Uptake, tissue distribution and elimination of C_4-C_7 alkylated phenols in cod. Dietary vs. waterborne exposure

Report AM-2003/001

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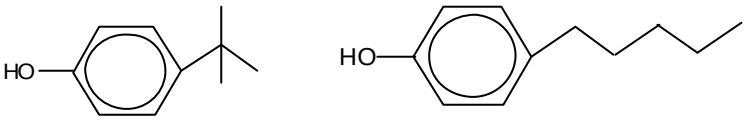

RF- Akvamiljø

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Scope:

The scope of the present study was to generate information about bioconcentration, tissue distribution and elimination of four alkylated phenols [C_4 to C_7] in Atlantic cod (*Gadus morhua*) from dietary and waterborne exposures. Each component was tested separately. A dose corresponding to 5 µg/kg fish was administred daily in dietary exposure and water exposure was at a nominal concentration of 0.008 µg/l.

Key-words:

toxicokinetics, bioconcentration factor , alkylated phenols, Atlantic cod, 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol, 4n-heptylphenol

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Summary

A previous study revealed effects on hormone balance and reproduction in cod exposed to a dietary dose of 5 µg alkylated phenols per kg fish.

The objective of the present study was to assess kinetics for uptake and elimination of 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol and 4n-heptylphenol in cod using water and diet exposure. Aditionally, tissue distribution of the compounds was estimated to obtain information of bioconcentration in specific tissues.

The study revealed that substantial amounts of alkylated phenols are taken up and bioconcentrated in the body tissues of fish exposed via seawater. Modelled bioconcentration factors range from 100 to 500.

Absorption efficiencies of the alkylated phenols via food are poor. The lowest value is observed for C_5-AP (8%) whilst for the other compounds absorption efficiency is between 12% to 14%.

In exposed fish most of the alkylated phenols are found in the gastro-intestinal system and the bile is apparently a major route of excretion of the compounds. The same pattern of tissue distribution was revealed for both water-borne exposed and dietary exposed fish.

In both types of exposures, the biological half-life of compounds is between 10 and 18 hours. Elimination rates of the different compounds are relatively comparable in both exposure pathways except for 4n-pentylphenol which seems to be eliminated at a slower rate in the dietary exposure.

Introduction and Objectives

Effects on hormone balance and reproduction in cod exposed to a dietary dose of 5 µg alkylated phenols per kg fish were revealed by Meier *et al*. (2002). In their study, the following compounds were investigated: 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol and 4n-heptylphenol. Assuming a bioconcentration factor of 600, a body burden of 5 µg/kg would correspond to a dose in the water of 0.008 µg/L

The norwegian oil industry association (OLF) asked for a confirmation of this consideration by testing the toxicokinetics of the four compounds in fish exposed either via food (daily dose 5 µg/kg fish) or seawater (0.008 µg/l) The objective of the present study was then to assess kinetics for uptake and elimination of 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol and 4n-heptylphenol in cod using these two exposure regimes. Aditionally, tissue distribution of the compounds was estimated to obtain information of bioconcentration in specific tissues.

Due to the low concentration to be tested, radioactive-labelled alkylated phenols were selected and both body burden and seawater concentration were estimated by mean of liquid scintillation counting.

In this report, results are presented and a description of the toxicokinetic patterns and values are given.

Terms and abbreviations

AP	Alkylated phenol
$C_5 – C_7$	Alkylated phenol designation referring to the number of carbon in the chain.
CPM	Counts Per Minutes (LSC measure of radioactivity)
^3H	Radioactive hydrogen (Tritium)
Hot material	Chemical component containing radioactivity
LSC	Liquid Scintillation Counting
Scintillation counting	Method for quantitative analysis of radioactive tracers
Tritium	Radioactive hydrogen [^3H]
Quenching	LSC correction to specific samples

EXPERIMENTAL DESCRIPTION

Organisms

Two size groups of farmed Atlantic cod (*Gadus morhua*), norwegian coastal cod, were employed. We used juvenile individuals (Sagafjord cod rearing station) with a mean weight of 30±15 g to obtain the kinetic parameters. Larger cods with a mean weight of 800 ± 100g (Vedavågen fish farm) were chosen to assess tissue distribution. Large fish were used to sample enough biological material for scintillation counting. All fish were vaccinated against vibriosis and stored indoor in a quarantine facility for at least 3 weeks before the start of the experiments. During the quarantine period, juvenile fish were weaned to feed on 2 mm pellets and the large fish were fed shrimps.

Exposure compounds

The four compounds were ^{3}H-labelled synthesized by RC Tritec AB, Teufen, Switzerland and purchased from IDA Chemie AS, Trondheim, Norway. All compounds were delivered in acetone as solvent. They consisted of 4-tert-butylphenol (16 Ci/mmol), 4n-pentylphenol (14 Ci/mmol), 4n-hexylphenol (18 Ci/mmol) and 4n-heptylphenol (21 Ci/mmol). All compounds had a radiochemical purity of 95% . The chemical structure of these compounds is shown in figure 1.

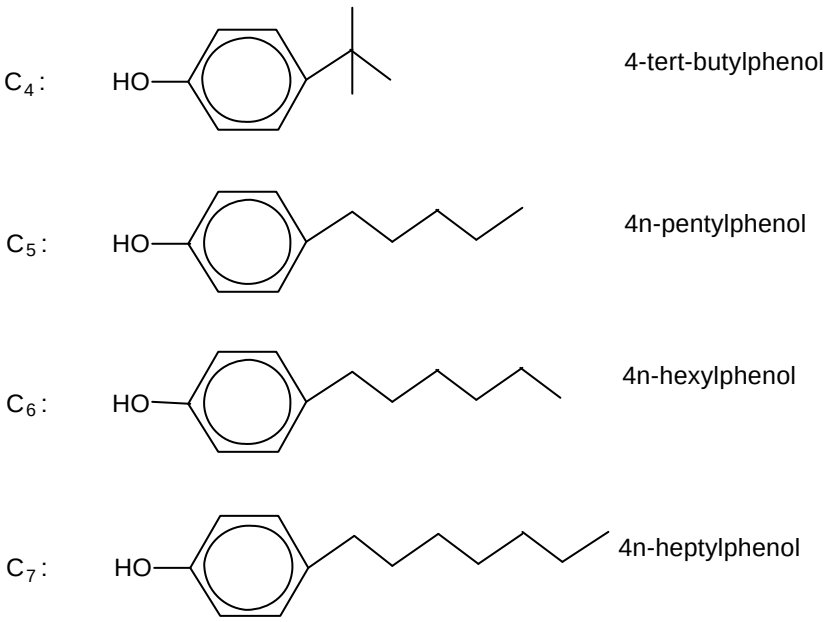

Figure 1. Structure of the alkylphenols used in the present study (untritiated form).

Experimental set-up

During the experiments, juvenile fish were maintained in 50 litres aquariums and large individuals were held in 250 litres tanks. Continuous water flow was kept at 0.5 l/min and 3.5 l/ min, respectively for the small and large fish. Natural seawater was directly pumped from an inlet at 80 meters depth and sand-filtered before supply to the exposure system. Temperature of seawater was 11±1° C throughout the experiment. We used 3 fish replicates per sampling time both for juvenile and large fish. At the start of the experiment, 17 juvenile individuals were placed in each 50 l aquarium (13 individuals for toxicokinetics study and 4 extra fish). Also, 6 large fish were used in each treatment for tissue distribution studies. For each sampling of juvenile fish, one fish from 3 different aquariums was taken whilst three large individuals from one tank were sampled for tissue distribution

The same design and sampling procedure was applied for both dietary and waterborne exposures. Experiments lasted for 16 days, 8 days of exposure and 8 days of elimination. Experiments were performed in two rounds. During each of them, two different chemicals were run in parallel. During the first round, the exposures of C_4-AP and C_5-AP were performed. Experiments with C_6-AP and C_7-AP exposures were run thereafter. A schematic overview of the experimental set-up for waterborne exposure is presented in Figure 2.

