## SCIENTIFIC OPINION

# Statement on oral toxicity of endosulfan in fish[1]

**EFSA Panel on Contaminants in the Food Chain (CONTAM)**[2, 3]

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## ABSTRACT

Endosulfan is a pesticide currently not authorised in the European Union and regulated as an undesirable substance in animal feed. The European Food Safety Authority (EFSA) was asked by the European Commission to assess recent information on the toxicity of endosulfan in fish and, if necessary, to update its opinion on endosulfan as an undesirable substance in animal feed, adopted on 20 June 2005 by the Scientific Panel on the Contaminants in the Food Chain (CONTAM Panel) and updated on 10 April 2006. The CONTAM Panel assessed six recent publications on exposure of Nile tilapia and Atlantic salmon to endosulfan via feed. Morphological changes in the liver were observed in Nile tilapia following 35 days of exposure to ≥ 0.001 mg/kg endosulfan in feed in tanks. However, such effects were not observed in Atlantic salmon exposed at 0.005 – 1 mg/kg endosulfan. Histological changes were observed in the intestine in Atlantic salmon following 49 or 112 days of exposure to ≥ 0.005 mg/kg endosulfan in feed in tanks, without a clear dose-effect relationship. Such changes in the intestine were not seen when Atlantic salmon were exposed for 95 days to up to 0.1 mg/kg endosulfan via feed in open sea-cages. Although a different sensitivity of Nile tilapia and Atlantic salmon to endosulfan could not be discounted, the CONTAM Panel noted that differences in the experimental conditions applied, leading to possible contamination of water with endosulfan, could also have influenced the results. The CONTAM Panel concluded that the recent information on toxicity of endosulfan in fish does not change the conclusions on toxicity in fish drawn in its previous opinion. The CONTAM Panel noted that further appropriately designed studies on oral toxicity in fish are needed to confirm the possible differences between fish species in sensitivity to endosulfan.



## KEY WORDS

Endosulfan, α-endosulfan, β-endosulfan, toxicity, residues in feed

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## SUMMARY

Endosulfan is a non-systemic organochlorine pesticide developed in the mid 1950s for a broad range of applications. The two diastereoisomers, α- and β-endosulfan, are the main components of the technical grades of endosulfan.

The release of endosulfan into the environment resulted in a ubiquitous contamination in many environmental compartments, due to its long-range transport and relatively high persistence and bioconcentration potential. The worldwide contamination, together with the toxicological and ecotoxicological profile of endosulfan led to its prohibition in the European Union for all uses as a plant protection product since 2005 (Commission Decision 2005/864/EC). Maximum levels for endosulfan in feed materials and compound feed have been laid down in Directive 2002/32/EC.

In 2005 the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA) adopted an opinion on endosulfan as an undesirable substance in animal feed. This opinion was subsequently revised in 2006 to take into account two additional studies on fish exposed to endosulfan in feed that had become available to the CONTAM Panel. The opinion pointed out the higher sensitivity of fish exposed to endosulfan through water in comparison to exposure via feed. Although the effects observed in dietary studies in fish were judged to be 'subtle, possibly adaptive, and not considered to represent adverse effects', the CONTAM Panel identified insufficient information regarding this route of exposure and recommended that additional oral toxicity studies on endosulfan in fish were needed.

The European Commission asked EFSA to assess recent scientific information on the toxicity of endosulfan in fish and, if necessary, to update its opinion on endosulfan as undesirable substance in animal feed.

The CONTAM Panel examined recent publications on exposure to endosulfan via feed and via water. Five publications from the same research group referring to studies in Atlantic salmon exposed to endosulfan via feed and one publication on Nile tilapia from another group were retrieved. The analysis of the available data on toxicity of endosulfan administered through the feed indicated morphological changes in the liver of Nile tilapia exposed at 0.001 mg/kg and 0.01 mg/kg endosulfan in feed, increasing in severity at 0.5 and 1 mg/kg endosulfan. In contrast, hepatic effects of comparable severity were not observed in Atlantic salmon exposed to 0.005-1 mg/kg endosulfan concentrations in feed. In Atlantic salmon, vacuolisation and fusion of the hind gut villi were the main effects following 49 or 112 days of exposure to ≥ 0.005 mg/kg endosulfan via feed in tanks, although no clear dose-effect relationship was observed in either study. No histological changes in the intestine or other adverse effects were observed in Atlantic salmon exposed for 95 days up to 0.1 mg/kg endosulfan in feed in open sea-cages.

