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Tolerance of Atlantic salmon (*Salmo salar*) to dietborne endosulfan assessed by haematology, biochemistry, histology and growth

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Abstract

The inclusion of plant-based ingredients in commercial fish feeds may pose a challenge because of the presence of undesirable substances, such as the pesticide endosulfan. Waterborne endosulfan is highly toxic to fish, whereas dietborne exposure has varied toxicity in different species. To investigate the systemic effects of endosulfan exposure, quadruplicate groups of Atlantic salmon (*Salmo salar*) were fed either 0 (control), 0.005 mg kg^{-1}; the European Union's maximum limit, or 10 or 20 times this level (0.05 and 0.1 mg kg^{-1} respectively) for 95 days. There were no significant differences ($P > 0.05$) in liver somatic index, spleen somatic index, condition factor or growth among treatments. There were no indications of liver damage in fish from any of the groups in the biomarkers measured: plasma aspartate aminotransferase, plasma alanine aminotransferase and histopathology. Similarly, there were no apparent treatment-related effects on the haematological parameters Hct, Hb, mean corpuscular volume, mean corpuscular haemoglobin concentration and mean corpuscular haemoglobin, and blood sodium, potassium, calcium and chloride levels were not significantly ($P > 0.05$) different among groups. Lipid digestibility, but not energy, protein, or glycogen digestibility, was significantly ($P < 0.05$) reduced at the highest exposure concentration. However, no significant differences were observed in lipid production value or lipid efficiency ratio. In contrast to previous studies, clinical histological abnormalities were not observed in the intestine, liver or spleen of endosulfan-treated fish.

KEY WORDS: alternative feed resources, Atlantic salmon, dietborne exposure, endosulfan, feed legislation, tolerance

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Introduction

The rapid growth of aquaculture in recent decades has led to increased demands for fish oil and fishmeal; however, their availability is limited. Consequently, much research and development has focused on sustainable, alternative, feed ingredients for fish feed. Fish feeds primarily based on ingredients of plant origin have been shown to be nutritionally adequate for carnivorous fish such as Atlantic salmon (Torstensen *et al.* 2008). Furthermore, the replacement of fish oil and fishmeal in fish feed with plant ingredients may reduce consumer exposure to several groups of organochlorine compounds such as polychlorinated biphenyls and dioxin (Berntssen *et al.* 2005). However, the inclusion of plant-based feed ingredients potentially exposes farmed fish to other groups of chemicals, such as polyaromatic hydrocarbons (Berntssen *et al.* 2010a) and organochlorine pesticides including endosulfan (Prasad & Chhabra 2001; Lorenzatti *et al.* 2004; Rubio *et al.* 2006). The European Union's maximum limit for endosulfan in complete feedingstuffs for fish is at least 20 times lower than the maximum content established for other feedingstuffs; however, the scientific basis for this discrepancy lacks transparency. While the transfer of endosulfan from fish feed to fish fillet has been shown to be limited (Berntssen *et al.* 2008), there remains controversy in the scientific literature regarding the susceptibility of the target organism (fish) to dietborne endosulfan.

Fish, in particular salmonids, are considered highly susceptible to waterborne endosulfan (EFSA 2005). In contrast, toxicity of dietborne endosulfan shows high variability among fish species and is dependent on the endpoints assessed.

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Exposure to concentrations up to 100 times higher than the current European Union's maximum limit for endosulfan in fish feed (0.005 mg kg^{-1}) did not cause acute toxicity in Atlantic salmon (Petri *et al.* 2006; Glover *et al.* 2007). However, histological alterations have previously been shown in the posterior intestine and liver of Atlantic salmon exposed to 0.004 mg endosulfan kg^{-1} for 35 days (Glover *et al.* 2007) and in the intestine and spleen after exposure for 16 weeks to 0.005 mg kg^{-1} (Berntssen *et al.* 2010b). Altered cellular morphology has also been observed in the liver and intestine of common carp (*Cyprinus carpio*) following 35 days of exposure to 0.0005 mg endosulfan kg^{-1} (Braunbeck & Appelbaum 1999). Furthermore, histopathological effects have been reported in the liver of tilapia (*Oreochromis niloticus*) orally exposed for 35 days to 0.001 and 0.1 mg endosulfan kg^{-1} (Coimbra *et al.* 2007). Although histological endpoints are clearly very sensitive to dietary endosulfan exposure in salmon, such changes can occur in the absence of measurable biochemical effects, or apparent growth performance (Petri *et al.* 2006; Glover *et al.* 2007; Berntssen *et al.* 2010b).

Mechanisms to cope with exposure to toxicants can alter energy metabolism, reducing energy available for growth and reproduction. Moreover, toxicant exposure may impair the digestion and absorption of nutrients further reducing the deposition of energy reserves. This may be particularly important following dietary exposure where the intestine is a major target organ (Berntssen *et al.* 1999). Digestive and absorptive functions of the intestine have previously been shown to be negatively influenced by dietary exposure to contaminants (Farmanfarmaian *et al.* 1985; Lanno *et al.* 1985; Berntssen & Lundebye 2001).

