC analysis of the methanol extracts of exposed roach fry revealed the presence of the parent compund (eluting at 84 min) and a number of polar metabolites (Fig. 1). When 7 DPH fry were exposed to *t*-OP for only 5 days, the parent compound made up 57 % of the total radioactivity (Tab. I, Fig. 1A). However when fry were exposed to *t*-OP for 12 or 19 days, the parent compound composed only 24 % or 21 % of the total radioactivity (Tab. I, Fig 1B and 1C). To determine whether the increased metabolism of the alkylphenol at longer exposure periods was due to long term changes in expression of metabolising enzymes or due to the developmental stage of the fry, 26 DPH fry were dosed with *t*-OP for 5 days. In this experiment the parent compound accounted for only 26 % of the radioactivity which suggested that the activity of metabolising enzymes in older (26 DPH) fry was much greater than that in 7 DPH fry.

In order to characterise the *t*-OP metabolites, a number of methanol extracts from 7 DPH fry dosed for 12 or 19 days were combined and the radioactive metabolites separated and purified on reverse phase-HPLC. The purified metabolites were subjected to β-glucuronidase hydrolysis and re-analysed by reverse phase-HPLC. Enzyme hydrolysis increased the retention time of metabolite I from 64.2 to 83.5 minutes, metabolite III from 59.1 to 81 minutes, and metabolite V from 29.5 to 53 minutes, indicating that all three metabolites were glucuronide conjugates. The HPLC retention time of the aglycone of metabolite I was the same as that of *t*-OP which suggested that it was the glucuronide conjugate of *t*-OP.

It is not clear when sexual differentiation takes place in roach fry, however in the carp (*Cyprinus carpio*) sexual differentiation takes place after 50 DPH [15]. In our study 7 to 31 DPH roach fry are likely to be either undifferentiated or undergoing sexual differentiation and therefore sensitive to exposure to xenoestrogens. Our study shows that at this stage roach express glucuronosyl transferase enzymes capable of conjugating and deactivating oestrogenic alkylphenols. Newly hatched salmonid fish have also been shown to form glucuronide metabolites of steroid precursors [16] which suggests that early stage fish can metabolise both oestrogens and xenobiotics which maybe derived either from maternal or external sources.

Conclusions

Our study shows that waterborne oestrogenic alkylphenols, such as *t*-OP, can bioconcentrate in early stage roach fry and thereby contribute to the oestrogenic burden during sexual development of the organism. Roach larvae aged > 12 DPH, express high levels of glucuronosyl transferase enzymes which maybe important in the deactivation and elimination of phenolic xenoestrogens.

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References

1. Jobling, S.; Nolan, M.; Tyler, C.R.; Brighty, G.; Sumpter, J.P. *Environmental Science and Technology* **1998,** *32*, 2498-2506.
2. Desbrow, C.; Routledge, E.J.; Brighty, G.C.; Sumpter, J.P.; Waldock, M. *Environmental Science and Technology* **1998,** *32*, 1549-1558.
3. Tyler, C.R.; Routledge, E.J. *Pure and Applied Chemistry* **1998,** *70*, 1795-1804.
4. Harries, J.E.; Sheahan, D.A.; Jobling, S.; Matthiessen, P.; Neall, P.; Sumpter, J.P.; Tylor, T.; Zaman, N. *Environmental Toxicology and Chemistry* **1997,** 16, 534-542.
5. Routledge, E.J.; Sumpter, J.P. *The Journal of Biological Chemistry* **1997,** *272*, 3280-3288.
6. Piferrer, F.; Donaldson, E. M. *Aquaculture* **1989,** *77*, 251-262.
7. Tyler, C. R.; Jobling, S.; Sumpter, J. P. *Critical Reviews in Toxicology* **1998,** *28 (4***),** 319-361.
8. Piferrer, F.; Carrilho, M.; Zanny, S.; Solar, I. I.; Donaldson, E. M *Aquaculture* **1994,** *119*, 409-423.
9. Gimeno, S.; Gerritsen, A.; Bowmer, T. *Nature* **1996,** *384 (6606*), 221-222.
10. Gray, M. A.; Nimi, A.J.; Metcalfe, D. *Environmental Toxicology and Chemistry* **1999,** *18*, 1835-1842.
11. Jobling, S.; Sheahan, D.; Osborne, J.A.; Matthiessen, P.; Sumpter, J.P. *Environmental Toxicology and Chemistry* **1996,** *15*, 194-202.
12. Servos, M.R. *Water Quality Research Journal of Canada* **1999,** *34*, 123-177
13. Ekelund R.; Bergman, A.; Granmo, A.; Berggren, M. *Environmental Pollution* **1990,** *64*, 107-120.
14. McLeese, D.W.; Zitko, V.; Sergeant, D.B.; Burridge, L.; Metcalfe, C.D. *Chemosphere* **1981,** *10*, 723-730
15. Gimeno, S.; Komen, H.; Venderbosch, P.W.M.; Bowmer, T. *Environmental Toxicology and Chemistry* **1997,** *31*, 2884-2890.
16. Yeoh, C.G.; Shreck, C.B.; Fitzpatrick, M.S.; Feist, G.W. *General and Comparative Endocrinology* **1996,** *102*, 197-209.