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“Transfer of toxaphene from feed to fish”

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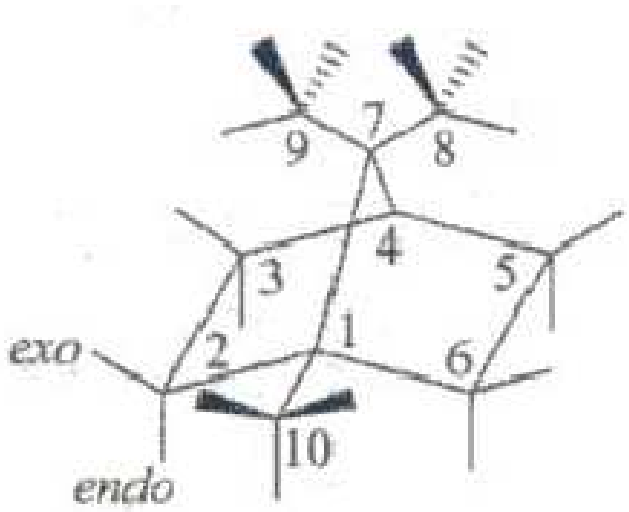

Abstract

The aim of the present study was to assess the carry-over of toxaphene congeners in Atlantic salmon. Toxaphene is a persistent organochlorine pesticide that is present in fish oils, and oily fish is the main source of exposure to humans. Currently there is focus on toxaphene legislation in both animal feed and food within the EU. Knowledge on carry-over of toxaphene from feed to edible tissue is needed to harmonise current legislation with regards to food safety.

Atlantic salmon with an initial weight of 0.3 kg were fed in triplicate either a traditional marine ingredient based feed or a feed with maximum replacement of fish oil and -meal by alternative feed ingredients. Salmon were fed in the sea water phase for 12 months until final weights of approximately 4 kg. There was a cross-over design for the last five months of the feeding trial and uptake- and elimination rates of toxaphene were determined.

The carry-over, as seen from biomagnifications and retention, of the toxaphene congeners that are currently included in EU legislation (26, 50 and 62) was two-fold higher than the additional congeners that the European Food Safety Authority (EFSA) recommend to include in monitoring programmes and in future legislation (40, 41, 44). Based on the uptake- and elimination rates from the cross-over trial, a simple one- compartmental model was used that described the carry-over of all toxaphene congeners from feed to fillet at different feed concentrations and aquaculture performance parameters (growth and feed intake). Model predictions show that steady state (maximum level) is not expected to occur during a normal production cycle in sea (12-16 months). The time of harvest after starting the seawater phase is consequently of importance with regards to the toxaphene concentrations found in the fillet of farmed Atlantic salmon. Using different model predictions (kinetic model and biomagnifications factor) it was estimated that feed levels of 6-12 µg/kg results in the maximum toxaphene level in farmed Atlantic salmon reported to date (approximately 20 µg/kg). This prediction was made for seawater-adapted Atlantic salmon reared for 16 months with an average growth and feed intake (0.65 g/day and 0.83 % BW/day, respectively). Due to the limited uptake of the novel congeners (40+41 and 44), inclusion of these congeners caused a relatively minor (~15%) increase in total fillet levels.

Carry-over of toxaphene (camphechlor) in fish feed

Introduction

To protect animal welfare and food safety, maximum limits for undesirable substances in animal feeds have been established by the European Union. Human exposure to toxaphene (a persistent organochlorine pesticide) occurs mainly through the consumption of contaminated fish, and high levels of campheclor have been reported in fish oil and fish meal. Toxaphene, also known as camphechlor, is a non-systemic insecticide and was previously widely used on crops and animals. It has been the most heavily applied pesticide in many parts of the world and replaced DDT in the early 1970s. The use of toxaphene is now phased out in most of the world. At least 202 different toxaphene compounds have been identified. Due to its persistence and lipid solubility it has been widely distributed in the environment and it is classified as a persistent organic pollutant (POP) as are dioxins and PCBs. The main source of camphechlor to animals from feed are fish oil and fish meal. Fish feed (particularly for carnivorous species) can contain significant amounts of fish meal and fish oil. The European Union revised the maximum limits (ML) for camphechlor in fish feeds (Directive 2005/86/EC), replacing the general ML for camphechlor in all animal feed (0.1 mg/kg) with a specific ML of 0.05 mg/kg for fish feed. The congeners that serve as indicators of camphechlor and that are included in the ML for fish feed are CHB 26, 50, and 62. Particular attention has been paid in risk assessment to the congeners CHB 32, 40, 41, 42 and 44, in fish samples, in addition to the three “indicator” congeners previously mentioned.