The aim of the current investigation was to investigate systemic effects of dietary endosulfan exposure in Atlantic salmon including nutritional performance and growth under conditions representative of commercial farming practice and to evaluate the effects of the current EU maximum level for endosulfan in complete feedingstuffs for salmonids. General health parameters (organ somatic indices, haematology, clinical chemistry) and histology were also assessed in the current study to enable comparison with previous studies on Atlantic salmon exposed to dietborne endosulfan (Petri *et al.* 2006; Glover *et al.* 2007; Berntssen *et al.* 2010b).

Materials and methods

Animals, diet, feeding and sampling

Postsmolt Atlantic salmon (*Salmo salar*) (08G strain, 0+) were pit-tagged and randomly distributed among 16 sea cages (5 × 5 × 5 m; 125 m^3; ~125 fish per cage) at the Gildesskål, Research Station, GIFAS, Gildeskål kommune, Norway. The 95 -day exposure period started in February 2009 and the fish were approximately 250 g. Prior to the start of the trial, fish were acclimated to the environmental conditions for 2 weeks. Fish were reared under a 24 -h light regime before the start of the trial and for the first 79 days of the experiment. Cages were illuminated by four 400 W IDEMA underwater lights that were positioned at the centre of each block of four cages at a depth of three metres For the remaining days of the experiment, fish were reared under daylight conditions without artificial light. The light regime throughout this trial is according to standard aquaculture practice. Fish were hand-fed till satiation two times daily, and feed intake was recorded for each sea cage. Total feed intake and mortality were recorded daily. Water temperature, salinity and oxygen saturation over the course of the trial varied from 4.6 to 8.4 °C, 30–34.2 g L^{-1}, and 8.7–12.2 mg L^{-1}, respectively. Following acclimatization, quadruplicate fish groups were given feeds spiked with either 0 (control), 0.005, 0.05, or 0.1 mg technical endosulfan kg^{-1}. The lowest concentration represents the current maximum limit for endosulfan in fish feed in the European Union (EC 2002), and 10 or 20 times this level (0.05 and 0.1 mg kg^{-1}, respectively) were selected to enable comparison with previous studies on Atlantic salmon (Petri *et al.* 2006; Glover *et al.* 2007; Berntssen *et al.* 2010b), and the highest concentration is the lowest maximum content established in the EU for other feedingstuffs (0.1 mg kg^{-1}; European Council (EC) 2002). The nominal endosulfan concentrations in the feed were confirmed by analysis and are given in Table 1. The feeds contained fish meal (Peruvian anchovy, 663 g kg^{-1} diet), wheat meal (Statkorn, Norway, 157 g kg^{-1} diet), fish oil (extracted from anchovy, Peru, 177 g kg^{-1} diet) and a standard vitamin and mineral mixture (3 g kg^{-1} diet), following the recommendations of the NRC (1993). Technical endosulfan (Sigma-Aldrich, Seelze,

Table 1 Analysed endosulfan concentrations (sum α-endosulfan and β-endosulfan mg kg^{-1}) in experimental feeds and estimated daily dose (ng kg^{-1} fish day^{-1})

Nominal feed concentration (mg kg^{-1})	Actual feed concentration (mg kg^{-1})	Daily dose (ng kg^{-1} fish day^{-1})
0	n.d.	–
0.005	0.00566	28.8
0.05	0.04740	239.3
0.1	0.10400	528.6

n.d., not detected (<0.50 μg kg^{-1}).

Germany, 99.8% purity; α + β-isomer ~ 2 + 1) was dissolved in fish oil, and the basal feed was spray-coated with the oil emulsion. A second coat of oil containing 1 g kg^{-1} yttrium (for digestibility measurements) without endosulfan was sprayed on the feed pellets to minimize endosulfan leakage. The pellets were dried at 40 °C for 24 h and stored at −20 °C until they were fed to the fish. After 0, 40, 73 and 95 days of exposure, six fish per sea cage ($n = 24$ per treatment) were terminally anaesthetized with an overdose of 3-amino benzoic acid ethylester (~1 g L^{-1}, pH 8.0). Length, weight and sex were registered, and blood samples were taken for haematology and biochemical analyses. Fish were subsequently killed by cervical transection of the spinal column, and liver and spleen were removed and weighed. Standardized sections of both liver and spleen and also mid- and hindgut were fixed in ice-cold 4% paraformaldehyde solution in phosphate-buffered saline and stored on ice until further processing. Remaining liver tissue was packed in aluminium foil and frozen at −20 °C. Finally, muscle samples were taken according to the Norwegian Quality Cut (NQC) and frozen at −20 °C. At the final sampling, faeces of all remaining fish from each cage were collected and pooled for analyses of yttrium, total protein, glycogen and lipid.