It is not possible to attribute the observed differences in toxicity between Atlantic salmon and the Nile tilapia to differences in species sensitivity to the substance. The CONTAM Panel noted that differences in the experimental conditions applied could also have influenced the results. Namely, a lower water flow rate was used in the experiments with Nile tilapia in comparison to the flow rates applied in the studies in Atlantic salmon. Furthermore, in the studies in Atlantic salmon several precautions were adopted to avoid leaching of endosulfan from the feed and faeces to water in order to limit the exposure of fish via the water, which is the more sensitive route. Possible contamination of the water with endosulfan was not controlled in the study in Nile tilapia.

The CONTAM Panel noted that the new studies on endosulfan exposure through the water confirmed the high toxicity of the substance in fish via this route of exposure.

The CONTAM Panel concluded that the recent information on toxicity of endosulfan in fish does not change the conclusions on toxicity in fish drawn in its previous opinion. The CONTAM Panel

recommended to perform further studies on oral toxicity in fish, in order to clarify the possible existence of fish species with higher sensitivity to endosulfan. In addition, dose response data are needed at low doses (i.e. in the region of the maximum level in feed) for fish species different from Atlantic salmon.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

On the request of the European Commission, the Scientific Panel on Contaminants in the Food Chain (CONTAM) adopted a scientific opinion on endosulfan as undesirable substance in animal feed on 20 June 2005.[4]

As regards the toxicity of endosulfan for fish, the CONTAM Panel concluded that 'Fish show high sensitivity to endosulfan exposure via water. Oral exposure studies have shown effects on thyroxin level and thyroid hormone metabolism at dietary concentration of 100 µg/kg (Nile tilapia), and ultra structural alterations of the liver and intestinal tract at dietary concentration of 0.5 µg/kg (common carp). These effects were subtle, possibly adaptive, and not considered to represent adverse effects'.

The CONTAM Panel recommended that 'Studies on carry-over, accumulation and oral toxicity of endosulfan, especially in farmed fish and laying hens, should be performed.'

In the meantime, new scientific information on the toxicity of endosulfan for fish via the diet have become available to the European Commission. Examples are:

- Petri et al. (2006). Sensitivity of Atlantic salmon (*Salmo salar*) to dietary endosulfan as assessed by haematology, blood biochemistry, and growth parameters. Aquatic Toxicology 80 (2006) 207-216.

- Glover et al. (2007). Assessing the sensitivity of Atlantic salmon (*Salmo salar*) to dietary endosulfan exposure using tissue biochemistry and histology. Aquatic Toxicology 84 (2007) 346-355.

- Berntssen et al. (2008). Accumulation and elimination kinetics of dietary endosulfan in Atlantic salmon (*Salmo salar)*. Aquatic Toxicology 86 (2008) 104-111.

- Lundebye et al. (2010). Tolerance of Atlantic salmon (*Salmo Salar*) to diet borne endosulfan assessed by haematology, biochemistry, histology and growth (in press).

- Berntssen et al. (2010). Assessing sensitivity of Atlantic salmon post smolt to dietary endosulfan using histology and markers of endocrine disruption, oxidative stress, and biotransformation.

## TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Art. 29 (1) of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority to consider to update the Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the European Commission related to endosulfan as undesirable substance in animal feed as regards the toxicity of endosulfan for fish, taking into account new scientific information which has become available since the previous assessment.

[4] Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the European Commission related to endosulfan as undesirable substance in animal feed (Question n° EFSA-Q-2003-066), adopted on 20 June 2005. EFSA Journal (2005) 234, 1-31. http://www.efsa.europa.eu/en/scdocs/doc/234.pdf

## EVALUATION

### 1. Introduction

Endosulfan is an organochlorine pesticide patented in the mid 1950s. Technical grade endosulfan is mainly composed of a mixture of two biologically active stereoisomers named α-endosulfan and β-endosulfan in a ratio of 70 % to 30 % respectively. As minor impurities technical grade endosulfan may also contain up to 2 % endosulfan alcohol and 1 % endosulfan ether.