The reduction of the ML for camphechlor in fish feed was partially based on occurrence data in fish feed and ingredients, the high sensitivity of fish to waterborne camphechlor exposure, and concern for human exposure to camphechlor by fish consumption. Oily fish is the main source of camphechlor exposure to humans (EFSA-Q-2003-068). The current limit for campheclor in fish fillet is 0.02 mg/kg ww while the new feed limit is 0.05 mg/kg feed. Assessment of the carry-over from feed to fillet is important to harmonise feed and food legislation in order to ensure food safety along the production chain.

Carry-over is a term used in feed and food safety legislation and refers to the transfer of contaminants from animal feed to edible tissue of the farm animal. Knowledge on carry-over is important for assessing which levels can be permitted in fish feed to guarantee the food

safety of farmed salmon. Currently, there is only one study in the peer-reviewed scientific literature on freshwater reared rainbow trout that addresses feed-to-fillet transfer of toxaphene (Karl *et al*. 2002). The carry over in the former study is expressed as percentage of contaminants in the edible part of the fish in relation to the total doses administered via feed, and is based on congeners 26, 50 and 62 but does not include the more recently recommended congeners to examine in fish (CHB 32, 40, 41, 42 and 44). The tissue (fillet) residue level of persistent organchlorines depends on absorption as well as elimination rates of the compound (Sijm *et al.* 1992). Hence studies on the carry-over of xenobiotics include the quantitative characterization of the uptake and elimination kinetics by use of tissue concentration-time profiles. Further important factors that determine final levels in fillet are feed intake and growth rate (Berntssen *et al*. 2007). After establishing the uptake and elimination rate, fillet levels at different feed concentrations, growth rates and feed intake can be predicted with a simple one compartmental kinetic model (Berntssen *et al*. 2007; 2008). Transfer kinetics depend on factors such as temperature and fish size. Carry-over studies on undesirable substances include uptake and depuration studies on market-size fish reared at ambient temperature.

General Objective

Asses the carry-over of background levels of toxaphene congeners (CHB 62, 50, 26, 32, 40, 41, 42 and 44) from feed to the edible tissue of Atlantic salmon (*Salmo salar* L.)

Specific objectives

1) Assess the feed to fillet biomagnifications factor and retention of toxaphene 62, 50, 26, 32, 40, 41, 42 and 44

2) Determine the uptake and elimination rates for dietary toxaphene 62, 50, 26, 32, 40, 41, 42 and 44

3) Establish a one compartmental model based on the combined uptake- and elimination rates, to estimate fillet levels at different growth and feed intakes, starvation periods, and feed levels.

Material and methods

The present FHF project used sample material from the ongoing IP-EU project "AQUAMAX" (016249-2) that includes nutritional assessment of alternative feeds in fish and mammalian models as well as monitoring of contaminants. The EU-project covers the trial conditions, while the FHF project covers the time course sampling for toxaphene material, analyses of all eight EFSA relevant toxaphene congeners at low detection limits, and modeling of carry-over from feed to fillet. The feeding trial was carried out at Matre Aquaculture Research Station (Matredal. Norway; 60°52'N. 05°35'E). The experimental conditions and feed composition are given in detail elsewhere (Torstensen *et al*. 2008). The experimental design is given in Figure 1. Atlantic salmon smolt with an initial weight of ~300 gram were fed with two different diets, in triplicate land-based tanks, over a period of 12 months until the fish reached a weight of ~ 4 kg. One diet was a traditional feed that was mainly based on fish meal and fish oil, the other diet was an alternative feed that had a high substitution of both fish meal and fish oil with feed ingredients of plant origin. The traditional feed had a relatively high level of toxaphene while the alternative feed had an approximately 2.5 fold lower toxaphene concentration. Fish were fed on these two diets for 8 months, and uneaten feed was collected and feed consumption was monitored. This part of the trial was used to assess fillet retention of the toxaphene congeners. After 8 months, a cross-over design was used to assess the assimilation and elimination parameters of toxaphene. Atlantic salmon that were previously fed on the "low toxaphene" vegetable oil-based diet were transferred to the "high toxaphene" fish oil-based diet. Conversely, fish previously fed on "high toxaphene" diet were transferred to the "low toxaphene" diet. Half of the fish were randomly fin clipped, and transferred to net pens that received the opposite diet, while the non-fin clipped fish were transferred to net pens and maintained on their original diet. The control fish were kept on the same diets and in the same tank conditions. The cross-over feeding lasted for five months and six fish from each net-pen were sacrificed at five sampling times. Pooled samples of whole fish from each experimental group (n=3 per group) were analysed for the 8 toxaphene congeners recommended by EFSA (EFSA, 2005). The diets were produced by Skretting ARC, Stavanger, and feed ingredient composition of the 9 mm pellet size diets are given in detail by Torstensen *et al*. (2008).