Haematology and blood biochemistry

Blood samples were drawn from the caudal vein of six fish from each cage (24 fish per treatment) and divided into two aliquots. The blood and plasma analyses were performed according to Sandnes *et al.* (1988). One aliquot was used for haematocrit, erythrocyte count and haemoglobin determination with a Cell-Dyn 400™ (Abbott Diagnostics, Santa Clara, CA, USA). Haematocrit (Hct), whole blood haemoglobin content (Hb) and red blood cell counts (RBC) were used to calculate mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) according to Houston (1997). The second aliquot was centrifuged, the plasma was snap-frozen in liquid nitrogen and stored at −80 °C until analysed. Plasma was analysed for aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), as indicators of liver cell damage, as well as total protein and creatinine using a Maxmat PL multianalyser (Montpellier, France) and kits (ASAT, D94610; ALAT, D94620; total protein, D95680; creatinine, D05540) from Dialab (Vienna, Austria).

Histology

Samples of liver, mid- and hind intestine were preserved in formalin; six fish were sampled from each cage ($n = 24$ per treatment) at exposure days 73 and 95. Samples were sent to the Institute of Aquaculture in Stirling, Scotland for examination, and the analyst was blinded to the treatment group to which the samples belonged. Samples were dehydrated in a graded alcohol series before embedding in paraffin wax, sectioning and processing. Routine observations were made on sections stained with haematoxylin and eosin.

Biochemical and chemical analysis

Feed and faecal samples were homogenized and freeze-dried (until successive weighing was unchanged) and analysed for total protein, lipid, glycogen, water content and yttrium, and endosulfan was measured in feed samples. Crude total protein, including both structural and soluble protein, was determined by nitrogen combustion of 0.5 g of freeze-dried material with a Dumas and Liebig nitrogen analyser (PE 2410, USA). Nitrogen was detected by thermal conduction and crude protein was calculated as Nx6.25 (Crooke & Simpson 1971). Casein (C-8654, Sigma, Dorset, UK) was used as reference material. Digestible carbohydrate in feed and faeces and glycogen in sampled tissues were determined using an enzymatic method briefly described by Hemre *et al.* (1989). Starch in 0.5 g of freeze-dried material was hydrolysed with the heat-stable enzymes amylase (Termamyl-120L, Novo-Industries, Bagsvaerd, Denmark) for 30 min at 80 °C and amyloglucosidase (EC 3.2.1.3., Ingelheim, Boehringer, Germany) for 30 min at 60 °C. Glucose was subsequently measured spectrophotometrically as NADPH at 340 nm after a hexokinase/glucose-6-phosphate dehydrogenase reaction in an automated analyser (Technicon, RA-1000, Wayne Bayer, NJ, USA). Glycogen concentration was calculated as the difference in glucose concentration before and after enzymatic breakdown. Dextrin was used as the reference material. Faecal lipid was determined gravimetrically as the sum of free and bound fat. Free or loosely bound fat was extracted with petroleum ether and dried at 103 ± 1 °C. The samples were subsequently hydrolysed with HCl in a Tecator Soxtec Hydrolysing unit to release the bound fat, which was extracted with petroleum ether and dried at 103 ± 1 °C. Dry weight and ash content were determined gravimetrically after freeze-drying the samples and dried to constant weight in an oven at 550 °C, respectively. Gross energy was analysed by adiabatic bomb calorimetry (IKA Laborteknik, Staufen, Germany).

Concentrations of endosulfan (sum α-endosulfan, β-endosulfan and endosulfan sulphate mg kg^{-1}) in feed were assessed by routine gas chromatography/mass spectrometry analyses as previously described by Petri *et al.* (2006). The

limit of quantification for endosulfan in feed was 0.0003 mg kg^{-1} for α-endosulfan and β-endosulfan and 0.0005 mg kg^{-1} for endosulfan sulphate, the levels of endosulfan sulphate were below the limit of quantification in all samples analysed. Measured endosulfan concentrations in the experimental feeds and daily doses that the fish were exposed to are given in Table 1. Previous studies conducted by this research group (Petri *et al.* 2006; Glover *et al.* 2007) detected endosulfan in the livers from Atlantic salmon fed 0.05 mg kg^{-1} and 0.5 mg kg^{-1} but not in those exposed to 0.005 mg kg^{-1}. Consequently, endosulfan concentrations were not measured in the fish in this study because it was assumed that the endosulfan burdens in the fish would be comparable to those measured in the former trials at the same feed concentrations.

The yttrium oxide concentrations in freeze-dried feed and faeces were analysed according to Otterå *et al.* (2003). Briefly, yttrium oxide was quantified by inductively coupled plasma mass spectrometry after wet digestion in a microwave oven (Otterå *et al.* 2003).

