Identification of 4-*n*-nonylphenol metabolic pathways and residues in aquatic organisms by HPLC and LC-MS analyses

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In order to better assess the risk associated with nonylphenol, a lipophilic contaminant of the aquatic environment having a strong tendency to bioaccumulate in organisms and exerting estrogenic effects in fish and invertebrates, we have investigated the capability of three types of freshwater species to metabolize labelled 4-*n*-nonylphenol. Reverse-phase radio-HPLC was used to isolate and quantify the metabolites. The identification of residues was based on co-chromatography with available standards or authentified metabolites and when possible by electrospray ionization LC-MS analyses. The residues found in rainbow trout (*Oncorhynchus mykiss*), pond snails (*Lymnea stagnalis*) and duckweeds (*Lemna minor*) were identified. These freshwater organisms are able to extensively biotransform 4-*n*-nonylphenol. Our attempts to draw the metabolic pathways of nonylphenol evidenced oxidative attack on the alkyl chain with the production of several hydroxylated compounds and related carboxylic acids. In addition, trout and duckweeds were able to conjugate the phenol moiety to glucuronic acid and carbohydrates respectively. Traces of sulphate conjugates were also detected in trout tissues whereas no conjugate was detected in pond snails.

Introduction

It has long been established that living organisms may accumulate a broad range of xenobiotics from the foodweb and/or the surrounding medium. The bioaccumulation of a contaminant is primarily dependent on: (1) its lipophilicity, (2) its stability in the medium, and (3) its capability to overcome biotransformation. The data reported by Ekelund *et al.* [1] suggest that nonylphenols (NPs) bear such characteristics. These chemicals are complex mixtures of aliphatic side-chain isomers mainly used for the production of nonylphenol polyethoxylates (NPnEOs), a class of non ionic surfactants with a wide range of applications [2]. Smaller quantities of NPs are used in plastic manufacturing processes and as spermicides [3,4]. NPs and NPnEOs enter the environment mostly through sewage treatment plants [5]. In the aquatic environment, NPs occur primarily as degradation products of NPnEOs. A wide range of NP and NPnEO concentrations were measured in water, including drinking water, and in sediment [6]. Parent NPnEOs are considered as non persistent, but some degradation products may have moderate persistence, especially in anaerobic conditions [5]. Ethoxylate chain length affect the chemical properties of the compounds, including water solubility and K_{ow}, therefore affecting their bioavailability and toxicity.

Acute toxicity of NPs and NPnEOs to aquatic organisms is usually achieved for high concentrations. However, effects on growth and reproduction were observed following chronic exposures to low NP and NPnEO concentrations [7]. In particular, NPs are known to exhibit estrogen-like activities in vertebrates, both *in vitro* and *in vivo*, and a number of studies have reported their estrogenicity in aquatic animals [7]. Exposure to NPs may thus influence the reproductive system of male fish, as demonstrated for the rainbow trout [8], Japanese medaka [9], carp [10], eelpout [11], atlantic salmon [12], mosquitofish [13]. Although NP was shown to bind *per se* to fish estradiol receptor [14,15], it has been suggested that hydroxylation of the aliphatic side chain might result in bioactivation of the parent compound [16]. In the amphibian *Xenopus laevis*, NP bound to the liver estrogen receptor, induced vitellogenin synthesis in primary cultured hepatocytes and caused *in vivo* effects on sexual development of larvae [17,18].

Invertebrates may also be affected by NPs. Arthropods have been recognized as the most sensitive group. In daphnids, NP was shown to significantly decrease the fecundity without effect on adult survival [19]. NP affected the production of *Daphnia galeata mendotae* female offspring, and prenatal exposure to NP caused morphological abnormality in the juveniles [20]. Life-cycle tests with non-biting midges (chironomids) showed that 4-NP exposure resulted in reduced survival and altered egg masses [21]. NPs and NPnEOs may also have effects on bivalve molluscs. In *Elliptio complanata*, NPs affected energy metabolism, possibly by uncoupling mitochondrial oxidative phosphorylation [22]. The development of the oyster *Crassostrea gigas* was

delayed by the exposure to 4-NP that also caused growth abnormality and a significant decrease in survival rate [23].

A few studies have shown effects of NPs and NPnEOs in aquatic plants. Inhibition of frond production, reduced growth and decreased photosynthetic activity were observed after exposure to NPs in the pond weed, *Lemna minor*, and floating fern, *Salvinia molesta* [24].

Bioaccumulation of NPs and NPnEOs has been demonstrated for a number of algae, plant, invertebrate, and fish species, and the bioconcentration/bioaccumulation factors ranged from 0.9 to 3400 [7]. The potential of such molecules to bioaccumulate in various organisms of the aquatic foodwebs was suggested from both laboratory [1] and field [25] exposure experiments. Bioaccumulation may contribute to increase internal NP concentrations, but these latter usually remain far beyond the levels that cause acute effects. However, non lethal effects of NPs, including their estrogenic effects, may result from biotransformation products. It is therefore of primary importance to aquire a better knowledge of the metabolic pathways of NPs in aquatic biota.