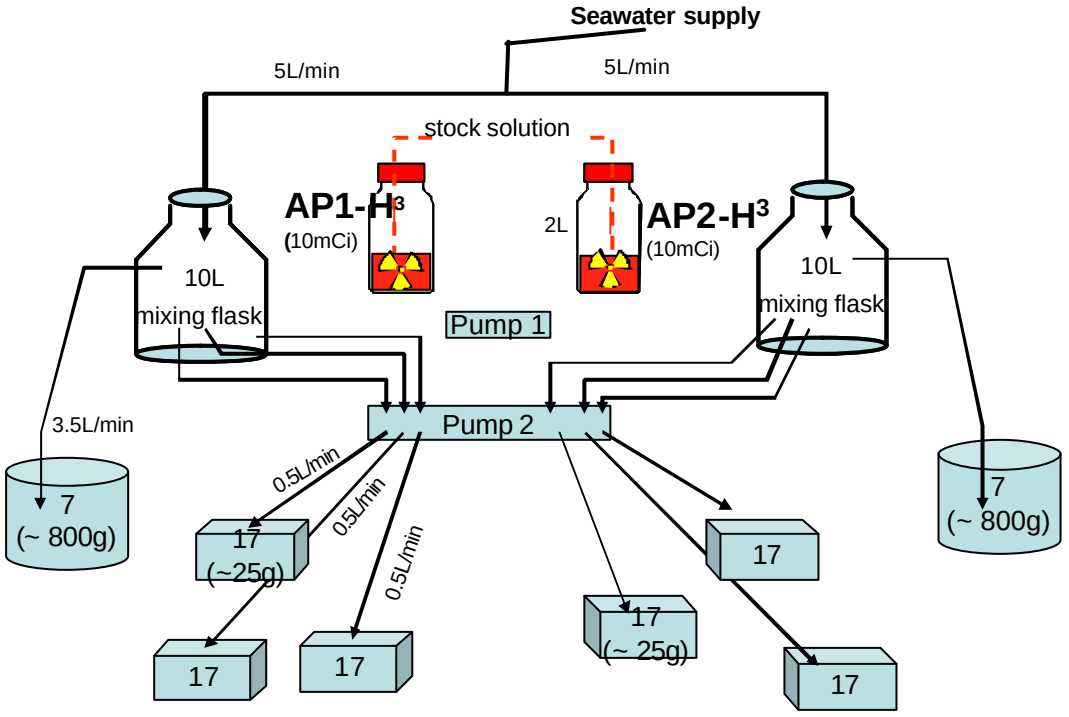

Figure 2. Experimental setup for water borne exposure of two components. Flows and number of individuals in each tank is indicated.

Administration of radioactive chemicals

Dietary pathway

Fish were exposed to each of the four alkylphenol compounds mixed into feed. One supply was applied daily, corresponding to 5 µg alkylphenol per kg fish. A mixture of radioactive compound and fish liver oil (1:10) was prepared and used to spike feed prior to the supply to fish. The daily ration of feed was 1% of total body mass of fish left in tanks. This amount was based on food consumption estimation prior to the experiment. For the juvenile fish, pellets (EWOS marine 2mm, 14% fat) soaked in the mixture, were used. For the larger fish, raw shrimps injected with the same mixture were given daily. Amounts provided were adjusted to fish biomass. To avoid unfavourable taste affecting appetite, the ration was first allowed to stay at room temperature for 5 minutes to evaporate some of the acetone prior to feeding.

The same food regime with uncontaminated feed was kept during the elimination period. To assure that all the feed given was ingested, feeding was inspected visually.

Waterborne pathway

A stock solution were prepared by mixing individual radio-labelled compound [total activity : 10 mCi] in 2 L acetone with the corresponding non-radioactive molecule in an appropriate amount to reach the final targeted seawater concentration (0.008 ppb) in the system. A high precision dosage peristaltic pump supplied each of the two solution separately in a dosage mixing bottle where it was mixed with seawater (see figure 2). Stiring was additionally used to help homogeneization of the compound in the mixing bottle. Acetone was used as carrier for each of the AP and the final concentration was kept at a maximum level of 30 ppm.

Juvenile fish were fed pellets and large fish were fed *Pandalus* shrimps using the same regime as the one described for dietary pathway during the whole duration of the experiment.

Sampling

Each fish were treated separately. They were first rinsed, and then weighted. Liver was sampled and analysed separately from the rest of the body tissues (“carcass”) in order to reduce the potential sub-sampling error that might have occurred upon homogeneization of the whole fish. Gut was removed from all fish prior to homogeneization of the carcass. The rationale for that was to make possible the assessment of uptake and elimination kinetics for fish exposed via dietary pathways. For comparison purposes, gut was then also removed from fish exposed via seawater. The analytical results of the two samples (liver and carcass) were finally added up to give a weighted body burden

(without gut). Samples of fish were taken at time intervals according to Table 1. The same sampling time was used for both dietary and waterborne exposures.

Samples for tissue distribution studies were taken only at the end of the exposure period (day 8) and at the end of the elimination period (day 16). Samples of bile, liver, intestine, intestine content, stomach, stomach content, gill, skin, muscle, gonad and a pooled sample of spleen, kidney, heart and brain were taken for LSC. The rationale for pooling the latter four was that generally low levels of alkylphenols were found in these tissues in the study of Tollefsen *et al.* (1998).

Water samples from the exposure tanks were collected at the same time as fish were sampled during both the exposure and elimination period (see Table 1).

Table 1. Sampling in the experiments, identical for both dietary and waterborne exposures.

		Sampling		
	Time	Fish for kinetics	Fish for tissue distribution	Water
	0	X		X
Exposure	6 h	X		X
	12 h	X		X
	1 d	X		X
	2 d	X		X
	4 d	X		X
	8 d	X	X	X
Depuration	6 h	X		X
	12 h	X		X
	1 d	X		X
	2 d	X		X
	4 d	X		X
	8 d	X	X	X

Analyses

Radioactivity analysis

Analysis of AP concentration was based on the ^{3}H radioactive tracer. Tissue distribution, bioconcentration and elimination kinetics in fish were analysed by liquid scintillation counting with automatic correction for quenching (Wallac LSC1415, Turku, Finland). Prior to the analysis of the samples, new quenching curves were added to the existing libraries of the LSC in order to refine quenching correction specifically to the samples. This was done both for body tissue and seawater samples. Upon fine tuning of the LSC, quenching correction standards (^{3}H capsules, Wallac, Turku, Finland) were added in scintillation vials with known amount of quencher (different weight of cod body tissue homogenates for biological samples and different volume of algae for seawater samples).

Water samples

Seawater samples were extracted for AP using the methodology described in Tollefsen *et al.* (1998). Samples of 100 ml were extracted and concentrated in a 20 ml scintillation glass vial. 10 ml Optiphase HiSafe 3 ionic cocktail (Wallac, Turku, Finland) was finally added prior to LSC analysis. This methodology was used during the first experiment with C_4-AP. However, we found out that measuring directly a volume of 7 ml seawater taken from the aquariums without prior extraction, gave similar results as with extraction of 100 ml samples. Hence, we further analysed 7 ml seawater samples to control waterborne exposure. This volume was directly added to 20 ml scintillation glass vial and 10 ml of HiSafe 3 cocktail was added before analysis. Yet, at the onset of elimination and further, we used the methodology with prior extraction because of the low levels of radioactivity in AP in samples during that period.