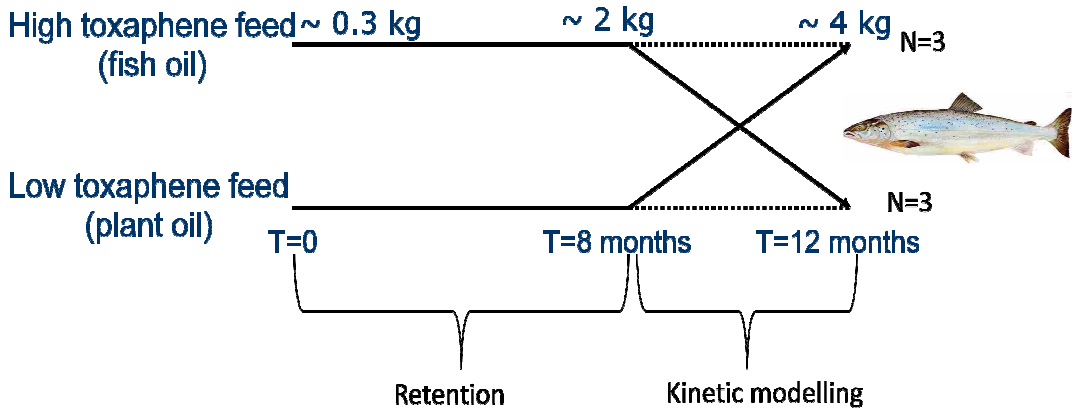

Figure 1. Schematic representation of the experimental design.

Toxaphene analysis

The analytical method was developed to a) include the congeners recommended by EFSA (40, 41, 42a, 44, 32) as well as the congeners included in EU legislation (26, 50, 62), and b) have a low limit of quantification. Samples of fish muscle were homogenized and freeze-dried before extraction. Samples of fish muscle and feed corresponding to approximately 0.5-1 g of fat were ground with the drying agent hydromatrix in a mortar. The samples were pressure solvent extracted on a Dionex accelerated solvent extractor (ASE® 300^{TM}, Dionex, USA) at 40 °C and 1500 psi with an 80/20 (v/v) mixture of Dichloromethane + hexane as extraction solvent. To quantify toxaphene, the samples were spiked with DE-TOX-414 as an internal standard. The sample extract was concentrated to approximately 0.75 ml (TurboVap II^{TM}, Zymark, USA), and dissolved in hexane. Further clean up was performed by adding 2 ml of concentrated sulphuric acid. The samples was concentrated to 1 ml and dissolved nonane added, the sample was further concentrated to 0.3 ml and spiked with recovery standard. Analysis was performed by GC/MS (Trace CG Ultra^{TM}/DSQ II^{TM} Single Quadrupole MS, Thermo, Bremen, Germany) in negative chemical ionization SIM mode, 1 µL of sample extract was injected in the splitless mode. The injector temperature and the transfer line temperature was kept at 225 °C and 300 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The GC was equipped with a fused silica capillary column (Rtx-5MS, 30 m x 0.25 mm x 0.25 µm, Restek, Bellefonte, USA). The column temperature program was as follows: 45 °C (1 min), 15 °C/min to 200 °C (0 min), 5 °C/min to 300 °C (5 min), 30 °C/min to 325 °C (5 min). Methane was used as MS reagent gas at a flow rate of 3.5 ml/min, and the source was kept at 230 °C. The quantification of toxaphene congeners was

performed using the toxaphene congener DE-TOX- 414 as an internal standard and a 5 point calibration curve with standard concentrations of 1, 5, 10, 25 and 50 ng/ml. For further quality control a procedure blank and a control sample was analyzed simultaneously with the samples to check for interferences or contamination form solvents and equipment.