Endosulfan was developed as a non-systemic insecticide with contact action, generally used in control of damage caused by aphids, ticks, mites and other insects on a broad range of crops and non crop vegetation such as cereals, maize, sorghum, oilseed crops, fruit, vegetables, olives, potatoes, cotton, tea, coffee, or ornamental plants. However, because of its potential for long-range transport, environmental persistence, bioconcentration and ecotoxicity, endosulfan is banned or restricted in many countries all over the world. Following a European Commission Decision 2005/864/EC, endosulfan is not included in the list of authorised active ingredients for crop protection in the European Union.

Endosulfan is mainly released into the environment as a consequence of its use as a pesticide. Its environmental persistence and its long-range environmental transport have resulted in it becoming a ubiquitous contaminant of atmosphere, soils, sediments, fresh and marine water worldwide.

Endosulfan exhibits toxicity towards mammalian and other organisms and bioconcentrates in various aquatic species. However, in contrast to other organochlorine pesticides, endosulfan has a less pronounced affinity to lipids, thus biomagnification in the terrestrial food chain is unlikely to occur.

In 2005 the EFSA's Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) adopted an opinion on endosulfan as an undesirable substance in animal feed (EFSA, 2005). In 2006 this opinion was revised in order to take into account two additional studies on fish exposed to endosulfan in feed that had become available to the CONTAM Panel. The opinion pointed out the high sensitivity of fish to endosulfan exposure through water, but also a relative lack of information regarding the toxicity of dietary exposure. At the time of the opinion revision, only two papers on dietary toxicity were available and they reported effects that were considered 'subtle, possibly adaptive and not considered to represent adverse effects'. In particular, effects on thyroid hormone metabolism were observed in Nile tilapia exposed to a dietary concentration of 0.1 mg/kg of endosulfan (Coimbra et al., 2005), and ultra structural alterations of the liver and the gut were observed in common carp at a dietary concentration of 0.0005 mg/kg (Braunbeck and Appelbaum, 1999). A specific recommendation was made by the CONTAM Panel to perform studies on oral toxicity in fish.

EFSA was asked by the European Commission to update the Opinion of the Scientific Panel on Contaminants in the Food Chain related to endosulfan as an undesirable substance in animal feed as regards the toxicity in fish, taking into account new scientific information which had become available since the previous assessment.

### 2. Legislation

Because of its toxicity and environmental impact endosulfan is banned or restricted in many countries.

In the European Union, endosulfan is not included in the list of authorised active substances in Annex I to Directive 91/414/EEC by Commission Decision 2005/864/EC,[5] because of the environmental fate, eco-toxicological profile and the operator's exposure risk. Consequently, all uses of endosulfan as plant protection product have been prohibited since 2005, with the exception of certain limited essential uses in some Member States for a limited period of time which has now expired.

Maximum residue levels for endosulfan are established by Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC.

However, these maximum residue levels are without prejudice to the maximum levels established for endosulfan by Directive 2002/32/EC[6] (see Table 1).

**Table 1:** Maximum levels of endosulfan in feed established by Directive 2002/32/EC.

| Undesirable substance | Products intended for animal feed | Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12 % |
|---|---|---|
| Endosulfan (sum of α- and β-isomers and of endosulfan sulphate expressed as endosulfan) | Feed materials and compound feed with the exception of: | 0.1 |
| | - maize and maize products derived from the processing thereof | 0.2 |
| | - oilseeds and products derived from the processing thereof, except crude vegetable oil | 0.5 |
| | - crude vegetable oil | 1.0 |
| | - complete feed for fish | 0.005 |

The maximum level of endosulfan in feed for fish is 0.005 mg/kg.

## 3. Assessment of recent scientific information on the toxicity of endosulfan in fish

A literature search was performed to retrieve scientific publications regarding endosulfan toxicity in fish. The search was limited to published papers starting from 2005, the year in which EFSA adopted the scientific opinion on endosulfan as an undesirable substance in animal feed, until December 2010. The literature search was performed on several databases (ISI Web of Knowledge, PubMed and Google Scholar). A more focused search was carried out with a greater emphasis on toxicity of endosulfan in fish exposed via feed. The assessment of the scientific information relevant to toxicity of endosulfan via feed and via water is summarised in the following sections.

### 3.1. Toxicity of endosulfan in fish by the oral route

The literature search identified one relevant publication in addition to the five identified by the European Commission. Repeated exposure to endosulfan via feed was studied in Atlantic salmon and Nile tilapia at concentrations ranging between 0.001 and 1 mg/kg feed.

[5] OJ L 317, 3.12.2005, 25-28.
[6] OJ L 140, 30.5.2002, 10-21.