Fish samples

Sub-samples of tissues were homogenized and transferred into 20 mL scintillation vials (see appendix for amounts). Carcass samples were homogenized using an ultra-turax model T25 blender and a subsample of 200±20mg was transferred in a scintillation vial. A subsample of 100±10mg was used for liver. A volume of 1 ml of tissue solubilizer (OptiSolv, Wallac, Turku, Finland) was added to samples, and these were kept at 50°C for at least five hours. They were allowed to cool down overnight in the dark. A bleaching treatment with H_2O_2 was then applied to reduce coloration of the samples (see appendix A). A volume of 19.5 ml Optiphase HiSafe 2 ionic cocktail (Wallac, Turku, Finland) was then added before counting with the LSC.

All the experiments were conducted in accordance to regulations given by the Norwegian Radiation Protection Authority (Statens strålevern).

Autoradiography

Samples were collected for whole body autoradiograpy, however due to a stop in the production of the film needed for this purpose this analysis had to be stopped.

Bioconcentration and elimination kinetics

A first-order kinetic model was used to describe kinetics of uptake and elimination in the fish (Spacie and Hamelink, 1982; Chaisuksant *et al*, 1997). In this model, internal concentration, *Cf*, at any time, *t*, is a function of the external concentration, *Cw*, the uptake rate constant, *k1*, and the elimination rate constant, *k2* as:

$$\frac{dCf}{dt} = k1Cw - k2Cf \qquad (1)$$

which gives after integration when *Cw* is constant:

$$Cf = \frac{k1Cw}{k2}\left(1 - e^{-k2t}\right) \quad (2)$$

We used model (2) to calculate uptake and elimination kinetic parameters in diet-exposed fish. However, equation (2) assumes that the external concentration, *Cw*, is constant. Yet, despite the use of a continuous flow, the concentration of alkylated phenols in the seawater measured in the exposure tanks decreased with time. This decline dictated the inclusion of a correction term, λ, in equation (1) which can then be expressed as

$$\frac{dCf}{dt} = k1Cw^{0}e^{-lt} - k2Cf \quad (3)$$

which after integration gives :

$$Cf = \frac{k1Cw^{0}(e^{-lt} - e^{-k2t})}{(k2 - l)} \quad (4)$$

where

Cf = concentration of the compound in the fish (µg.kg^{-1} wet weight or mg.kg^{-1} lipid weight);

Cw^{0} = concentration of compound in sea water at the start of the exposure (µg.l^{-1});

*k*1 = uptake clearance rate constant of compound from sea water (L seawater.kg^{-1} organism.day^{-1});

*k*2 = elimination rate constant (day^{-1});

λ = rate constant for the decline of compound in the sea water (day^{-1});

t = time (day).

The term e^{-lt} describes the rate of compound non available to the fish because of the (assumed exponential) decline in the seawater. It represents the loss from a bioavailable pool (Landrum 1989; Landrum *et al*. 1994; Baussant *et al*. 2001). Note that when λ approaches 0, e^{-lt} will approach 1 and equation (4) is equal to equation (2).

Model (2) was used to estimate *k1* and *k2* parameters in water-borne exposures.

In both models, the elimination rate constant values, *k2*, in (2) can be determined from the slope of the linear regression line for plots of ln*Cf versus* time fit to a first-order decay as:

$$\ln Cf = \ln Cf^{0} - k2t$$

where Cf 0 is the concentration of compound in the fish at the beginning of the elimination period. k2 was determined from the data on residue concentrations in fish with time during the elimination period.

These values were used to determine uptake rate constants, *k1*, in terms of wet weight, by fitting the fish uptake data to either model (1) or (3) by mean of nonlinear regression analysis. However, a fundamental assumption of the model is that biotransformation is negligible as it will results in the decrease of the equilibrium level by increasing the rate of elimination and will change the proportion of parent and biotransformed compound (Spacie *et al.*, 1983; Barron 1990).

If steady-state is reached, an estimation of the bioconcentration factor (*BCFss*) can be obtained by:

$$BCFss = \frac{k1}{k2} \qquad (5)$$

Thus, equation (5) assumes that, when steady-state is reached, uptake and elimination of the compound equilibrates. Equation (5) can be related to the experimental BCF (*BCFexp*) which is expressed by

$$BCF\,\exp = \frac{Cf}{Cw}$$

The time-dependent bioconcentration factors, BCFexp, were fit by non-linear regression to equation (6) to yield the steady-state BCF (*BCFss*):

$$BCF = BCFss\left(1 - e^{-k2t}\right) \qquad (6)$$

The biological half-life ($t_{1/2}$) was calculated using the $k2$ or λ coefficients as:

$$t_{1/2} = \frac{\ln 2}{k2} \qquad (7)$$

Statistical analysis

Data were expressed as mean of the three replicates (both seawater and body tissues) ± standard deviation. Regression analyses for the estimation of the elimination rate constants (slope of the regression line) were considered significant at $p \leq 0.05$. The significance of the fitting of the model to the uptake data was tested by nonparametric measures of association using the Spearman rank test at $p \leq 0.05$. All calculations were realized using JMP software (*Windows*, ver 3.2.2, SAS Institute, Cary, NC, USA)

RESULTS

Bioconcentration and elimination kinetics

Waterborne pathway

Figures 3 to 6 show kinetics for uptake and elimination of 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol and 4n-heptylphenol, respectively. Despite a continuous pumping of the chemicals in the flow of seawater, a reduction of seawater concentration was observed with time. The uptake of all these compounds occurred relatively rapidly following apparently a first-order kinetic. The first-order kinetic model described in (4) fitted indeed well to experimental data for all compounds. The λ values varied 0.30 and 0.09 day^{-1} with corresponding half-life between 8 and 2 days (see table 2). This coefficient decreased with increasing log K*ow* values and, hence, suggested that model (2) could also have been used for C7-AP because of smaller reduction of bioavailability with time for this compound. However, the use of model (4) was justified for the 3 other compounds because of a decrease of bioavailibilty with time until the end of the exposure. The apparent time-dependent BCF showed that steady-state was reached after 2 days of exposure for C4-AP and C5-AP but decreased thereafter. Also, a maximum value was reached after 2 days for C6-AP and C7-AP, decreased thereafter and BCF remained relatively stabile from day 4 (see Appendix).

Reduced but detectable amounts of AP in the seawater were analysed at the onset of the elimination period. However, APs were not detected after one day. Initial elimination of the compounds in body tissues was rapid and linearly dependent of time ($p<0.01$). Background levels were reached after 1 to 2 days of elimination for all the compounds.