Carry-over calculations

The relative carry-over was addressed in terms of, a) biomagnification calculations, b) retention and c) kinetic rate models

a) Biomagnification factor

Biomagnification factor (BMF) is based on the notion that an equilibrium will be obtained between levels in feed and in the organism. In ecotoxicology, biomagnification expresses the relative increase of a pollutant along the food chain. In aquaculture biomagnification factor is used to express the relative carry-over of several contaminants from feed to fish (e.g Serranno *et al.* 2003). Persistent organic pollutants (POPs) are often fat soluble, and the ability of different POPs to biomagnify is curvilinear related to their lipophility (Fisk *et al.* 1998). Biomagnification factors are lipid corrected and expressed as the ratio between the concentration in fillet (lipid-based) and in diet (lipid-based) (formula 1) at the end of the trial (12 months).

$$(1)\quad BMF = \frac{Conc.\ fillet_{\ lipid\ based}}{Conc.\ feed_{\ lipid\ based}}$$

b) Retention

Retention calculations are based on the amount of contaminant fed and which is retained in the muscle. Retention calculations vary considerably among different studies, and are strongly depend on fish size, dose, and length of exposure (Berntssen and Lundebye, 2008). Retention was calculated as the percentage of contaminants in the edible part of the fish in relation to the total dose consumed as in Formula 2. Since feed collection was only possible in the first 8 months (during cross-over two groups were pooled per tank) retention was assessed over a 8 month period.

$$(2)\ \mathrm{Re}tention(\%) = \frac{(Conc.fillet_{t=end}(\mu g\ kg^{-1}) * mass_{t=end}(kg)) - (Conc.fillet_{t=0}(\mu g\ kg^{-1}) * mass_{t=end}(kg))}{Conc.\ feed\ (\mu g\ kg^{-1}) * amount\ \ feed\ consumed\ (kg)}$$

c) Kinetic rates and models

Fillet levels at different time points were corrected for growth and control levels. Growth rates were calculated by fitting fish weight to the equation; ln fish weight= $a+b*t$, where a is a constant, b the growth rate (g day^{-1}), and t the time of experiment. All fillet concentrations (C_{fillet}) were multiplied by the factor (1+b*t) to correct for growth dilution, which was minimal in the present experiment due to short exposure and depuration durations. The elimination constant (k_{el}), which includes non-metabolic and metabolic elimination, was determined by fitting concentration data to a first-order decay curve; ln $\underline{C}_{fillet}$ = $\underline{a}$ + $\underline{k}_{el}$*t. Elimination half-lives ($t_{1/2}$) are ln2/ $\underline{k}_{el}$. The uptake rates were calculated by fitting (Statistica, Statsoft Inc., Tulsa USA, 1993) the concentration data to the integrated form of the kinetic rate equation (3) for constant dietary exposure (Sijm *et al*. 1993).

$$\alpha = \frac{C_{fillet}(t)\cdot k_{el}}{F\cdot C_{feed}[1-\exp(-k_{el}\cdot t)]} \quad (3)$$

where $\underline{C}_{feed}$ is the total toxaphene concentration (µg g^{-1} wet weight) in feed; α is the uptake rate constant; and F is feeding rate (g feed g^{-1} fish d^{-1}). Fillet concentrations were modelled by using formula (1) re-written as equation (4), which is a simple model-based one compartment first-order rate kinetics (Sijm *et al.* 1993).

$$C_{fillet}(t) = \frac{\alpha F t}{K_{el}+b} Cfeed\,(1-e^{-(k_{el}+b)t}) + C_{fillet0}e^{-(k_{el}+b)t} \quad (4)$$

where b is the growth rate and $C_{fillet0}$ is the fillet level at the start of the exposure.

Results and discussion

Levels in feed and fish

The levels in fillet of Atlantic salmon fed on "high" and "low" diets for 12 months are given in Table 1.