Atlantic salmon (*Salmo salar*)

Petri et al. (2006) treated pre-smolt Atlantic salmon with diet containing 0, 0.005, 0.05 and 0.5 mg/kg technical endosulfan for 49 days in fibreglass tanks (water flow-through rate of 12 L/min, uneaten feed and faeces were collected during the experiment). Measured endosulfan concentrations were 0, 0.004, 0.050 and 0.710 mg/kg feed for the 0, 0.005, 0.05 and 0.5 nominal concentration groups, respectively. Two further groups were administered 0.033 mg/kg and 0.017 mg/kg of α- and β-endosulfan in feed, respectively (measured concentrations were 0.030 and 0.018 mg/kg feed for the groups exposed to the α- and β-isomer, respectively). Fish were fed twice a day with an approximate feeding rate of 1.5 % of body weight. Daily doses were estimated to be 60, 758 and 10,621 ng/kg fish/day for the 0.005, 0.05, 0.5 mg/kg technical endosulfan treatment group, respectively, and 279 and 441 ng/kg fish/day for the α- and β-isomer treatment groups, respectively. On day 0, 14, 35 and 49 of the treatment period fish were sampled, and weight and body length were recorded. Blood samples were taken for haematology and biochemistry analyses and liver and spleen were weighed. A blood smear to record morphological parameters for white blood cells was prepared from the blood of 3 fish/group on day 35; endosulfan concentration in the liver was determined at the end of the treatment period. Adverse effects of minor severity were observed only at the highest concentration tested of 0.5 mg/kg technical endosulfan. These adverse effects included a slight but statistically significant reduction in the body condition factor (weight/length$^3$) in comparison to the control group. Haematology showed statistically significant increases in haemoglobin, haematocrit and mean corpuscular haemoglobin after 35 days, but not after 49 days of exposure, in comparison to controls. Morphological analysis of white blood cells did not reveal any consistent changes, although morphological alterations in the majority of the observed lymphocytes were recorded in blood smears from two out of three fish sampled from the 0.5 mg/kg group. Endosulfan was detected in the liver of fish exposed to 0.05 mg/kg and to 0.5 mg/kg endosulfan, and to 0.033 mg/kg of α-isomer, but not to 0.005 mg/kg endosulfan, 0.017 mg/kg of β-isomer, or to the control diet. Endosulfan in the water samples, analysed weekly during the study period, resulted always below the detection limit of 0.01 µg/L.

Additional examinations of the fish from the Petri et al. (2006) study were performed and reported by Glover et al. (2007). At each sampling time (0, 14 and 35 days) 12 fish/group were sampled and hepatic glutathione peroxidise (GPx) activity, intestinal and branchial sodium/potassium adenosine triphosphatase ($Na^+$, $K^+$-ATPase) activities and the hepatic α-tocopherol levels were measured. Hepatic glutathione-S-transferase (GST) and 7-ethoxyresorufin-O-deethylase (EROD) activities were measured on day 14 and 35 in 12 fish from the control group and from the highest exposure group only. Histopathology of liver and of mid and hind intestine was performed on six fish from each treatment group at 0, 14 and 35 days of exposure. Similarly to the previous study, statistically significant changes were observed mainly in the 0.5 mg/kg endosulfan group: increased hepatic EROD activity and decreased $Na^+$, $K^+$-ATPase activity in the intestine were observed at 35 days of exposure. Decreased $Na^+$, $K^+$-ATPase activity was recorded also in the gill at 14 days of exposure at 0.5 mg/kg, but full recovery was noted at 35 days. Liver histopathology showed changes possibly related to an increased metabolic demand, such as depletion of glycogen stores coupled with lipidosis at all the tested doses. Signs of atrophy and necrosis in liver cells were found in the mid dose endosulfan group (0.05 mg/kg feed) and in the α-isomer and β-isomer groups, but not in fish treated at 0.5 mg/kg endosulfan in feed. Histological examination revealed also injuries to the intestinal mucosa (vacuolation and fusion of intestinal villi), mainly limited to the hind gut in all treatment groups in comparison with the controls. Although the most severe effects in the intestinal mucosa were observed in five of the six fish examined from the 0.5 mg/kg endosulfan group, no clear dose- and time-relationships were observed in the incidence of changes in the hind gut.