The present study was designed to study the ability of aquatic organisms belonging to a simplified aquatic food chain (plants, macroinvertebrates, fish) to metabolize 4n-NP. The metabolic pathways of ^{3}H-4n-NP was previously described in the rainbow trout, *Oncorhynchus mykiss* [26,27]. After oral exposure to ^{3}H-4n-NP, radioactivity was widely distributed in tissues but mainly concentrated in the digestive tract and liver [28]. In the present study, NP residues were identified in rainbow trouts orally exposed to ^{3}H-4n-NP. In addition, the nature of NP residues was investigated in the freshwater snail, *Lymnaea stagnalis*, and the duckweed, *Lemna minor*, following water exposure to ^{3}H-4n-NP.

Materials and methods

Chemicals and biochemicals

4n-NP and 3-(4-hydroxyphenyl)propionic acid were obtained from Fluka (Buchs, Switzerland) and 4-hydroxybenzoic acid was from Merck (Darmstadt, Germany). 4-Hydroxycinnamic acid, caffeic acid, β-glucuronidase from bovine liver (type B-1), sulphatase from Aerobacter aerogenes (type VI), β-glucosidase from almonds, MS 222 (tricaine methanesulfonate) and D-saccharic acid 1,4-lactone were purchased from Sigma (Saint-Quentin-Fallavier, France). [R-2,6-^{3}H]-4n-NP was synthesized as previously described [26]. All solvents were of analytical grade.

Exposure experiments

Immature rainbow trout (mean weight 200 g) were supplied by the Institut National de la Recherche Agronomique pisciculture at Donzacq (France) and maintained in dechlorinated tap water at 14 ± 1 °C. Fish were fed on commercial trout feed and starved 48 h before experiment. Three trout were anaesthetized with 100 mg/l MS222 (tricaine methanesulphonate) and force-fed a gelatin capsule containing 1.5 g feed and ^{3}H-4n-NP (1850 KBq, 5 mg). After 48 h, fish were killed with an overdose of anaesthetic. The liver was excised and the gall bladder removed prior to homogenization of the viscera.

Pond snails (*Lymnea stagnalis*) were bred at the INRA Aquatic Ecology and Ecotoxicology Experimental Unit (Rennes, France) at $21° \pm 2$ °C in dechlorinated tap water. The experiment was conducted in a 500 ml glass beaker. The beaker contained 300 ml dechlorinated tap water, so that the volume of air allowed the animals to breathe. ^{3}H-4n-NP (370 KBq, 100 ppb) was dissolved in 10 µl ethanol and diluted in the 300 ml of water before the snails ($n = 11$) were transferred to the beaker. The animals were not fed during the experimental period and after 12 h of exposure, they were sacrificed by freezing in liquid nitrogen.

Stocks of duckweeds (*Lemna minor*) were maintained in sterile culture in 250 ml Erlenmeyer flasks containing 100 ml of Hoagland medium. Cultures were grown under continuous light at 25 °C. Duckweeds (average wet weight 200 mg) were transferred to a glass Petri dish containing 20 ml of sterile Hoagland medium. ^{3}H-4n-NP (37 KBq) was dissolved in 5 µl ethanol and diluted in the culture medium. After 6 days exposure, the duckweeds were transferred to 20 ml clean Hoagland medium. After 6 days of depuration, plants were harvested and the excess of water drained off before homogenization.

Analytical procedure

Figure 1 gives the general procedure for isolation and analysis of metabolites from trout, snail and duckweed tissues. Analyses were performed individually for trout and snails and on pooled samples for duckweeds. Trout digestive tracts were cut into small pieces and frozen in liquid nitrogen prior to homogenization with a Dangoumeau grinder. Snails, trout livers and duckweeds were ground using a Potter homogenizer. Tissues or organisms were homogenized in 10-50 mL methanol and centrifuged at 10000 *g* for 10 min at 5 °C. This step was repeated twice on the residual material and the three supernatants were combined. The extractable radioactivity was measured directly by counting an aliquot of the pooled supernatants in a Tricarb 2200CA (Packard Instruments Co., Downers Grove, IL, USA) liquid scintillation counter using Ultima Gold (Packard Instruments Co.) as the scintillation cocktail. The radioactivity remaining in the residue after extraction was quantified by oxidative combustion with a Packard oxidizer model 306 followed by liquid scintillation counting. Aliquots of the methanol extracts from trout livers and duckweeds were evaporated to dryness under nitrogen stream, and dissolved in the appropriate HPLC mobile phase before chromatography analysis. Two purification steps were added for trout viscera and snails, before HPLC analysis, namely a methanol: chloroform partition and an isooctane: acetonitrile partition (Fig. 1).
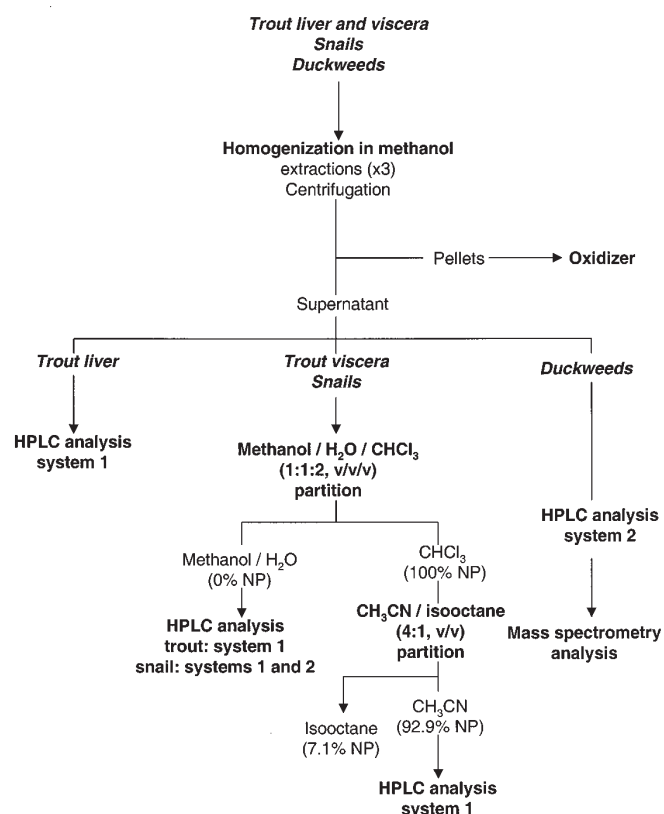
Figure 1. Flowchart for extraction and analysis of residues from trout liver and viscera, snails and duckweeds. % NP indicates partitioning of radioactivity in each phase when extraction is performed on a water sample containing 1KBq ^{3}H-4n-NP.