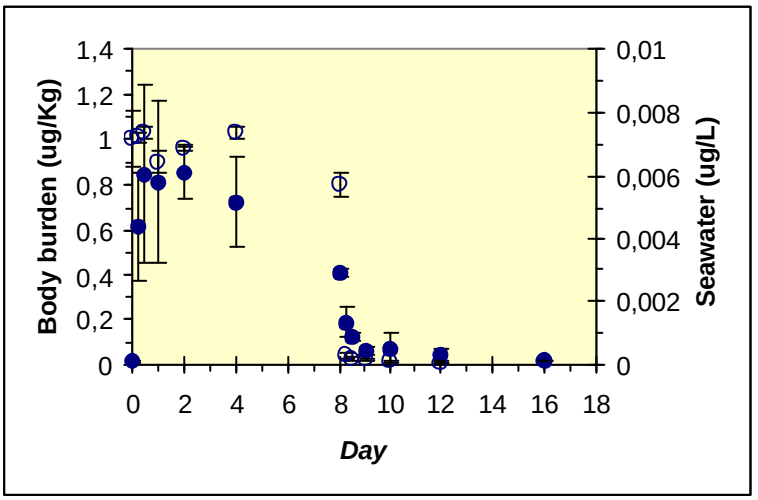

Figure 3 Exposure concentration (○), bioconcentrationand elimination (●) in juvenile cod of [^{3}H]-4-tert-butylphenol and metabolites. Values (mean ±SD) are based on three individual samples.

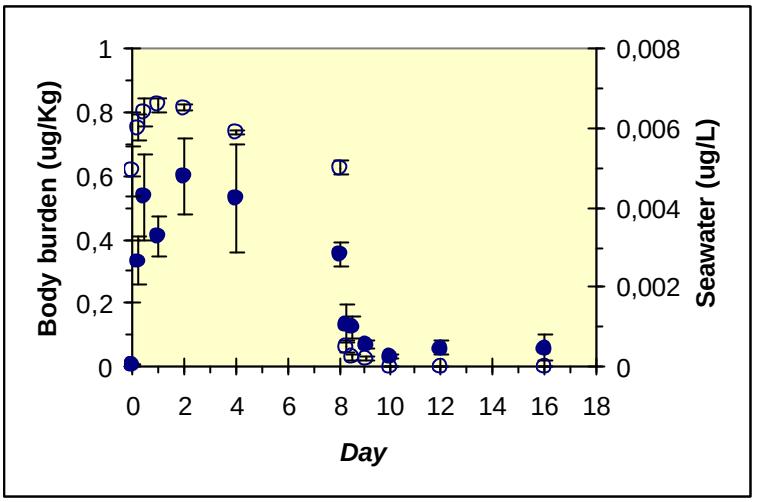

Figure 4 Exposure concentration (○), bioconcentrationand elimination(●) in juvenile cod of [^{3}H]-4n- pentylphenol and metabolites. Values (mean ±SD) are based on three individual samples.

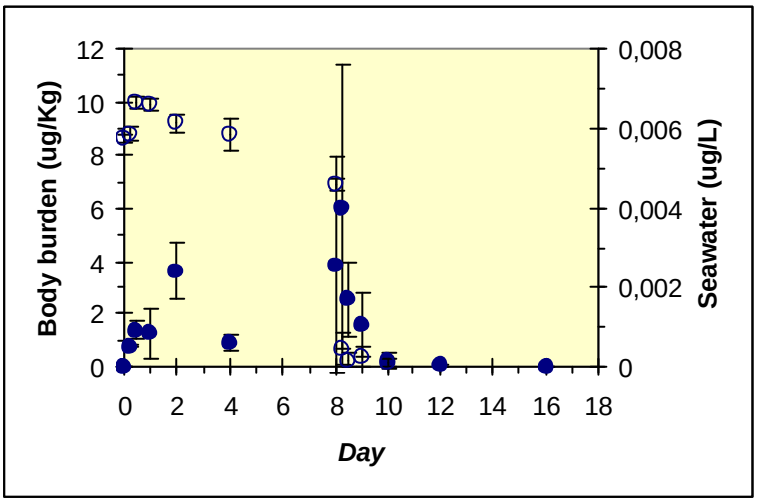

Figure 5 Exposure concentration (○), bioconcentrationand elimination (●) in juvenile cod of [^{3}H]-4n-hexylphenol and metabolites. Values (mean ±SD) are based on three individual samples.

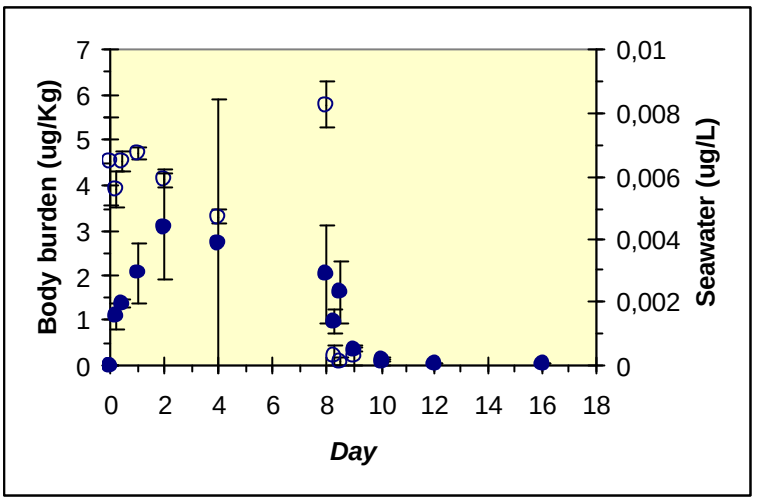

Figure 6 Exposure concentration (○), bioconcentration and elimination (●) in juvenile cod of [^{3}H]-4n- - heptylphenol and metabolites. Values (mean ±SD) are based on three individual samples.

Because of the change of BCF over time after 2 days of exposure, we estimated the steady-state BCF by considering only the data for the two first days in equation (6). BCFss varied between 100 and 500.

Dietary pathway

Figures 7 to 10 show the corresponding dietary uptake and elimination for the four alkylated phenols. Since the first feeding occurred at day 0 and the first samples taken at 6 h and 12 h thereafter, samples analysed at these two sampling intervals reflect the administration of feed shortly after the sampling whilst all the other samples have been taken with the same interval of time from feeding. Because of that, the samples at 6h and 12h were ommited in the calculation of kinetic rate of absorption.

Uptake via feed (absorption) followed a first-order kinetics fitting significantly to model (2)but model fitting to 4n-pentylphenol was not significant (p=0.22) and poorely significant for 4n-hexylphenol (p=0.056). Steady state was apparently reached after day 2 although a decrease was observed over time for 4n-pentylphenol. The initial elimination was rapid and linear after feeding with non-contaminated food ($p \leq 0.001$) but slower for 4n-pentylphenol ($t_{1/2}$=38h; p=0.03). The values were in good agreement with those found from fish exposed via seawater and background levels were found after 2 days.

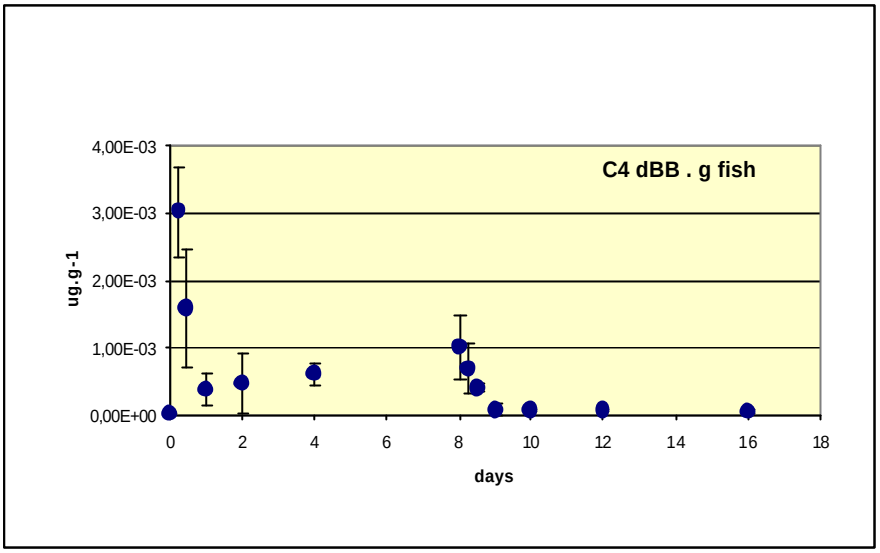

Figure 7 Bioconcentration and elimination of [^{3}H]-4-tert-butylphenol and metabolites in dietary exposed (5ppb body burden / day) juvenile cod. Values (mean ±SD) are based on three individual samples.