Table 1. Toxaphene concentrations (µg kg^{-1} wet weight) in the feed and in fillets from Atlantic salmon after the12 month feeding trial. For values that could not be quantified the limit of quantification (LOQ) is given as <LOQ.

level (mean±SD)	CHB 26	CHB 32	CHB 40+41	CHB 42a	CHB 44	CHB 50	CHB 62	Sum 26, 50, 62
feed low	0.3	< 0.2	0.3	< 0.5	< 0.1	0.5	0.2	1.0
feed high	0.8	< 0.2	0.8	< 0.5	0.2	1.3	0.5	2.6
fillet low	0.48±0.05	< 0.2	0.33±0.01	< 0.2	0.12±0.01	0.86±0.01	0.39±0.02	1.7±0.07
fillet high	1.96±0.14	< 0.2	1.35±0.07	< 0.2	0.67±0.06	3.66±0.31	1.6±0.18	7.3±0.63

The levels of sum toxaphene 26, 50, 62 in feed was 2.6-fold different among the two diets, whereas the levels in fillets after 12 months of feeding had a 4.3-fold difference. The levels in the feed and fillet were in the lower range compared with monitoring data reported for toxaphene in commercial growth feeds and market-size Atlantic salmon fillets (Maage *et al*. 2007; www.NIFES.no). This can be at least partly attributed to the high limit of quantification (1, 2.5, 1.5 for congeners 26, 50, 62, respectively), and the reporting of LOQ in the sum of toxaphene congeners when one or more of the congeners could not be quantified. None of the levels in feed or fillet exceeded the current EU maximum levels for toxaphene in fish feed and meat, both set at 50 µg/kg (no maximum level currently exists for fish).

Biomagnification and retention

The biomagnifications factor for the different toxaphene congeners for fish fed on the "high toxaphene" diet (feed based on fish meal and oil) and the "low toxaphene" diet (feed based on plant meal and oil) are given in Figure 1.

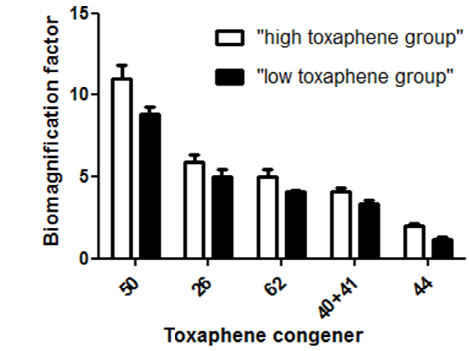

Figure 2. Biomagnification factor in Atlantic salmon fed on high and low toxaphene diets

Highest biomagnification was observed for congener 50, followed by 26, 62, 40+41 and 44, respectively. The congeners that are currently included in legislation (26, 50, 62) have highest biomagnifications potential while the additional congeners recommended by EFSA (40, 41, 44) had the lowest biomagnification. For congeners 32, and 42a no biomagnification calculation could be made, because the levels found in salmon fillet were under the level of quantification (LOQ). Similarly, in surveillance data on farmed Atlantic salmon currently on the market (www.NIFES.no) the congeners 32 and 42a were not quantifiable (below the LOQ). The bimagnification was dose dependent as seen from the higher BMF in the group fed on “high toxaphene” diets compared to “low toxaphene” diets. This caused the differences in total toxaphene 26, 50, 62 levels in fish fillet (4.5 fold) to be greater than that found for fish feed (2.6 fold) between the two dietary groups. As for biomaginification, highest retention was observed for congener 50, followed by 62 and 26. The retention for 40+41 and 44 was significantly lower compared to the 50, 62 and 26 congeners. The retention of toxaphene congener, 26, 50, 62, 40, 41, and 44 was 53±4, 73±6, 61±8, 34±4, and 33±7%, respectively. The retention of dietary toxaphene 26, 50, and 62 in freshwater rainbow trout fillet was 28, 35, and 26 %, respectively (Karl *et al*. 2002). Similar to the present study, highest retention was observed for congener 50. The level of retention in the feeding trial by Karl *et al*. (2002) was nearly two fold lower than in the present study. This can be attributed to the lack of feed collection in the study on rainbow trout, causing an over estimate of the feed intake.

Accumulation and elimination during cross over

The accumulation of toxaphene congeners that are currently included in legislation (26, 50, 62) are given in Figure 2A, and accumulation of the congeners recommended by EFSA are given in Figure 2 B. All data is corrected for growth dilution in control levels. As for

biomagnification and retention, highest accumulation was found for toxaphene 50 followed by 26 and 62, respectively. The congeners 40+41 and 44, had a lower accumulation.