redissolved in acetonitrile-ammonium acetate buffer (1:4, v/v) and injected into HPLC.

Radio-HPLC and identification of NP metabolites

HPLC was performed on a P-1000 Spectra Physics system equipped with a 250×4.6 mm Nucleosil C18 (5 µm) reversed-phase column (Bishoff Chromatography, Germany) protected by a guard column ODS Hypersil (5 µm). Separations of NP metabolites were performed using two HPLC systems differing in the pH of the ammonium acetate buffer (20 mM): pH 5 (system 1) or pH 3 (system 2). The mobile phases were composed of (A) ammonium acetate buffer and (B) 90 % acetonitrile and 10 % ammonium acetate buffer and were pumped at a flow rate of 1 ml/min. The run was set as follows: 0-40 min, linear gradient from 100 % A to 35 % B; 40-55 min, linear gradient from 35 % B to 100 % B; 55-65 min, 100 % B. Chromatographic peaks were monitored by on-line radioactivity detection with a Radiomatic Flo-one A-200 apparatus (Packard Instrument Co.) using Flo-scint 2 (Packard Instrument Co.) as scintillation cocktail. Identification of the metabolites was established on the basis of co-chromatography with authentic standards or metabolites unambiguously identified by mass spectrometry in previous studies [26,27]. Where conjugates were concerned, the samples were analyzed before and after hydrolysis. When necessary, metabolites were isolated by coupling the HPLC system with a model FC-204 fraction collector (Gilson Medical Electronics, Middleton, WI, USA). The collected fractions were evaporated *in vacuo* at 45 °C and redissolved in methanol and stored at –18 °C until mass spectrometry (MS) analysis.

Electrospray ionization-mass spectrometry (ESI-MS) analyses were achieved on a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Quest, Les Ulis, France) equipped with an electrospray source. Solution samples (typically 10 ng/µl), were prepared in 50-50, (v/v) mixtures of methanol-H$_2$O (CH$_3$OD-D$_2$O for H/D exchange experiments) and were infused into the electrospray source at a flow rate of 3 µl/min. The electrospray needle voltage and heated capillary temperature were set to 5.0 kV and 230 °C, respectively. The heated capillary voltage was – 16 V and the tube lens offset was 0 V. The instrument was used in the negative-ion mode and scanned over a mass range from m/z 50 to 600. MS-MS operating parameters were determined on the $[M-H]^-$ ion of each intact metabolite and standard compound to enhance structural information.

The remaining part of the methanol extracts from trout, pond snails and duckweeds was evaporated to dryness and dissolved in water before treatment by β-glucuronidase, β-glucosidase, sulphatase or HCl hydrolysis. HCl (6 M) hydrolysis of the duckweed extract was carried out at 100 °C. After 1 h, the reaction was stopped and HCl was diluted with distilled water. Radioactivity was extracted from the aqueous phase using chloroform (1:1, v/v). The chloroform fraction was then evaporated to dryness under nitrogen stream, redissolved in acetonitrile-ammonium acetate buffer (pH 3.0) (1:4, v/v) and analyzed by HPLC (system 2). The extracts redissolved in water for enzymatic treatment, were diluted in 0.2 M sodium acetate buffer (pH 5.0) for β-glucosidase and β-glucuronidase hydrolysis or tris-HCl buffer (0.5 M, pH 7.4, MgCl$_2$ 10 mM) for sulphatase hydrolysis. β-Glucosidase and β-glucuronidase hydrolysis were carried out at 30 °C for 2 h and 37 °C for 12 h, respectively. For sulphatase hydrolysis, D-saccharic acid 1,4-lactone was added in order to inhibit β-glucuronidase activity and the reaction was carried out at 37 °C for 2 h. Methanol was added to stop the reactions and the precipitated proteins were removed by centrifugation. An aliquot of the supernatant was evaporated to dryness under nitrogen stream,

Results and discussion

Rainbow trout

Since previous studies indicated very low residue levels in most of the tissues of trout dietary exposed to ^{3}H-4n-NP [28], analyses were only performed on the liver and digestive tract. In our experimental conditions, 11.0 ± 1.8 % of the radioactivity was still present in the whole body after

48 h and 1.8 and 2.1 % remained in the liver and the digestive tract, respectively (data not shown).