Calculations

Growth and nutritional indices were calculated as follows:

Condition factor (CF) = body weight (g)/$Length^3$ (cm) × 100

Hepatic somatic index (HSI) = liver weight (g)/body weight (g) × 100

Spleen somatic index (SSI) = spleen weight (g)/body weight (g) × 100

Food conversion ratio (FCR) = feed intake (g)/fish weight gain (g)

Thermal unit growth coefficient (TGC) = $(FBW^{1/3} - IBW^{1/3})/\sum(T \times D) \times 100$;

where FBW and IBW are final and initial body weight, T is water temperature and D is days

Protein efficiency ratio (PER) = weight gain (g)/protein intake (g)

Protein production value (PPV) = fish protein gain (g)/ protein intake (g)

Lipid efficiency ratio (LER) = weight gain (g)/lipid intake (g)

Lipid production value (LPV) = fish lipid gain (g)/lipid intake (g)

Specific growth rate (SGR) was calculated according to the formula of Houde & Schekter (1981): $SGR = [\ln(W_2) - \ln(W_1)] \times (t_2 - t_1)^{-1}$; where W_2 and W_1 are weights on day t_2 and t_1, respectively.

Apparent digestibility (AD) of nutrients and energy was calculated using the formula described by Maynard & Loosli (1969): $AD = 100 - (Y_d \times CX_f) \times (Y_f \times CX_d)^{-1} \times 100$ where d is diet, f is faeces, Y is yttrium concentration and CX is nutrient- or energy concentration.

Statistical analysis

To account for variance among cages within a dietary treatment, as well as variance among fish within an experimental unit, nested ANOVA was performed on all individual blood parameters, organ somatic indices, CF and body weights, followed by Tukey's HSD *post hoc* test. For parameters based on cage level (digestibility and nutrient efficiency ratios), normal ANOVA was performed followed by Tukey's HSD *post hoc* test. Data were checked for homogeneity of variance by the Levene test and for normality of distribution by the Komogorov–Smirnov test. Statistics were performed using the program Statistica (Statsoft Inc., Tulsa, OK, USA).

Individual growth data of pit-tagged fish were analysed using individual fish weight in a multi-level (mixed effects) model (Pinheiro & Bates 2000) with the feed endosulfan concentration (as a categorical variable), sampling day and their interaction (a measure of the growth effects by different feed endosulfan concentrations in relation to the control) as the predictors i.e. individual growth trajectories were modelled. The intercept (initial weight estimate) and the slope of the day term (growth rate estimate) were allowed to vary among individual fish within a cage, giving two additional levels of random variation to the residual error. This model takes into account the correlation structure in the data generated by measuring individuals in different cages. The modelling was conducted with the lme4 package (Bates *et al.* 2008) of the R language and environment for statistical computing and graphics (R Development Core Team 2008).

Results

Growth parameters

There were no mortalities in any of the treatments. No significant differences in weight, CF, liver somatic index or SSI were observed amongst the dietary groups (Table 2). Fish in all groups almost doubled their weight during the 95 -day feeding trial (Table 2). The output of the model of growth trajectories showed the initial weight of the control fish was about 245 g (intercept), and the differences of the initial weights of the other groups in relation to the zero dose were

Table 2 Growth (body weight) and growth indices (condition factor, CF; hepatic somatic index, HSI; spleen somatic index, SSI) of Atlantic salmon following dietary exposure to endosulfan for up to 95 days

Parameter and day	Endosulfan concentration			
	0 mg kg^{-1}	0.005 mg kg^{-1}	0.05 mg kg^{-1}	0.1 mg kg^{-1}
Body weight (g)				
Day 0	245 ± 21	246 ± 21	245 ± 22	246 ± 22
Day 40	290 ± 25	303 ± 29	294 ± 23	293 ± 30
Day 73	372 ± 33	365 ± 47	375 ± 47	371 ± 46
Day 95	446 ± 53	451 ± 51	452 ± 55	454 ± 53
CF				
Day 0	1.11 ± 0.06	1.11 ± 0.06	1.10 ± 0.07	1.10 ± 0.07
Day 40	1.10 ± 0.06	1.12 ± 0.06	1.09 ± 0.07	1.11 ± 0.05
Day 73	1.15 ± 0.06	1.12 ± 0.07	1.13 ± 0.07	1.13 ± 0.07
Day 95	1.14 ± 0.07	1.13 ± 0.07	1.13 ± 0.07	1.13 ± 0.06
HSI (%)				
Day 0				
Day 40	1.18 ± 0.13	1.20 ± 0.14	1.23 ± 0.19	1.19 ± 0.16
Day 73	1.17 ± 0.15	1.14 ± 0.12	1.12 ± 0.12	1.12 ± 0.09
Day 95	1.08 ± 0.17	1.11 ± 0.13	1.08 ± 0.11	1.09 ± 0.13
SSI (%)				
Day 0				
Day 40	0.010 ± 0.004	0.012 ± 0.005	0.012 ± 0.005	0.010 ± 0.003
Day 73	0.011 ± 0.004	0.012 ± 0.006	0.010 ± 0.004	0.011 ± 0.005
Day 95	0.013 ± 0.006	0.013 ± 0.005	0.012 ± 0.005	0.012 ± 0.004