Post-smolt Atlantic salmon kept in saline water (salinity of 3.3 – 3.5 %) in fibreglass tanks (15 L/min of water flow rate) were exposed to 0, 0.005, 0.05 and 1 mg/kg technical endosulfan in the diet for 16 consecutive weeks by Berntssen et al. (2010). Fish received feed continuously via automatic feeders at an approximate rate of 0.7 % of body weight. After 0, 4, 8 and 16 weeks of exposure, fish were sampled and weight and length after terminal anaesthesia were recorded. Blood was taken for

haematological and biochemical analyses, including the determination of vitellogenin and sex-binding protein levels. Liver and spleen weights were recorded. Hepatic EROD activity, GPx activity and α-tocopherol levels were determined and histological examinations were performed on liver, spleen and intestine. No effects on mortality, feeding behaviour, weight, condition factor and spleen somatic index (SSI)[7] were observed. A reduced hepatic somatic index (HSI)[8] was observed at the highest dose at the end of the experiment. EROD activity was increased at all dose levels after 16 weeks of exposure in comparison with controls, achieving statistical significance at 0.005 and 0.05, but not at1 mg/kg. No changes were observed among treatment and control groups in vitellogenin concentration, sex steroid binding capacity of plasma, hepatic glutathione peroxidase activity and hepatic α-tocopherol levels. Haematological and plasma biochemical parameters were not statistically different between the control and treatment groups. In the endosulfan-exposed fish occasional liver cells with lipidosis, nuclear atrophy or condensed nuclei were noticed. The hind gut of endosulfan-exposed fish showed histological changes such as vacuolization on the tips of the villi, occasional fusion of the villi and erosion of the underlying areolar tissue. Changes were observed at all concentrations, but no dose-dependence could be established in the incidence and severity of these findings. Spleen showed a significant increase in the red pulp in the 0.005 and 0.05 mg/kg endosulfan groups, and the incidence of granular lipofuscin-like deposits was significantly higher in the two highest endosulfan treatment groups compared to controls. However, these histological changes in spleen did not cause any haematological disturbances, since red blood cell count, haemoglobin and haematocrit in endosulfan-treated fish were not different from controls. According to the authors, the hepatic and splenic changes were considered of minor severity in view of the absence of adverse changes in plasma and haematological analyses.

Lundebye et al. (2010) studied the toxicity of endosulfan via the diet in post-smolt Atlantic salmon exposed for 95 days under realistic farming conditions. The fish were distributed among six sea cages and treated with nominal concentrations of 0, 0.005, 0.05 and 0.1 mg/kg technical endosulfan in feed (actual concentrations: 0, 0.00566, 0.04740 and 0.10400 mg/kg). Fish were hand-fed till satiation twice a day and feed intake was recorded for each sea cage. The feed administered both to the control and the treatment groups was coated with oil containing 1 g/kg yttrium, used as a tracer for the performance of digestibility measurements. Daily doses of 28.8, 239.3 and 528.6 ng/kg fish/day were estimated for the 0.005, 0.05 and 0.1 mg/kg treatment groups, respectively. On day 0, 40, 73 and 95 of the exposure period, 24 fish/group were sampled. At each sampling time, weight, length and sex were recorded and blood samples were taken for haematology and blood chemistry analyses; liver and spleen were subsequently collected and weighed. Liver, spleen, mid- and hind-intestine sections were prepared for histopathological examinations. At the end of the treatment period, faeces of all remaining fish from each cage were collected, pooled and examined for yttrium, total protein, glycogen and lipid. There were no mortalities during the treatment period and no statistically significant changes were observed in weight, length, condition factor, HSI or SSI at any sampling time among the treatment and control groups. With the exception of a statistically significant, transient decrease in erythrocyte count observed at 0.005 and 0.05 mg/kg, but not at 0.1 mg/kg, and a statistically significant increase in plasma protein at 0.05 mg/kg observed on day 40 only, no changes were observed in haematology and blood chemistry analyses. Lipid digestibility was found to be statistically significantly reduced at 0.1 mg/kg in comparison to 0.005 mg/kg. However, no differences among treatment and control groups were observed in nutritional indices such as feed conversion ratio, specific growth rate, lipid efficiency ratio, and lipid production value. Histopathology examinations did not reveal any treatment related changes in liver, spleen and intestine, in contrast with previous studies in Atlantic salmon (Glover et al., 2007; Berntssen et al., 2010).