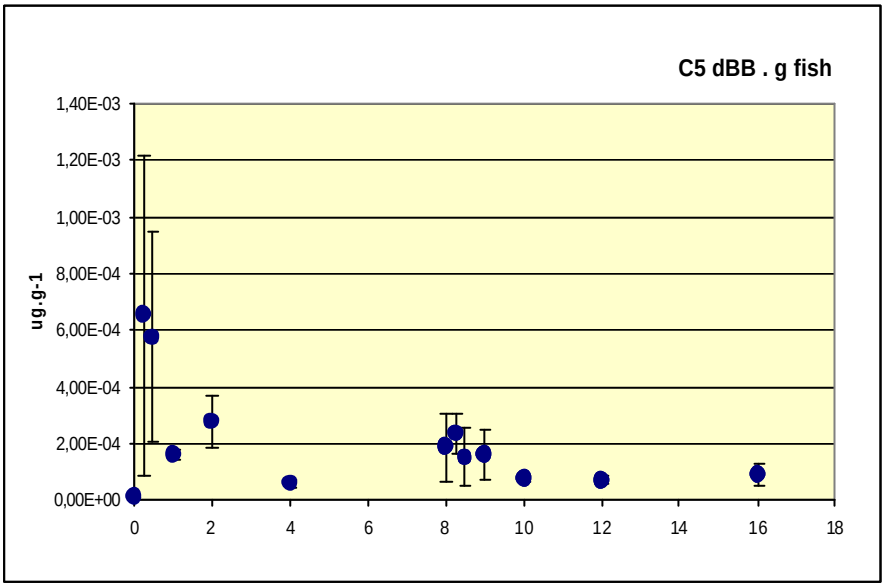

Figure 8 Bioconcentration and elimination of [^{3}H]-4n-pentylphenol and metabolites in dietary exposed (5ppb body burden / day) juvenile cod. Values (mean ±SD) are based on three individual samples.

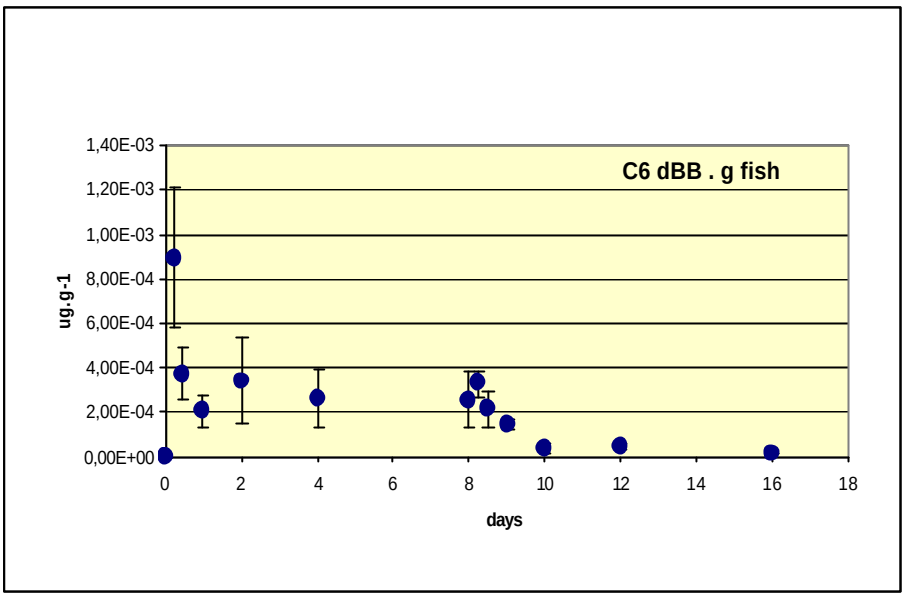

Figure 9 Bioconcentration and elimination of [^{3}H]-4n-hexylphenol and metabolites in dietary exposed (5ppb body burden / day) juvenile cod. Values (mean ±SD) are based on three individual samples.

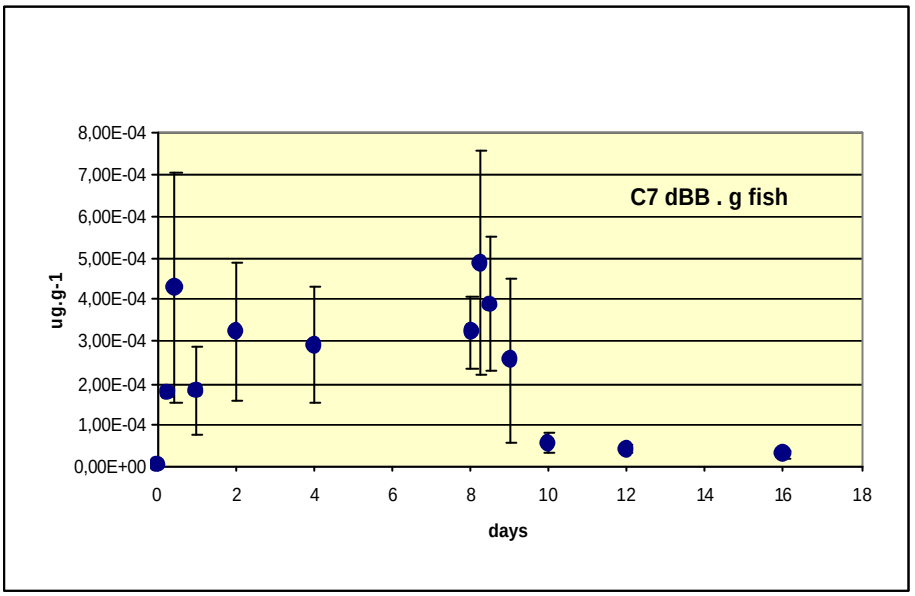

Figure 10 Bioconcentration and elimination of [^{3}H]-4n-heptylphenol and metabolites in dietary exposed (5ppb body burden / day) juvenile cod. Values (mean ±SD) are based on three individual samples.

Generally, the estimated BCF values (absorption efficiencies) were much lower in dietary exposure than in seawater exposure. A maximum value of 0.14 was calculated for C4-AP and a minimum value of 0.08 for C5-AP (Table 2).