In the liver, 95.7 % of the radioactivity was extracted and the remaining non extractable 4.3 % (Tab. I) could be attributed to bound residues. This result is consistent with the data reported by Coldham *et al.* [29]; in trout injected with a single dose of ^{3}H-4-NP, they found that the liver contained a low level (2 %) of covalently bound residues. Rainbow trout liver extracts were analyzed with the HPLC system 1 previously used for the separation of biliary metabolites (Fig. 2A, [26]). The chromatogram obtained from the liver extracts (Fig. 2B) showed the presence of 83.64 ± 10.19 % of nonylphenol-glucuronide and 6.28 ± 1.15 % of unchanged NP. These results are in agreement with those of Coldham *et al.* [29] who reported that nonylphenol-glucuronide was the predominant extractable residue in trout liver. Such findings are consistent with the fact that nonylphenol-glucuronide was described as the major metabolite in bile of trout [26,29,30] and salmon [31], most of the metabolites produced in the liver being excreted in the bile.

In the digestive tract, non-extractable radioactivity accounted for 35.8 % (Tab. I). Viscera extracts contained significant levels of lipids because the adipose tissue associated to the intestine was not removed before homogenization. After chloroform/methanol partitioning, 78.5 ± 1.1 % of the extracted radioactivity was found in the upper phase and analyzed with the HPLC system 1. The profile (Fig. 2C) pointed out nonylphenol-glucuronide as the major residue (47.12 ± 3.28 %). Coldham and co-workers [29] reported similar data from HPLC analysis of ^{3}H-4-NP residues in trout pyloric caecae. Five other metabolites were identified based on their retention time before and after enzymatic hydrolysis (Fig. 2C). These compounds were carboxylic acids resulting from the β-oxidation of the alkyl chain. 7-(4-Hydroxyphenyl) heptanoic, 5-(4-hydroxyphenyl) pentanoic, 3-(4-hydroxyphenyl) propionic and 4-hydroxybenzoic acid were detected as such or as glucuronide conjugates (Fig. 2C). V1 and V2 metabolites were hydrolyzed by sulphatase treatment and de-conjugated V2 coeluted with 7-(4-hydroxyphenyl) heptanoic acid. Based on these data, V2, which amounted to 17.89 ± 1.23 % of the extracted radioactivity was identified as the sulphate conjugate of
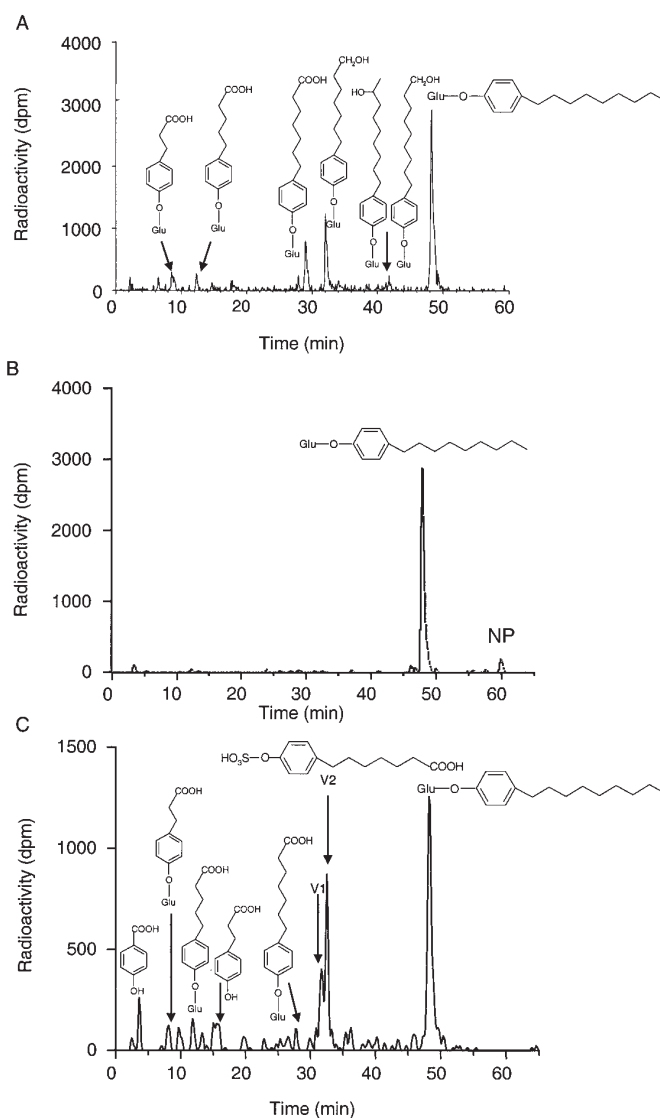
Figure 2. Comparison of the radio-HPLC profiles of trout biliary metabolites (A) and extractable residues of ^{3}H-4n-NP from liver (B) and viscera (C).

Table I. Extraction of ^{3}H-4n-NP residues from trout liver and viscera, pond snails and duckweeds. Means ± SD were obtained from three animals.