No significant differences were measured among treatments at the $\alpha = 0.05$ level using ANOVA; for initial growth $n = 500$, for final growth $n = 350$, 375, 391 and 373, respectively, for the four treatments, 0, 0.005, 0.05 and 0.1 mg endosulfan kg feed^{-1}; for CF $n = 500$ for day 0, $n = 40$ for day 40 and 73 and $n = 350$, 374, 391 and 373 for day 95, respectively, for the four treatments; for HSI and SSI $n = 40$ for day 40 and 73 and $n = 154$, 156, 158 and 158, respectively, for the four treatments for day 95.

insignificant, about 0.5 g. The daily growth rate of the control fish from the model indicates that they grew approximately 2.1 g day^{-1}. The interaction terms measuring the differences in daily growth rates of the endosulfan groups relative to the control fish showed that the endosulfan-exposed fish tended to grow slightly faster than the zero dose fish. However, all estimates were equal or less than their standard errors and thus the differences cannot be considered significant.

Haematology, blood and serum biochemistry

The haematological parameters Hct, Hb, MCV, MCHC and MCH did not reveal any differences among the groups at any time point (Fig. 1). Red blood cell count was significantly reduced in the 0.005 mg kg^{-1} and 0.05 mg kg^{-1} groups compared to the control and the 0.1 mg kg^{-1} at day 40 (Fig. 1b), there were no differences among treatments on day 73 or 95. No disruption in the regulation of ion metabolism (sodium, potassium, calcium or chloride) was observed in any of the treatments at any time point (Table 3). Similarly, the ALAT and ASAT activities and creatinine levels were not statistically different among the dietary treatments (Table 4). There was a transient statistically significant increase in the plasma total protein level at day 40 in fish from the highest endosulfan concentration (Table 4) compared to the control group; however, this was not evident at sampling day 73 or 95.

Nutritional parameters

There were no significant effects of endosulfan on energy, glycogen or protein digestibility (Table 5). Lipid digestibility was significantly reduced in the highest endosulfan group (0.1 mg kg^{-1}) compared to the lowest exposure group (0.005 mg kg^{-1}). PER, PPV, LER and LPV were not significantly affected in the fish following 95 days of exposure (Table 6). Furthermore, there were no significant differences in either food conversion rate or SGR among the dietary treatments (Table 6).

Histology

Histology was assessed in samples from day 0, 40 and 95 in all four treatments: 0, 0.005, 0.05 and 0.1 mg kg^{-1}. Gross liver pathology was apparently absent, and there was no evidence of bleeding, endothelial damage, or necrosis. Some areas of the liver from several fish contained moderate

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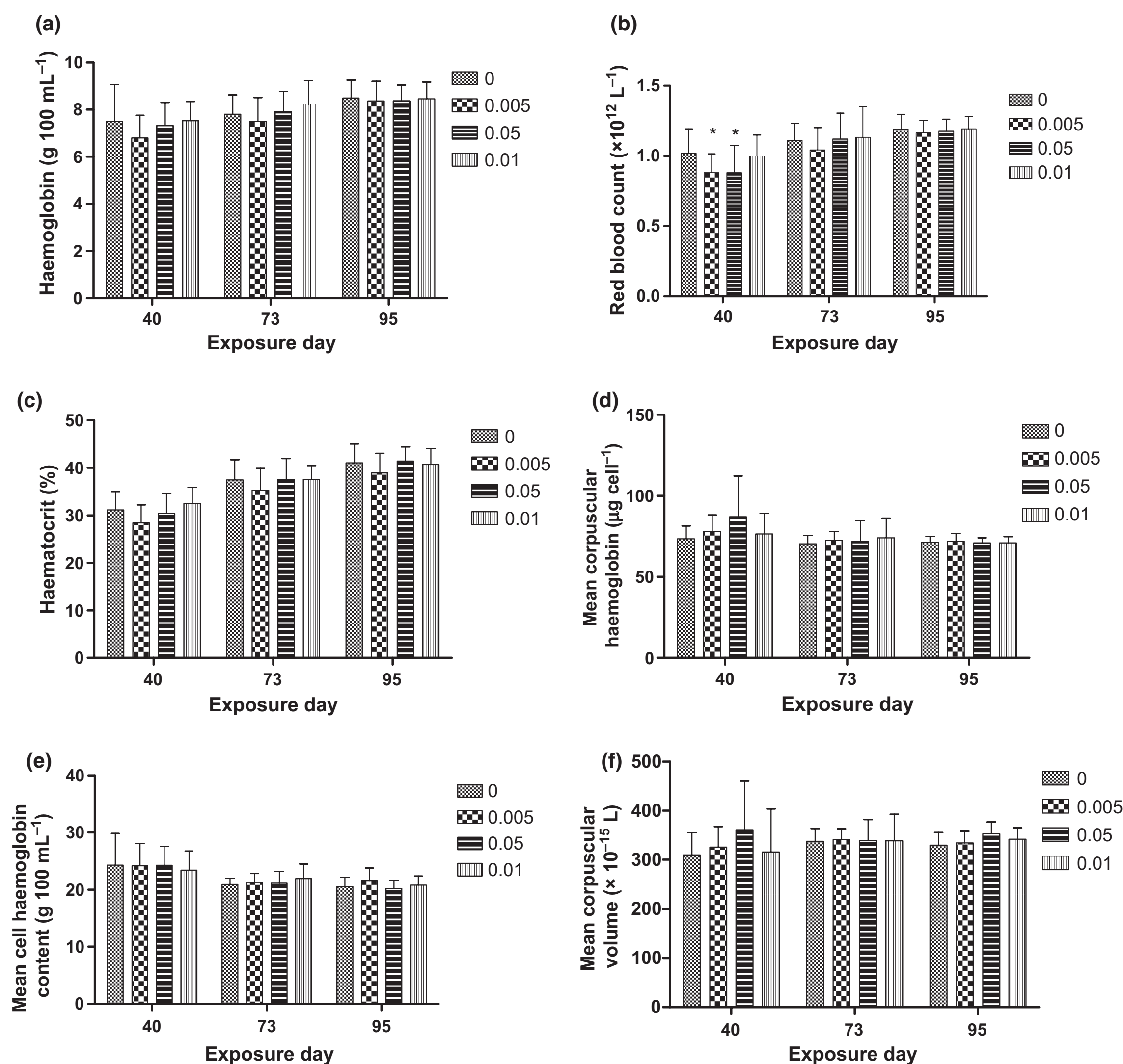