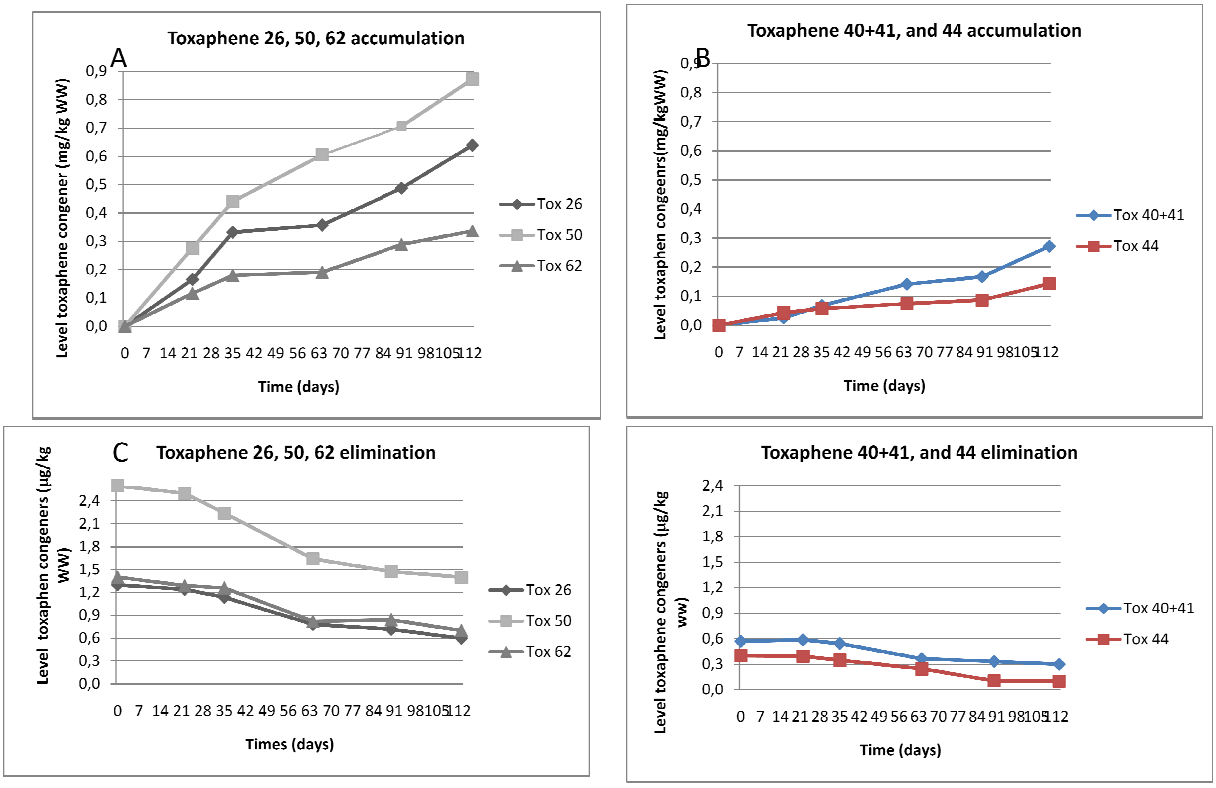

Figure 2. Levels of toxaphene congeners, corrected for growth and control levels, during accumulation and elimiantion period

The elimination patterns of toxaphene 26, 50, 62, and 40+41, 44 are given in Figure 2 C and D, respectively. The elimination was slow, during the five month period only approximately half of the toxaphene congener was eliminated. This was found for all congeners examined.

Uptake and elimination kinetics and modelling

The uptake and elimination rates are given in Table 2. The elimination rates, given as half-life, did not differ substantially among the congeners. The half-life varied from 113 to 177 days, which was shorter than previously reported for sum toxaphene 26 and 50 in lake trout in a natural ecosystem where a half-life of 232-322 days was found, depending on the dose recieved (Delorme *et al*. 1999). The uptake rates were significantly higher for congeners 26, 50 and 62 compared to 40+41 and 44. The differences in retention and biomagnification among the toxaphene congeners were consequently not explained by differences in elimination and metabolism, but were related to differences in uptake rates.