Table 2. Uptake (*k1*), elimination (*k2*) rate constants and biological half-life (*t1/2*) in cods estimated with a first-order kinetic model. BCFss = Modeled bioconcentration factor . BCFexp = Experimental Bioconcentration Factor (Body burden/Seawater concentration) at day 8 of experiment (end of exposure). λ is the rate constant for change in bioavailable concentration. λ and $t_{a1/2}$ are given in days. $t_{b1/2}$ in hours

Compound	Code	log K_{ow}	k_1	n	l	$t_{a1/2}$	k_2	n	$t_{b1/2}$	BCF_{ss}	BCF_{exp}
Seawater exposure											
4-tert-butylphenol	C4-AP	3.04-3.31	233 ± 32	21	0.30	2	0.91 ± 0.2	15	18	194 ± 25	125 ± 16
4n-pentylphenol	C5-AP	-	159 ± 17	21	0.15	5	1.08 ± 0.14	15	15	107 ± 10	90 ± 18
4n-hexylphenol	C6-AP	3.60	712 ± 138	21	0.12	6	1.60 ± 0.42	15	10	450 ± 58	592 ± 174
4n-heptylphenol	C7-AP	4.00	643 ± 96	20	0.09	8	1.32 ± 0.15	15	13	509 ± 44	520 ± 197
Diet exposure											
4-tert-butylphenol	C4-AP	3.04-3.31	0.20 ± 0.03	15	-	-	1.44 ± 0.27	15	12	0.14 ± 0.020	0.20 ± 0.09
4n-pentylphenol	C5-AP	-	0.042 ± 0.003	15	-	-	0.57 ± 0.15	13	29	0.08 ± 0.007	0.06 ± 0.018
4n-hexylphenol	C6-AP	3.60	0.13 ± 0.016	15	-	-	1.10 ± 0.18	15	15	0.12 ± 0.014	0.10 ± 0.051
4n-heptylphenol	C7-AP	4.00	0.132 ± 0.007	15	-	-	1.04 ± 0.25	15	16	0.13 ± 0.013	0.13 ± 0.035

Tissue distribution

Quantitative expression of tissue distribution of bioconcentrated APs were provided by radioactivity analysis in dissected organs in the large fish. The distribution pattern of ^{3}H-AP in these fish is shown in figures 11 to 18, respectively for C_4- to C_7-AP via waterborne and dietary at end of exposure and elimination. On these figures, the *y*-axis is expressed as ^{3}H cpm and normalised to 1 g of tissue.

Generally, a large amount of the radioactivity was found in all gastrointestinal system i.e. bile, intestine, intestine content and stomac content at the end of exposure. Very little was however found in liver. Also, there were much more activity in bile from fish exposed via seawater than in bile from fish exposed via food. Compared to bile levels, there were much more activity in intestine and intestine content of fish exposed dietary than fish exposed waterborne. The other tissues had substancially lower levels of activity in fish exposed to both pathways. Muscle, pooled sample of spleen/heart/kidney/brain and gonads contributed little to the total radioactivity.

A similar distribution pattern was revealed at the end of the elimination period. The bile, intestine and intestine content of fish exposed to C_5-AP in seawater had relatively large amount of activity left.

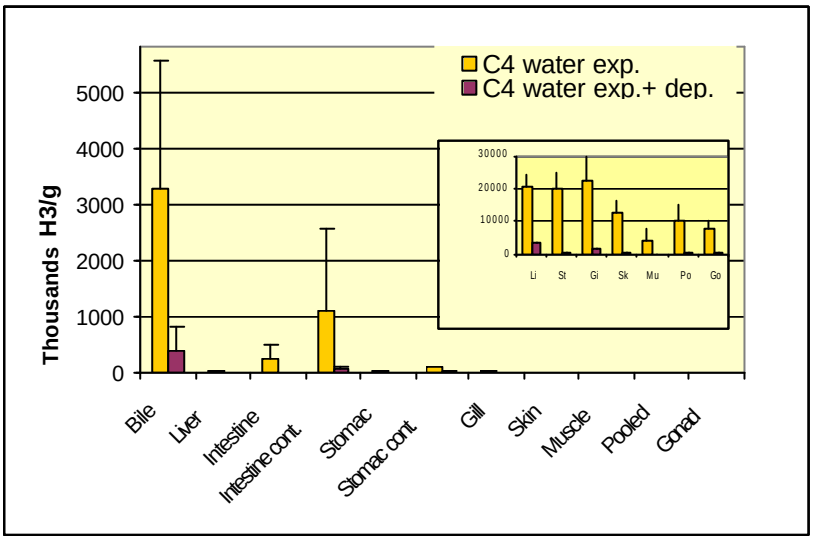

Figure 11 Tissue concentration of [^{3}H]-4-tert-butylphenol and metabolites in water exposed cod (0,008 ppb for 8 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axis).

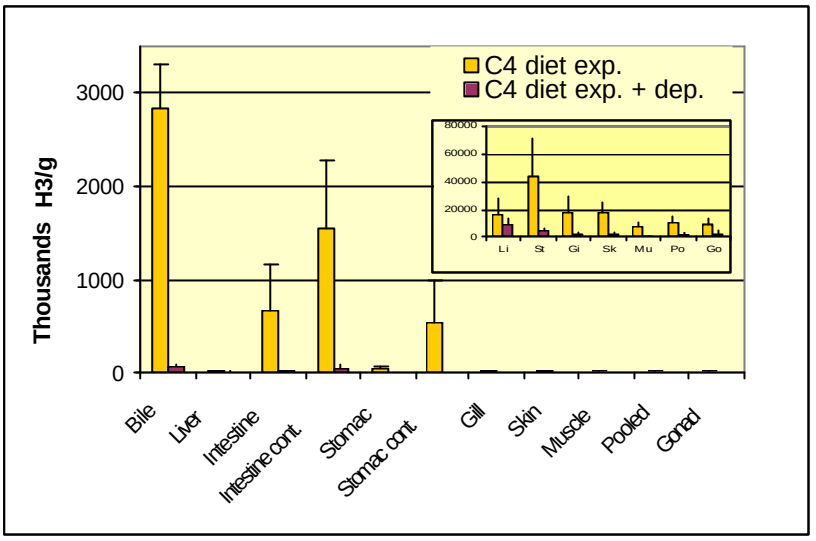

Figure 12 Tissue concentration of [^{3}H]-4-tert-butylphenol and metabolites in dietary exposed cod (5 ppb BB for 8 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axi).

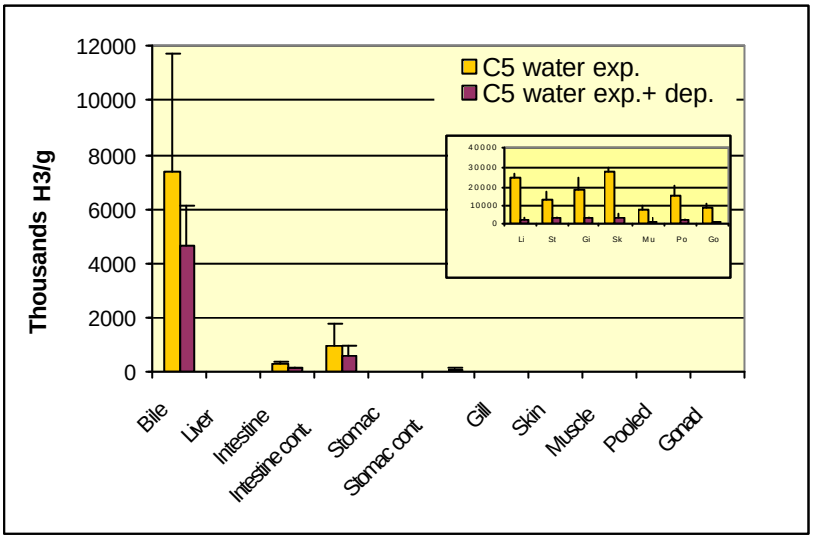

Figure 13 Tissue concentration of [^{3}H]-4n-pentylphenol and metabolites in water exposed cod (0,008 ppb for 8 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axis).