Figure 1 Effect of up to 95 days of dietborne endosulfan exposure (0.005, 0.05 or 0.1 mg kg^{-1}) on haemoglobin content (a) erythrocyte count (b), haematocrit (c), mean corpuscular haemoglobin (MCH) (d), MCH content (e) and mean corpuscular volume (f) in Atlantic salmon. Significant differences (*) from the control at each sampling point were assessed at the $\alpha = 0.05$ level using nested ANOVA followed by Tukey's HSD test, mean ± SD, $n = 24$.

numbers of small extracellular vacuoles indicative of hepatocytes turnover. Granulomas were noted in two of the livers that may represent a response to previous infection, or represent an antigenic response to injected vaccine. Vaccine-related peritonitis was evident in most of the samples, with chronic inflammation evident in spleens, some areas of livers and intestines. This response was not limited to the peritoneal surface and foci appeared systemically within viscera such as the liver. Some spleen samples had increased yellow-brown pigment depositions that are normal features and may be lipofuscein, which is a product of lipid metabolism. No apparent abnormalities were observed in the intestinal samples. The effects observed in liver and spleen did not appear to be treatment related, and no apparent differences were observed between the endosulfan-exposed fish compared to the controls.

Discussion

The current study was conducted in realistic farming conditions, and the growth performance and feed conversion ratio in Atlantic salmon are comparable to those typically found in

Table 3 Blood chemistry in Atlantic salmon following dietary exposure to endosulfan for up to 95 days

Sampling day	Treatment (mg kg^{-1})	Na^+ (mmol L^{-1})	K^+ (mmol L^{-1})	Ca^{2+} (mmol L^{-1})	Cl^- (mmol L^{-1})
0	0	166 ± 2	5.6 ± 0.6	1.5 ± 0.2	149 ± 2
40	0	181 ± 5	2.8 ± 1.2	2.2 ± 0.1	154 ± 6
	0.005	183 ± 3	2.9 ± 1.0	2.3 ± 0.1	156 ± 3
	0.05	182 ± 4	2.9 ± 1.0	2.2 ± 0.1	155 ± 4
	0.1	181 ± 5	2.3 ± 0.9	2.2 ± 0.1	153 ± 4
73	0	180 ± 4	4.2 ± 1.0	2.2 ± 0.2	154 ± 4
	0.005	181 ± 5	4.5 ± 0.8	2.3 ± 0.2	155 ± 5
	0.05	180 ± 5	4.2 ± 0.8	2.2 ± 0.2	154 ± 4
	0.1	181 ± 4	4.3 ± 0.9	2.3 ± 0.1	155 ± 4
95	0	183 ± 5	2.3 ± 1.8	2.2 ± 0.2	157 ± 5
	0.005	181 ± 4	3.0 ± 1.6	2.1 ± 0.2	155 ± 6
	0.05	182 ± 6	1.7 ± 0.9	2.2 ± 0.2	154 ± 5
	0.1	183 ± 4	2.7 ± 1.3	2.3 ± 0.1	157 ± 4

No significant differences were measured at the $\alpha = 0.05$ level using nested ANOVA, mean ± SD, $n = 20$ for the initial sampling and $n = 24$ for all other time points.