Organisms		Total	extractable				non extractable
			Methanol/CHCl$_3$ partition		Iso-octane / acetonitrile partition		
			Methanol/H$_2$O	CHCl$_3$	acetonitrile	Iso-octane	
Trout	liver	95.7 ± 0.7 %	/	/	/	/	4.3 ± 0.6 %
	digestive tract	64.2 ± 4.4 %	78.5 ± 1.1 %	21.5 ± 0.9 %	80.1 ± 6.9 %	19.9 ± 5.6 %	35.8 ± 3.6 %
Snails	whole body	96.8 ± 1.9 %	57.5 ± 9.4 %	42.5 ± 7.6 %	70.6 ± 1.4 %	29.4 ± 1.1 %	3.2 ± 1.5 %
Duckweeds	whole organisms	52.6%	/	/	/	/	47.4%

7-(4-hydroxyphenyl) heptanoic acid. The nature of V1 remains to be elucidated. In 1998, Coldham *et al.* [29] reported the *in vitro* production of a sulphate conjugate of hydroxylated 4-NP in isolated trout hepatocytes. The present study confirms *in vivo* the involvement of sulphation in 4n-NP metabolism in fish. Analysis of the radioactivity extracted with the lipid fraction (chloroform) was performed after acetonitrile - isooctane partition, and corresponded to the parent compound. This significant level of unchanged NP may be a consequence of the *per os* administration route and a storage in the adipose tissue. However the presence of some residues of unabsorbed ^{3}H-4n-NP in the intestinal lumen cannot be excluded.

Pond snails

Radioactivity remaining in the snail was measured after oxidative combustion of the whole body. It reached only 1.97 ± 0.68 % of the radioactive dose after 12 hours of waterborne exposure and lower levels were detected at 24, 48 and 72 h (data not shown). After 12 h of exposure, 96.8 ± 1.9 % of the radioactivity remaining in snail was extracted and only 3.2 ± 1.5 % (Tab. I) could be attributed to bound residues. These results suggest a weak uptake of NP and rapid metabolization and excretion of metabolites. In pulmonate freshwater snails, the uptake of waterborne chemicals is mainly through the body surface. Significant uptake may also occur through the foot sole for chemicals that are adsorbed onto immersed substrates. Only few data on the metabolism of xenobiotic compounds in freshwater gasteropods are available and they mostly focused on pesticides. Takimoto *et al.* [32] found similar half life for fenitrothion in two freshwater snails and in fish (about 0.5 day) and reported a rapid excretion of its metabolites. Kanazawa [33] observed a lower accumulation of diazinon in snails than in fish species. In contrast, the uptake of atrazine by fish and freshwater molluscs is fast; the uptake directly from waters occurs quickly and is much higher than the rate of uptake from food, but only a slight accumulation is observed [34]. In *Llymnae palustris*, atrazine was transferred more rapidly than hexachlorobenzene (HCB) from water, but the maximal internal level was greater and maintained for longer in HCB-exposed snails than in the atrazine-exposed groups. At the equi-concentration of 5 µg/L, the maximal internal amount of pesticide residues was 17.4-fold lower for atrazine than for HCB [35]. Bioconcentration of xenobiotics in freshwater snails may also be influenced by tissue lipid content [32]. Thus, lower lipid content may explain, at least in part, less efficient accumulation of lindane in *Lymnaea palustris* (0.8 % lipid relative to total fresh weight [36]) as compared to *Ancylus fluviatilis* (12 % lipid relative to total fresh weight [37]). In *Lymnaea palustris* exposed to lindane, biphasic depuration kinetics were observed when the animals were transferred to pesticide-free water, with half-lives of 0.7 h and 130.2 h for the fast and slow phases, respectively [36].

After chloroform/methanol partitioning of snail extracts, 57.5 ± 9.4 % of the extracted radioactivity was found in the aqueous phase (Tab. I). More than 70 % of the radioactivity

extracted in chloroform was subsequently present in acetonitrile and corresponded to unchanged NP (data not shown). The radioactivity associated with the aqueous phase was analyzed by the HPLC systems 1 (Fig. 3A) and 2 (Fig. 3B). The metabolic profiles showed between four and six peaks depending on the HPLC system used, and was not affected by β-glucuronidase or sulphatase hydrolysis. These results suggest that metabolites correspond to unconjugated compounds. The major metabolite amounted to 46.8 % of the analyzed radioactivity, and co-eluted with authentic 4-hydroxybenzoic acid at 3.8 min and 12.0 min on HPLC system 1 and 2, respectively. The other chain-shortened carboxylic acids detected were 4-hydroxycinnamic acid and 3,4-dihydroxybenzoic acid, accounting for 11.3 % and 3.5 % of the analyzed radioactivity, respectively. Less polar metabolite(s) which accounted for 11.3 % of the analyzed radioactivity could be related to ω-1 hydroxylated NP and/or 9-(4-hydroxyphenyl) nonanoic acid, which co-eluted at 50.0 min on both HPLC systems. These results show that NP is extensively biotransformed in the pond snail and that
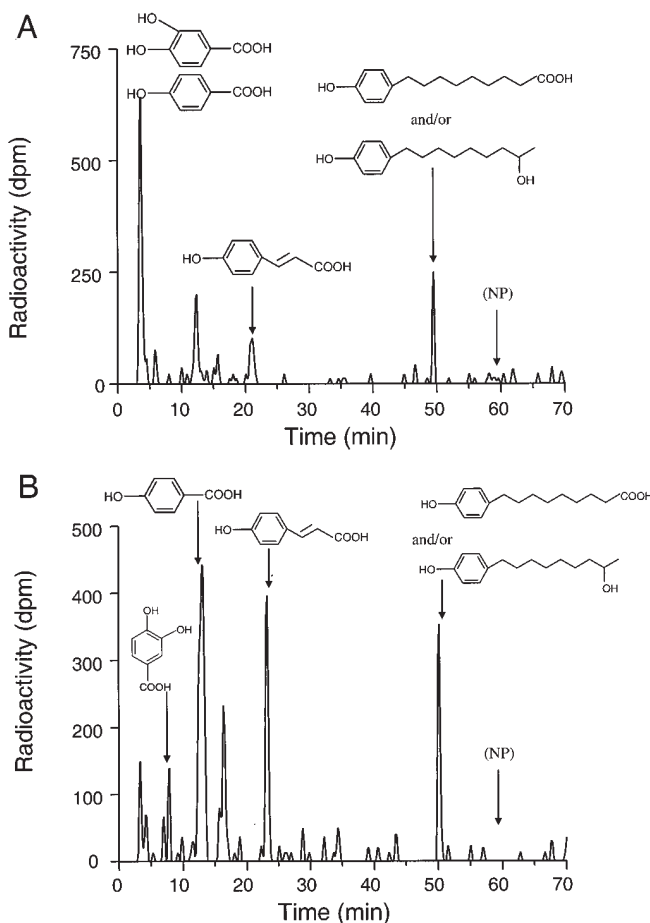