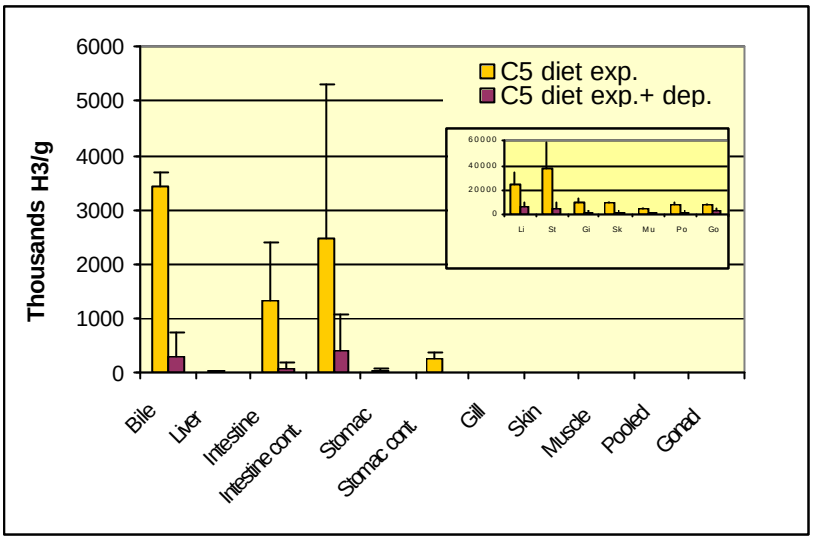

Figure 14 Tissue concentration of [^{3}H]-4n-pentylphenol and metabolites in dietary exposed cod (5 ppb BB for 8 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axis).

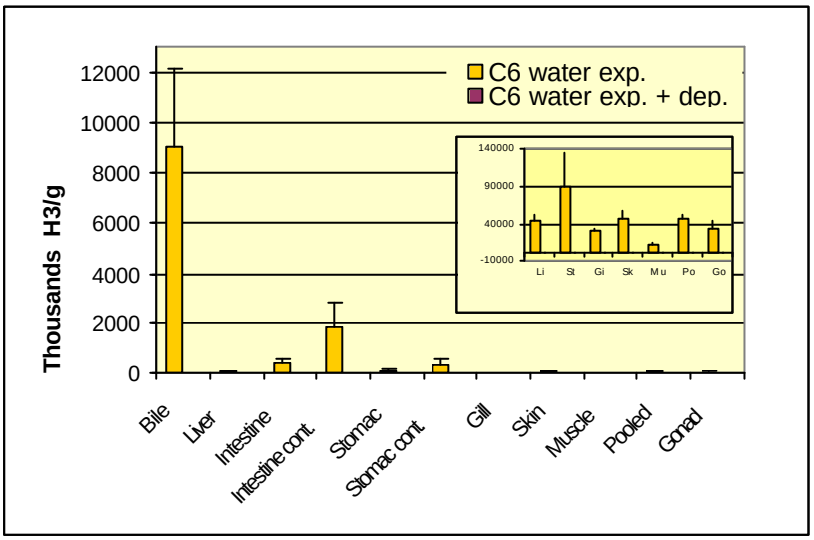

Figure 15 Tissue concentration of [^{3}3H]-4n-hexylphenol and metabolites in water exposed cod (0,008 ppb for 7 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axis).

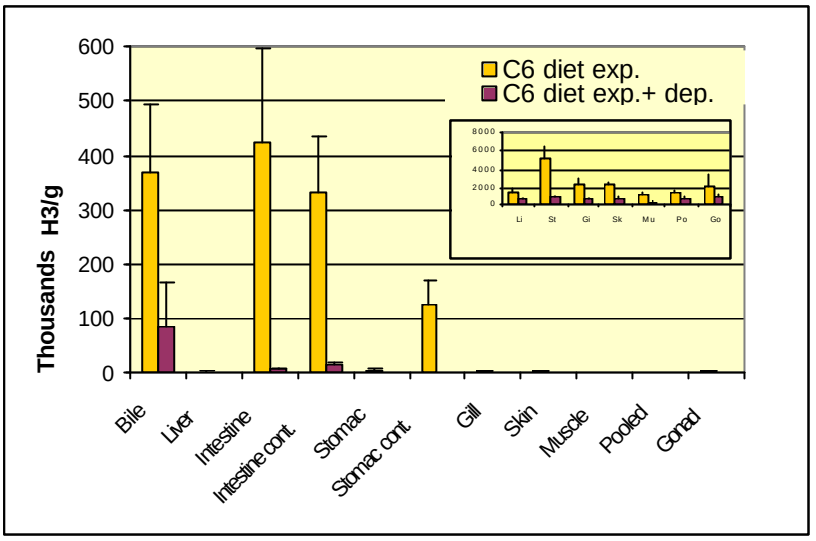

Figure 16 Tissue concentration of [^{3}H]-4n-hexylphenol and metabolites in dietary exposed cod (5 ppb BB for 8 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axis).

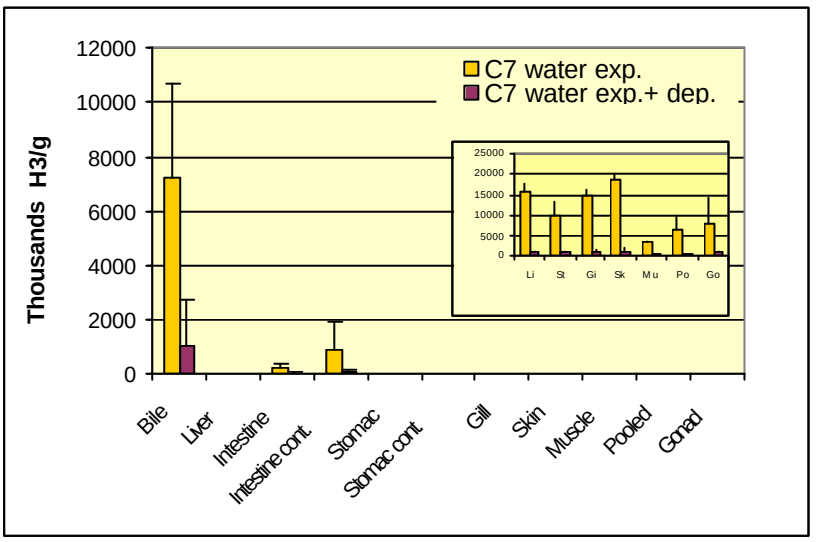

Figure 17 Tissue concentration of [^{3}H]-4n- heptylphenol and metabolites in water exposed cod (0,008 ppb for 8 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axis).

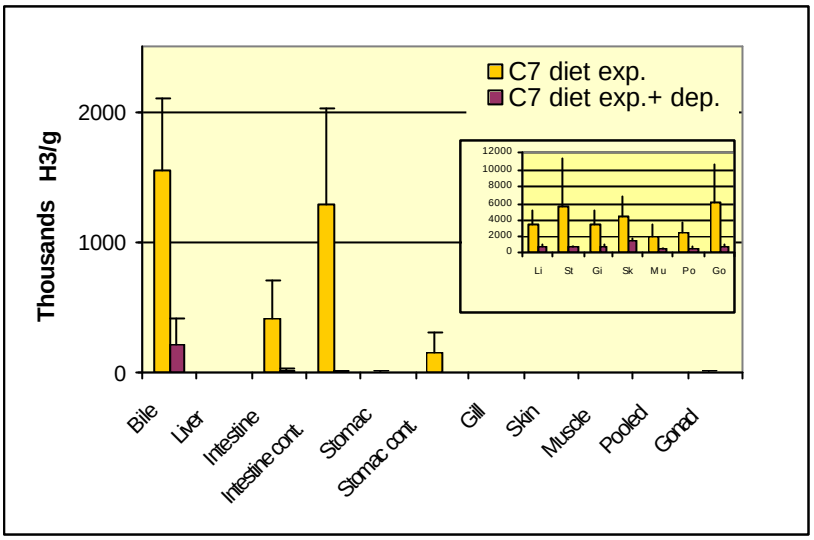

Figure 18 Tissue concentration of [^{3}3H]-4n- heptylphenol and metabolites in dietary exposed cod (5 ppb BB for 8 days) and after 8 days of depuration. Values (mean ±SD) are based on three individual samples. Tissues with low levels of contaminants are presented in additional superimposed graph with expanded scale (unit on *y*-axis).