Table 2. Estimated elimination rate constants (Half-life; Kel), and uptake rates (α) for toxaphene congeners (mean±SD). Values with the different superscripts are significantly (P<0.005) different from each other (ANOVATukey's *t*-test)

	CHB 26	CHB 40+41	CHB 44	CHB 50	CHB 62
Elimination					
Half-life (T1/2, days)	113±22	114±22	177±151	129±54	112±37
elimination rate (kel, day-1)	6.3±1.3	4.2±3.4	5.9±3.8	5.9±2.2	6.6±2.1
Uptake					
uptake rate (α, day)	0.77±0.18[a]	0.20±0.078[b]	0.29±0.051[b]	0.78±0.21[a]	0.62±0.21[a]

Figure 3 gives the predicted accumulation of the sum toxaphene 26, 50, and 62 (Figure 3A) as well as sum toxaphene 26, 50, 62, 40+41, and 44 (Figure 3B). The model used is a one compartmental kinetic model as described in formula (4) using the kinetic parameters (uptake and elimination rates) reported in Table 2.

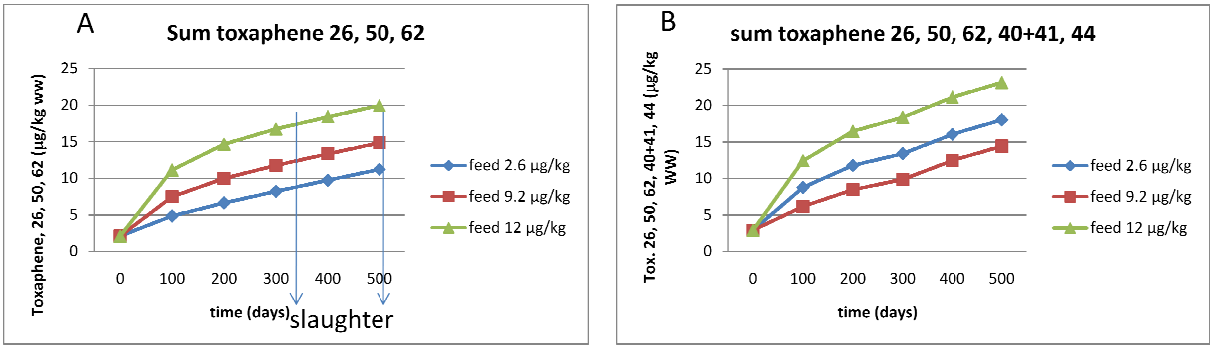

Figure 3. Model predictions of toxaphene congener accumulation at different feed concentrations (2.6, 9.2, and 12 µg/kg) over time.

The toxaphene concentrations chosen were 1) the levels found in the fish-based feed used in the feeding trial (2.6 µg/kg), 2) the maximum level documented in the National fish feed and feed ingredients monitoring programme (9.2 µg/kg; Maage *et al*. 2007), 3) estimated feed levels (12 µg/kg) that gave fillet levels with the maximum toxaphene level reported to date in farmed Atlantic salmon (www.nifes.no). Figure 3A shows that no steady state conditions were obtained during the experimental period (~300-500 days after seawater transfer). The inclusion of all analysed congeners, including the additional ones recommended by EFSA (40+41 and 44) gave minor increases in the total toxaphene levels (Figure 3B). Since model prediction depend on input data such as feed intake and growth, predictions were also made using different aquaculture conditions and compared to calculated biomagnification. The lowest feed concentration which led to a fillet level of 20 µg/kg was 6 µg/kg (data not

shown). The maximum concentration of toxaphene reported in farmed Atlantic salmon to date is 16.9 µg/kg wet weight (www.nifes.no). Hence the level of 20 µg/kg was chosen to model the carry-over of toxaphene from feed to fillet in farmed salmon. There is currently no EU maximum limit for toxaphene in seafood, however the maximum limit for toxaphene (sum of congeners 26, 50 and 62) in meat is 50 µg/kg.

Conclusion

The carry-over, as seen from biomagnification and retention, of the toxaphene congeners that are currently included in EU legislation (26, 50, and 62) was two-fold higher than the additional congeners that EFSA recommend to include in monitoring programmes and future legislation (40, 41, 44). Using different model predictions is was estimated that feed levels of 6-12 µg/kg result in toxaphene levels in fish fillets that represent the maximum concentration in farmed Atlantic salmon reported to date (approximately 20µg/kg). This prediction was made for seawater-adapted Atlantic salmon reared for 16 months at average growth and feed intake (0.65 g/day and 0,83 % body weight/day, respectively). Due to the lower uptake and concentration of the "novel" congeners (40+41 and 44), their inclusion led to a minor (~15%) increase in total fillet levels.

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