Table 4 Serum biochemistry in Atlantic salmon following dietary exposure to endosulfan for up to 95 days

Sampling day	Treatment (mg kg^{-1})	ALAT (units L^{-1})	ASAT (units L^{-1})	Creatinine (mg dL^{-1})	Protein (g L^{-1})
0	0	25.4 ± 14.7	575 ± 130	0.38 ± 0.07	23.8 ± 3.7
40	0	16.9 ± 7.7	381 ± 149	0.75 ± 0.60	25.7 ± 6.8
	0.005	17.4 ± 8.3	307 ± 63	0.58 ± 0.13	25.5 ± 4.8
	0.05	15.9 ± 10.5	342 ± 134	0.74 ± 0.44	27.4 ± 6.0
	0.1	14.7 ± 8.7	367 ± 137	0.67 ± 0.13	34.5 ± 5.9*
73	0	34.5 ± 20.1	557 ± 150	0.81 ± 0.29	35.8 ± 8.1
	0.005	29.4 ± 7.6	562 ± 112	0.74 ± 0.27	32.7 ± 3.9
	0.05	29.4 ± 8.2	651 ± 144	0.78 ± 0.32	37.3 ± 8.2
	0.1	30.5 ± 18.5	769 ± 467	0.83 ± 0.28	32.1 ± 5.9
95	0	31.2 ± 9.0	465 ± 154	0.94 ± 0.62	33.9 ± 8.0
	0.005	34.8 ± 18.8	494 ± 222	0.91 ± 0.67	36.0 ± 8.5
	0.05	28.4 ± 6.0	465 ± 103	0.62 ± 0.17	35.5 ± 5.7
	0.1	39.2 ± 16.8	479 ± 102	0.56 ± 0.12	34.8 ± 6.8

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase.
* Significant differences from the control at each sampling point were assessed at the $\alpha = 0.05$ level using nested ANOVA followed by Tukey's HSD test, mean ± SD, $n = 20$ for the initial sampling and $n = 24$ for all other time points.

Table 5 Energy, lipid, glycogen and protein digestibility (%) in Atlantic salmon exposed to dietborne endosulfan for 95 days

Treatment (mg kg^{-1})	Energy digestibility (%)	Lipid digestibility (%)	Glycogen digestibility (%)	Protein digestibility (%)
0	87.2 ± 0.77	72.2 ± 5.5[ab]	74.3 ± 2.1	84.8 ± 0.90
0.005	87.4 ± 0.54	75.5 ± 7.7[a]	75.6 ± 2.4	85.3 ± 0.48
0.05	87.6 ± 0.31	73.8 ± 2.5[ab]	73.2 ± 0.8	85.6 ± 0.43
0.1	87.3 ± 0.57	63.4 ± 3.8[b]	72.7 ± 1.8	84.9 ± 0.64

Significant differences were measured at the $\alpha = 0.05$ level using ANOVA followed by Tukey's HSD test, mean ± SD, $n = 4$, $P < 0.05$.

commercial farming. Atlantic salmon exposed to endosulfan concentrations of up to 0.1 mg kg^{-1} feed for more than 3 months did not exhibit any signs of toxicity in this trial. The absence of gross liver damage in this study was confirmed by no negative effects on liver somatic index, and no increase of plasma ASAT and ALAT enzymes that usually are indicative of liver injury (Sandnes *et al.* 1988). This observation is supported by the findings of Berntssen *et al.* (2010b) that Atlantic salmon exposed to 1 mg endosulfan kg^{-1} for 4 months had significantly lower liver somatic

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Table 6 Nutritional indices for Atlantic salmon–fed diets containing graded levels of endosulfan for 95 days

Parameter	0 mg kg^{-1}	0.005 mg kg^{-1}	0.05 mg kg^{-1}	0.1 mg kg^{-1}
FCR[1]	0.91 ± 0.02	0.91 ± 0.01	0.89 ± 0.02	0.90 ± 0.01
SGR[2]	0.61 ± 0.02	0.62 ± 0.03	0.63 ± 0.02	0.64 ± 0.02
TGC[3]	2.47 ± 0.09	2.50 ± 0.11	2.52 ± 0.07	2.54 ± 0.09
PER[4]	1.42 ± 0.17	1.46 ± 0.13	1.64 ± 0.11	1.58 ± 0.08
PPV[5]	0.30 ± 0.03	0.31 ± 0.02	0.34 ± 0.02	0.33 ± 0.02
LER[6]	2.12 ± 0.10	2.12 ± 0.10	2.12 ± 0.10	2.12 ± 0.10
LPV[7]	0.15 ± 0.04	0.19 ± 0.02	0.17 ± 0.04	0.17 ± 0.03