Nile tilapia (*Oreochromis niloticus*)

Coimbra et al. (2007) studied the impact of high and low dietary doses of endosulfan in adult Nile tilapia. Two different experiments were carried out. In the first experiment fish were administered a

[7] SSI = (spleen weight/b.w.) × 100
[8] HSI = (liver weight/b.w.) × 100

diet containing 0, 0.5 or 1 mg/kg technical endosulfan for 21 days. Due to the severe liver lesions observed in the first experiment, a second experiment was carried out with larger (by total weight and length) fish exposed to 0, 0.001 or 0.1 mg/kg endosulfan for 35 days. Fish were maintained in 70 litre tanks at a low flow rate (3 L/h) and fed daily with an approximate feeding rate of 2 % of body weight (b.w.). At the end of experiments, fish were anesthetised before sacrificing. Total weight and length were determined, blood was collected from the caudal vein for plasma analysis and liver was weighed. Liver samples were subsequently prepared for histological examination and determination of EROD activity. No changes in mortality, feeding behaviour, weight, length and condition factor were observed in either experiment among treatment and control groups. In the first experiment HSI was statistically significantly decreased at 1 mg/kg endosulfan in comparison to controls. In the second experiment no statistically significant difference between the exposure groups and the control group was observed, but a significantly lower HSI was observed in the 0.001 mg/kg group in comparison to the 0.1 mg/kg group. In both experiments all fish exposed to endosulfan presented at least one type of liver alteration, such as hepatocyte damage and vacuolisation, rupture of blood vessel endothelium, increased eosinophilic granular cell number and melanomacrophage aggregation. No abnormalities were observed in the control Nile tilapia. According to the authors, liver lesions appeared to be dose-related, with the most severe damage induced by the highest endosulfan concentration. The CONTAM Panel noted however that no quantitative information on the incidence of morphological changes in the liver is reported in the publication. In the first experiment, endosulfan-exposed fish did not exhibit altered EROD activity, while in the second experiment EROD was significantly increased in the 0.001 mg/kg group only. The thyroid hormone concentrations in fish in the first experiment were not altered, while in the second experiment fish exposed to 0.1 mg/kg of endosulfan showed lower thyroxine (T4) plasma concentrations than control Nile tilapia.

In conclusion, the studies on Atlantic salmon indicate only minor adverse effects in the intestine of fish exposed to 0.005 mg/kg of endosulfan in feed in exposure tanks, whereas no significant adverse effects were observed in fish exposed up to 0.1 mg/kg endosulfan in feed in open-sea cages. On the other hand, the study in Nile tilapia showed morphological changes in the liver at 0.001 and 0.01 mg/kg endosulfan in feed, increasing in severity at 0.5 and 1 mg/kg endosulfan in feed. It should be noted that in the experiments with the Nile tilapia a lower flow rate of water in the tank was used and possible contamination of the water with endosulfan from the diet or faeces was not controlled.

### 3.2. Toxicokinetic studies of endosulfan in fish following oral administration

Berntssen et al. (2008) studied the uptake and elimination of α- and β-endosulfan in post-smolt Atlantic salmon. Fish were exposed to a mixture of 0.7 mg/kg α-endosulfan and 0.3 mg/kg β-endosulfan in feed for 92 days and then maintained for an additional 56 day period. A concurrent control group was included in the test. On day 0, 2, 8, 15, 29, 57 and 92 of the treatment period, and 0, 3, 7, 14, 28 and 56 of the post-treatment period, fish were sampled, anaesthetised, and weight and body length were recorded. Fish were filleted, analysed for liver weight and for content of α- and β-endosulfan and endosulfan sulphate in the fish fillet. Both α- and β-endosulfan rapidly accumulated in the fillet with a final concentration, measured at the end of the exposure period, of 65±5 and 50±8 µg/kg for α- and β-isomers, respectively. No steady state concentration was achieved during the treatment period. In contrast, endosulfan sulphate levels were found to reach a steady state concentration from 28 days of the treatment period onwards, amounting to about 1 % of the total endosulfan level at the end of the treatment period. About 90 % and 75 % of the accumulated levels of α- and β-endosulfan, respectively, were rapidly eliminated during the 56 days of post-treatment, whereas endosulfan sulphate levels remained unchanged during the treatment and post-treatment period. A statistically significantly higher uptake and lower elimination rate were observed for the β-isomer in comparison to the α-isomer. The calculated biomagnification factor (BMF) was 0.05 ± 0.003 and 0.10 ± 0.026 for the α- and β-isomer, respectively.