Figure 3. Radio-HPLC analyses of extractable residues of ^{3}H-4n-NP from pond snails with HPLC system 1 (A) and HPLC system 2 (B). HPLC conditions are described in the materials and methods section.

terminal oxidations and β-oxidation of a linear alkyl chain are metabolic pathways occurring in this species. Similarly, Takimoto *et al.* [32] reported that freshwater snails exhibited excretory oxidation activity. However, these authors described the conjugation of phenols with sulphate in *Cipangopaludina japonica* and mainly with glucose in *Physa acuta*. We did not demonstrate these pathways with *Lymnea stagnalis* using NP as substrate.

Duckweeds

After 6 days of exposure and 6 days of depuration, 70.2 % of the radioactive dose was still present in the duckweeds. Non-extractable radioactivity amounted to 47.4 % and may be attributed to bound residues (Tab. I). After longer depuration periods (12 and 18 days) similar amounts of both extractable and non-extractable radioactivity were found in the duckweeds (data not shown) suggesting that NP and/or its metabolites were retained into the organisms. These results were expected because plants lack a well-developed excretory system and because plant cells are characterized by the presence of vacuoles where xenobiotic metabolites may be sequestered. Bound residues are common and generally found incorporated or associated with polymers of plants, including lignin, various carbohydrate polymers and proteins [38].

Extractable residues were separated using HPLC system 2. A typical profile of metabolites in duckweeds is given in figure 4A, showing more than ten peaks and no trace of the parent compound. None of the metabolites were affected by β-glucosidase, β-glucuronidase or sulphatase hydrolysis but the profile was totally modified by HCl treament (Fig. 4B), suggesting the presence of conjugated compounds in duckweeds. Glucose appears to be the most common xenobiotic conjugation reaction in plants. However, conjugations with other carbohydrates such as arabinose and xylose were frequently described [38].

Analyses were performed on intact metabolites using ESI-MS. The major metabolite L2, accounted for 33.5 % of the extracted radioactivity and eluted at 20.5 min on HPLC system 2. ESI-mass spectrum (negative ionization) of metabolite L2 (Fig. 5A) yielded a [M-H]$^-$ ion at m/z 279. The corresponding MS/MS spectrum exhibited fragment ions at m/z 163, 133 and 119. Analyzed in the same conditions, authentic 4-hydroxycinnamic acid showed a [M-H]$^-$ ion at m/z 163 and a [M-H-CO$_2$]$^-$ fragment ion at m/z 119, suggesting that L2 aglycone could correspond to 4-hydroxycinnamic acid. The m/z 133 fragment ion was tentatively attributed to a deoxypentose as a possible structure among various conjugating carbohydrates. Therefore, H/D exchange experiments were carried out in order to get more information by counting the number of exchangeable hydrogens of the molecule. In a deuterated medium, the molecular species was shifted from a [M-H]$^-$ species at m/z 279 to a [Md$_3$-D]$^-$ at m/z 281, indicating that L2 contained three exchangeable hydrogen atoms. Thus two deuterium atoms may be located on the carbohydrate moiety. This was confirmed by the MS/MS spectrum of the [Md$_3$-D]$^-$ ion which yielded
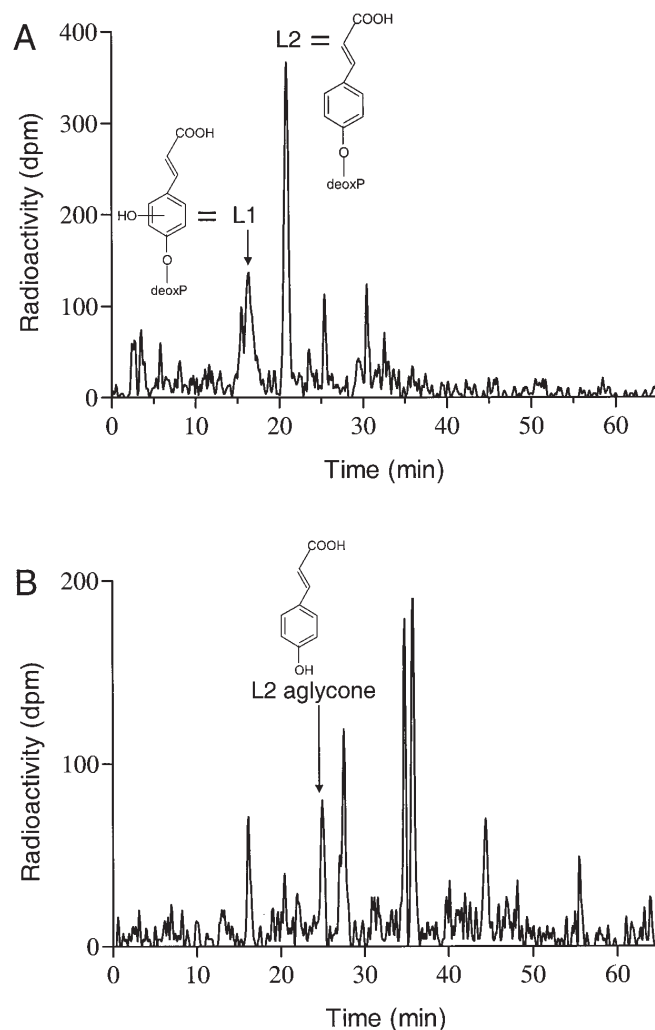