DISCUSSION and CONCLUSIONS

Uptake from water

Uptake rates from seawater generally increases with increasing log K*ow* but appears to level off for the larger molecules with log K*ow*≈ 4. The lowest value is indicated for C_5-AP. Substantial amounts of AP compounds are taken up and bioconcentrated in the body tissues of fish exposed via seawater. Modelled BCFs range from 100 to 500. The modelled BCF obtained with C_7-AP (509) is similar to the value observed by Tollefsen *et al.* (1997) for the same compound (555).

In their study on hormonal effects in cod to the same alkylated compounds, Meier *et al.* 2002 assumed an average BCF of 600 which is in the same magnitude as observed in the present study.

Dietary uptake

Uptake rates via food are much slower than via seawater. We found that absorption efficiencies of the alkylated phenols are poor. The lowest value is observed for C_5-AP (8%) whilst for the other compounds absorption efficiency is between 12% to 14%.

There are few previous studies of dietary uptake of APs. Pedersen *et al.* (2003) showed that a very small amount (1-2 ‰) of 4-tert octylphenol administered orally over 11 days experimental period was retained in muscle and liver at the end of the exposure. Yet, for other organic compounds like PAH, the general pattern is that rather little is absorbed via dietary pathways. Similar observations on limited absorbtion from diet are exemplified by Niimi and Palasso (1986); Niimi and Dookhran (1989). These authors found that dietary absorption efficiencies of PAHs were between 1% and 14% in rainbow trout. Bioavailability of ingested contaminants are in the same magnitude as observed in the present study. Randall *et al.* (1998) came to the same conclusion from a theoretical and experimental standpoint. They even argued that under natural conditions, the uptake of toxicants from food can be ignored when estimating toxicant body concentration.

Tissue distribution

The present results showed that bile was apparently a major route of excretion of the studied alkylated phenols. Similar results are reported by Tollefsen *et al.* (1997) and Arukwe *et al.* (2000) for 4-heptylphenol and nonylphenol, respectively.

Also, the same pattern of tissue distribution was revealed for both water-borne exposed and dietary exposed fish.

From the litterature, it appears that organic compounds like PAH ingested in food are largely metabolized by efficient enzymatic activities in the gut epithelium and are hence poorly absorbed (Neff and Burns, 1996). Spacie *et al* (1983) also indicated that

biotransformation can considerably increase the elimination rates, hence decreasing significantly the BCFs of organic compounds.

Elimination rates

Elimination rates of the different compounds are rapid and relatively comparable in both exposure pathways except for 4n-pentylphenol which seems to be eliminated at a slower rate in the dietary exposure. Generally, in fish, the reported half-lives are very short and are attributed to both rapid enzyme kinetics and fast diffusion rates (Meador *et al*. 1995).In both types of exposures, the biological half-life of compounds is between 10 and 18 hours. Similar levels were observed by Arukwe *et al.* (2000) for nonylphenol in Atlantic salmon. Pedersen and Hill (2002) found values of $t_{1/2}$ between 15 hours and 1.7 days for 4-tert-octylphenol in different tissues of a cyprinid fish (bile excluded). Biological half life value for 4n-heptylphenol found in the present study is very similar with and the one observed by Tollefsen *et al.* (1998).

Bioconcentration factors

The time dependent bioconcentration factors were apparently changing over time in water-borne exposures. Steady-state was apparently reached after 2 days but BCFs started to decrease after that time. This period may correspond to the time needed by the enzymatic systems for a significant biotransformation of organic compounds like alkylated phenols and PAH (Djomo *et al*. 1996; Baussant *et al*. 2001). In that study, we can not state the time at which biotransformation becomes significant but we do measure high radioactivity levels in the bile at day 8 of exposure which strongy suggest that biotransformation is effective in cods exposed to alkylated phenols. The prediction based on these relatively short-term kinetic studies do not include predictions of biotransformation potential. Hence, there are not appropriate for estimating steady-state tissue residues unless the variable rate of biotransformation can be modelled more precisely (Spacie *et al*. 1983; Feijtel *et al*. 1997).

The main conclusion to draw from this short-term study is that the bioconcentration from seawater is much higher (500%) than via aborption through the gut wall (10%). In the study of Meier *et al.* (2002), it was assumed that a daily dose of 5 ppb via food would be equivalent to the amount fish cod would accumulate if they had been exposed to a concentration of 0.008 ppb in the seawater based on a BCF value of 600. The present results together with previous findings suggests that Meier *et al.* 2002 could have overestimated the actual body burden in their study. Consequently, the observed effects may have occurred at a lower body burden level than the 5 µg AP/kg fish that was assumed.

Since approximately 10% of what is ingested is really absorbed, the estimated body burden of fish fed 5 ppb would be 0.5 ppb. Based on this estimation and considering a BCF of 600, this would consequently correspond to an exposure via seawater concentration of approximately 0.0008 ppb.

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Appendix

Lipids

To assure the quality of the data for possible comparisons with other results, the data should be possible to lipid normalize. Therefore, 8 individuals were analysed according to the method of Folch et al. (1957) and following recommendations by Christie (1993). In addition a subset of samples for lipid analyses will be stored at Akvamiljø until 01/01/2004.

Sample	Tissue	Sampling date	% lipid
C4SW1	Liver	011102	56,028
C7SW1	Liver	271102	49,946
C4SD1	Liver	011102	56,531
C7SD1	Liver	271102	58,395
			Mean 55,22
4SW1	Carcass	011102	0,830
C4SD1	Carcass	011102	0,944
C7SW1	Carcass	271102	0,948
C7SD1	Carcass	271102	0,660
			Mean 0,85

Short description of sample preparation for scintillation counting

- Sample organism and dissect
- Take a tissue sample and weigh.

bile	0,05 g
liver	0,1 g
intestine	0,1 g
intestine content	0,1 g
stomach	0,1 g
stomach content	0,1 g
gill	0,1 g
skin	0,05 g
muscle	0,2g
gonad	0,1 g
pooled sample of spleen, kidney, heart and brain	0,1 g

- Add 1 ml OptiSolv (tissue solubilizer) and allow digestion of sample at 50°C for ≈ 5 hours. Let the sample cool down in the dark overnight
- Add dropwise 0.5 ml H_2O_2 30% (for bleaching)
- Cap glass vial loosely and wait for at least 30 min at room temperature
- Add 15 – 19 ml of HiSafe 2 : 0.5 M HCl (9:1 v/v). Add first HCl, mix with solution before adding HiSafe 2. Shake vigorously to obtain a yellowish solution, to avoid milky solution, we decreased the amount of 0.5 M HCl to 0.3 ml and add 19.5 ml HiSafe 2
- Put the sample inside the counter and wait another 30 min for light and temperature stabilization. Then count.

Time dependent bioconcentration factors (BCF).

The observed decrease in BCF over time suggests a delay in induction of detoxification enzymes within the first 2 days.