### 3.3. Toxicity of endosulfan in fish through water

The acute toxicity of endosulfan through water was studied in several species. Lethality was generally observed to occur at concentrations between 0.4 and 8 µg/L. Values for the 96-hr $LC_{50}$ determined in different species are reported in Table 2. Capkin et al. (2006) found that survival of the fish was significantly increased with increasing fish size and that water temperature and alkalinity can also influence the resistance to endosulfan.

Sub-lethal effects following acute exposure included abnormal behavioural responses, impaired enzymatic activities, and changes in biochemical parameters and histopathological lesions in the gills, liver and brain. Siang et al. (2007) noted abnormal behaviours such as imbalanced position, restlessness of movement, erratic swimming, tremor, flashing[9] and lethargy. Similarly, Ballesteros et al. (2009a) recorded a decreased swimming mobility in *J. multidentata* exposed to sub-lethal concentrations of 0.072-1.4 µg/L endosulfan in a commercial formulation containing 35 % of the active substance.

Activity of several enzymes was impaired by acute exposure to sub-lethal concentrations of endosulfan. Acetylcholinesterase (AchE) activity was found to decrease in axial muscles but not in the brain in *J. multidentata* exposed at 0.144 - 1.4 µg/L (Ballesteros et al., 2009a). The CONTAM Panel noted that endosulfan is not an AchE inhibitor and no dose-response relationship was observed in the Ballestreros et al. (2009a) study. Conversely, Sarma et al. (2010) observed a decreased AchE activity in the brain, together with decreased adenosine triphosphatase activity, decreased vitamin C levels and increased glucose levels in *C. punctatus* exposed to 8.1 µg/L of a technical grade of endosulfan. Ballesteros et al. (2009b) observed a statistically significant decrease in GST activity in gills, muscle, intestine and liver following 24 hour exposure to endosulfan. In the same study GST activity was increased in the brain, and increased activity of glutathione reductase (GR) and GPx was observed both in the brain and gills. The reduced enzymatic activity observed by Ballesteros et al. (2009b) was accompanied by an increased level of lipid peroxidation in the brain and in the liver, suggesting that oxidative stress could be responsible for endosulfan toxicity.

Following acute exposure, haematological analyses showed a statistically significant, concentration-dependent decrease in the phagocytic index and in the fraction of active phagocytic cells in *O.niloticus* exposed to 4 and 7 µg/L endosulfan in a commercial formulation in comparison to controls (Girón-Pérez et al., 2008) and a significant decrease in the erythrocyte count, leukocyte count, haemoglobin and haematocrit in *M. albus* when exposed to ≥ 0.05 µg/L endosulfan in a commercial formulation containing 33 % of the active substance (Siang et al., 2007).

Acute exposure to endosulfan caused histological lesions in the gills (lifting and cell hypertrophy of the epithelium of the secondary lamellae) and liver (hydropic degeneration and dilatation of sinusoids and focal areas of necrosis with leukocyte infiltration) in *J. multidentata* exposed to 0.45, 0.76, 1.26 and 2.1 µg/L of a commercial formulation of endosulfan (Ballesteros et al., 2007). Sarma et al. (2010) observed areas of mild necrosis in the apical lobe of the cerebrum and focal areas of gliosis in *C. punctatus* exposed to 8.1 µg/L endosulfan.

Repeated exposure to endosulfan through the water was studied in several species. Thangavel et al. (2010) exposed freshwater teleosts (*Sarotherodon mossambicus*) to a commercial formulation of endosulfan (36 %) for five days at a concentration of 1.0 µg/L endosulfan to study the possible effects

[9] The term flashing describes an erratic swimming behaviour, commonly considered as a sign of irritation or discomfort.