Figure 4. Radio-HPLC profiles of extractable residues of ^{3}H-4n-NP from duckweeds before (A) and after (B) HCl hydrolysis.

fragment ions at m/z 163 and 135, which could correspond to the deprotonated form of 4-hydroxycinnamic acid and a dideuterated deoxypentose, respectively. Fragment ions at m/z 164 and 134 were also observed and could be attributed to the result of a deuterium atom migration from the carbohydrate to the aglycone moiety. Based on these data, 4-hydroxycinnamic acid-deoxypentose conjugate was proposed as a possible structure for L2 metabolite.

Metabolite L1 accounted for 28.64 % of the extracted radioactivity. ESI-mass spectrum (negative ionization) of metabolite L1 (Fig. 5B) showed a quasi-molecular [M-H]$^-$ ion at m/z 295 and fragment ions at m/z 179, 135 and 133. The presence of the fragment ion at m/z 133 suggested that L1 was conjugated to a deoxypentose carbohydrate as previously described for metabolite L2. L1 and L2 aglycones showed a similar fragmentation pattern. Fragment ions at m/z 179 and m/z 135 suggested a dihydroxycinnamic acid
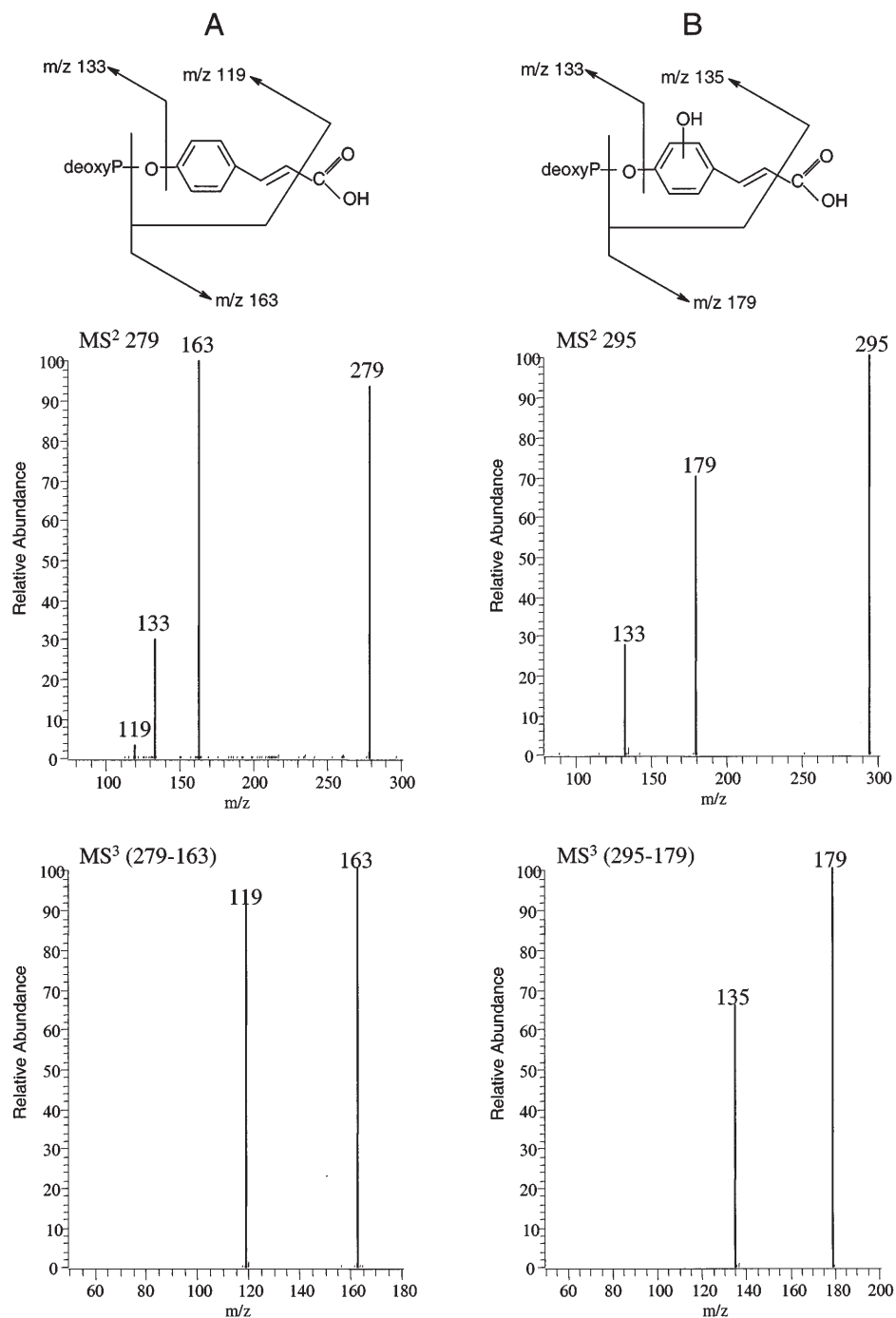
Figure 5. ESI-MS and MS/MS spectra (negative ionization) of L1 (B) and L2 (A) metabolites of ³H-4n-NP extracted from duckweeds.