No significant differences were measured among treatments at the $\alpha = 0.05$ level using ANOVA, mean ± SD, $n = 4$.
[1] FCR, food conversion ratio; [2] SGR, specific growth rate; [3] TGC, thermal unit growth coefficient; PER, [4] protein efficiency ratio; [5] PPV, protein production value; [6] LER, lipid efficiency ratio; [7] LPV, lipid production value.

index; however, apparent markers of liver damage (ASAT, ALAT and histopathology) were absent. In contrast, altered cellular ultrastructures were detected in the liver and intestine of common carp (*C. carpio*) exposed to 0.0005 mg endosulfan kg^{-1} (Braunbeck & Appelbaum 1999). Similarly, Coimbra *et al.* (2007) found increased vacuolization and numbers of eosinophil granular cells in hepatocytes from tilapia (*O. niloticus*) orally exposed for 35 days to 0.001 and 0.1 mg endosulfan kg^{-1}. Histological examination of the liver from Atlantic salmon fed 0.004 mg kg^{-1} endosulfan for 7 weeks showed loss of hepatic glycogen stores and some lipidosis (Glover *et al.* 2007). Glover *et al.* (2007) attribute the loss of glycogen to an increase in energy metabolism in response to endosulfan exposure. Braunbeck & Appelbaum (1999) also argued that changes in liver morphology were consistent with stimulated metabolism; however, this study which examined individual growth found to the contrary that the endosulfan-exposed fish tended to grow slightly faster than the control fish. The digestibility of lipids in the highest exposure group was significantly reduced in this study. In contrast, there were no effects of endosulfan exposure on energy, protein, or glycogen digestibility, indicating that the overall intestinal function was not affected. A previous study on the effect of endosulfan-exposed carp showed a reduction in lipid transport vesicles, chylomicrons, in the epithelial lining, which suggests a disturbance of intestinal lipid absorption (Braunbeck & Appelbaum 1999). The reduction in lipid digestibility in this study was not reflected by reduced feed lipid utilization as seen from the LER and the LPV.

Gross histological changes in the spleen were not evident in this study, in contrast to a previous study on Atlantic salmon that quantified red cell infiltration in the red pulp accompanied by accumulation of lipofuscin-like deposits in the spleen from fish fed 0.005 mg endosulfan kg^{-1} diet for 4 months (Berntssen *et al.* 2010b). This was supported by the haematological profiles of endosulfan-treated fish, because no effects were observed in haemoglobin content or haematocrit; however, there was a transient reduction in erythrocyte count in fish exposed to 0.005 and 0.05 mg kg^{-1} feed for 40 days. Previously, a transient elevation of haematocrit, haemoglobin and MCH was observed in Atlantic salmon exposed to 0.5 mg endosulfan kg^{-1} feed for 7 weeks (Petri *et al.* 2006). Alterations in erythrocyte metrics are linked to changes in oxygen demand (Houston 1997), hence the aforementioned changes may reflect an increased oxygen demand as a result of exposure to endosulfan. However, the haematological parameters in the exposed fish were restored to control values by day 49, indicating that their long-term toxicological significance was limited (Petri *et al.* 2006).

There were no clinical gross histological tissue abnormalities in either the anterior or posterior intestine related to endosulfan exposure in this study. In contrast, Glover *et al.* (2007) found cellular injuries to the intestinal mucosa, with villi tips showing vacuolation or fusion in endosulfan-exposed fish. Similarly, Berntssen *et al.* (2010b) found some cellular injury to the intestine in Atlantic salmon exposed to 0.005 mg endosulfan kg^{-1}; however, these effects were not concentration dependent, and the lowest endosulfan dose showed more pronounced histological changes than the intermediate and highest dose (0.05 and 1 mg kg^{-1}). Intracellular space distension, increased mucus cell precursor production and lack of chylomicrons absorption have been reported in carp following dietary exposure to as low as 0.0005 mg endosulfan kg^{-1} (Braunbeck & Appelbaum 1999). This discrepancy in findings compared to this study may be related to different gut morphology and sensitivity between herbivorous carp and carnivorous salmon. Furthermore, Braunbeck & Appelbaum (1999) conducted a detailed ultrastructural assessment on cell organelles, while this study assessed clinical tissue abnormalities by light microscopy.

Conclusion

In conclusion, there were no dose-dependent effects on blood biochemistry, haematology, organ somatic parameters or growth in Atlantic salmon following dietary endosulfan exposure for more than 3 months at the concentrations used in this study; up to 0.1 mg kg^{-1}. Lipid digestibility, but not glycogen, energy or protein digestibility, was significantly reduced in fish fed 0.1 mg kg^{-1} endosulfan. However, this was not accompanied by altered dietary lipid utilization assessed by LER and LPV. There were no apparent treatment-related clinical histological abnormalities in liver, spleen or intestine. These findings together with other recent studies support a re-evaluation of the current EU maximum level for endosulfan of 0.005 mg kg^{-1} in complete feeding-stuffs for salmonids.

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