structure for L1 aglycone. The ESI-mass spectrum (negative ionization) of authentic caffeic acid was similar to the ESI-mass spectrum of L1 aglycone. A quasi-molecular $[M-H]^-$ ion at m/z 179 and a $[M-H-CO_2]^-$ fragment ion at m/z 135 were observed. Based on these data, dihydroxycinnamic

acid-deoxypentose conjugate was proposed as chemical structure for metabolite L1. Bokern *et al.* [39] investigated the metabolites of 4n-NP in wheat cell suspension cultures. The metabolites were shown to be β-glucosides and β-glucuronides of 4-(monohydroxy) and 4-(dihydroxy)-

nonylphenols which were partially further conjugated with malonic acid. Hydroxylations occurred at all C atoms of the alkyl side chain, except C1 and C9. They reported the involvement of the same metabolic pathways in NP biotransformation for a wide range of terrestral plant species [40]. In agreement with these data, the detection of chain-shortened carboxylic acids (4-hydroxycinnamic acids) indirectly demonstrates that hydroxylation of the alkyl chain of 4n-NP also occurs in aquatic plants, but in this case at C9 position. As previously described in fish [26], C9-hydroxylated NP is converted by dehydrogenation into the corresponding carboxylic acid which undergoes an incomplete β-oxidation reaction. Furthermore, we detected, a metabolite hydroxylated on the phenol ring, namely dihydroxycinnamic acid. Such compounds are endogenous substances in plants and they may polymerize with other substrates into lignin. Consequently a possible endogenous origin of these compounds can not be totally excluded. In duckweeds, Krause and Strack [41] characterized glucose esters conjugates of hydroxycinnamic acids using a co-HPLC method. In our experiment, using mass spectrometry analysis, 4-hydroxycinnamic and 3,4-dihydroxycinnamic acids conjugated to a deoxypentose carbohydrate were tentatively identified. However, further studies are necessary to confirm this original metabolic pathway.

Conclusion

As summarized in figure 6, the present study confirmed that NP is extensively biotransformed in fish in which several metabolic pathways and several organs are involved. However, significant levels of unchanged NP and bound residues are retained in viscera and adipose tissue, and are likely to be further released with potential subsequent estrogenic effects. Bioaccumulation and storage may be even more important in field conditions, where fish are chronically exposed to highly branched NP isomers, more resistant towards metabolism. In the pond snail, phase I metabolic pathways are involved in 4n-NP biotransformation. For branched isomers of 4-NP, β-oxidation is impaired, and because of unefficient phase II metabolic pathways, metabolization and excretion of NP are probably weak. This needs to be confirmed in further studies. As shown for several aquatic macrophytes, NP residues can reach high values in duckweeds in spite of their high capability to biotransform NP. This is due to the fact that in plants, metabolites are retained within the tissues and are detectable after a long time following the exposure period. Regarding these data, the potential of duckweeds as bioindicators of NP pollution in aquatic ecosystems should be considered.
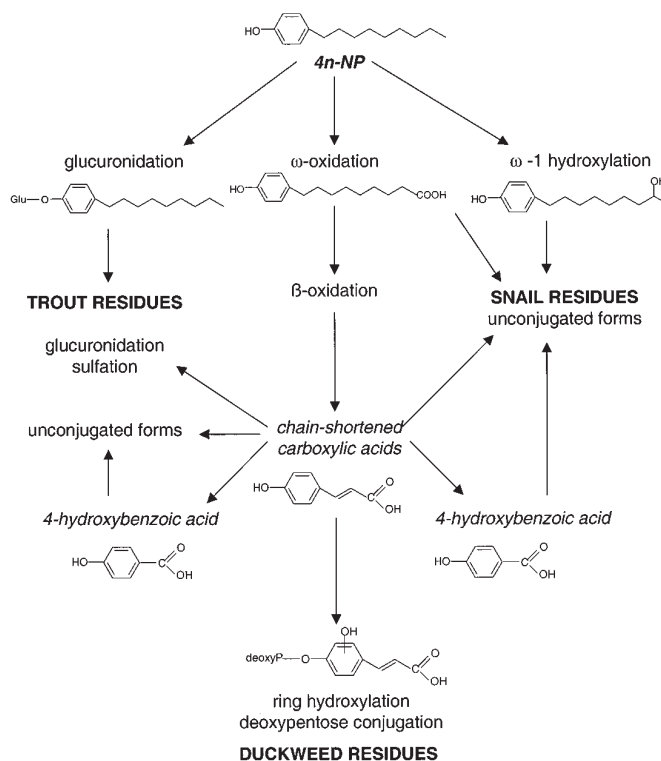
Acknowledgements

Figure 6. Proposed pathways for the formation of ³H-4n-NP residues in trout, pond snails and duckweeds.

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