**Table 2:** 96-hr $LC_{50}$ in fish exposed to endosulfan through the water.

| **Species** | **Test item** | **Fish weight mean ± SD (g)** | **$LC_{50}$ value (µg/L)** | **95 % Confidence limits** | **Reference** |
|---|---|---|---|---|---|
| Rio de la Plata onesided livebearer (*Jenynsia multidentata*) | Commercial formulation (35 % endosulfan) | Males: 0.58 ± 0.21<br>Females: 1.12 ± 0.5 | Males: 0.7<br>Females: 1.3 | 0.5-0.9<br>0.7-1.9 | Ballesteros et al., 2007 |
| Rainbow trout (*Oncorhynchus mykiss*) | Commercial formulation (32.9 % endosulfan) | 10.61 ± 1.69 | 1.75 | 1.58-1.96 | Capkin et al., 2006 |
| Asian swamp eel (*Monopterus albus*) | Commercial formulation (33 % endosulfan) | 150-180[a] | 0.42 | 0.35-0.50 | Siang et al., 2007 |
| Common carp (*Cyprinus carpio*) | n.a. | n.a. | 2 | n.a. | Salvo et al., 2008 |
| Striped dwarf catfish (*Mystus vittatus*) | Commercial formulation (35 % endosulfan) | 15.20 ± 4.11 | 2 | n.a. | Sharma et al., 2007 |
| Spotted snakehead (*Channa punctatus*) | Commercial formulation (35 % endosulfan) | 12 - 25 [a] | 7.75 | 5.59-10.74 | Pandey et al., 2006 |

n.a: not available
[a]range

on hormonal levels. Several transient changes were observed in the group exposed to endosulfan in comparison to controls. In particular, a statistically significant decrease in triiodothyronine (T3) and T4 levels was observed after 12 hours of exposure. Serum cortisol levels were significantly decreased after 1, 6 and 12 hours. Prolactin was found to significantly increase at the 12 hr-measurement only. Finally, a large increase was observed in insulin levels achieving a peak after 12 hours. All changes disappeared after 24 hours or 5 days.

After 15 days of exposure of carps (*Cyprinus carpio*) to 1 µg/L endosulfan, Salvo et al. (2008) observed a decrease in liver weight and in relative liver weight, together with histological changes in the liver (proliferation of endoplasmic reticulum and glycogen depletion). At 1 µg/L endosulfan did not affect the cholinesterase activity in the brain and axial muscle in this study.

Juveniles of *Labeo rohita* were exposed for 15 days to a concentration of 0.25 µg/L of endosulfan following a static bioassay method with 24 h renewable water medium. NADPH cytochrome c reductase (NCCR) was analysed. A statistically significant increase in the NCCR activity was observed in liver tissues of fish exposed to endosulfan in comparison to controls (Ramaneswari and Rao, 2008). The CONTAM Panel noted that the NCCR increase was observed in a single dose study, it was of modest magnitude and of borderline significance.

Altinok and Capkin (2007) exposed rainbow trout (*Oncorhyncus mykiss*) to 0, 0.6 and 1.3 µg/L endosulfan (as a commercial formulation containing 33 % of the active substance) in water for 21 days. Histological lesions were observed in gills (lifting of lamellar epithelium; epithelial necrosis, hypertrophy and hyperplasia; fusion of lamellae and presence of eosinophilic material), liver (hepatocyte necrosis, hypertrophy and vacuolisation), spleen (presence of melanomacrophage centres and necrosis of white pulp) and kidney (enlarged sinusoids; necrosis of haematopoietic tissue, glomerular and tubular cells) of fish exposed at both concentrations of endosulfan and examined at the end of the exposure period.

Carp (*Labeo rohita*) were exposed to 0 or 10 µg/L endosulfan for 50 days by Saravanan et al. (2010). At the end of the exposure period, statistically significant changes in blood chemistry (increased glucose, cholesterol, amino acids levels; increased alanine aminotranferase (ALT) and aspartate aminotransferase (AST) activity; increased T3 and decreased thyroid stimulating hormone (TSH) levels) were observed in fish exposed to endosulfan in comparison to controls. Biochemical changes consistent with blood chemistry results were observed in the liver. Histopathological analysis showed lesions in the gills (erosion and vacuolation of primary and secondary lamellae) and in the liver (hepatocyte enlargement, vacuolisation and necrosis) in fish exposed to endosulfan in comparison to controls.

The Comet assay was applied to evaluate the genotoxicity of endosulfan in fish. In one study, Pandey et al. (2006) exposed freshwater teleosts (*Channa punctatus*) to a commercial formulation containing 35 % endosulfan at concentrations ranging from 4 to 12 µg/L for 96 hours and DNA strand breaks were measured in gills and kidney by means of the Comet assay. In the exposed fish, the level of DNA breaks statistically significantly increased in a concentration-related fashion in the gill tissues. A similar trend, although with lower magnitude and less clear concentration-effect relationship, was observed in the kidney.

Genotoxicity was also evaluated by the Comet assay by Sharma et al. (2007) in gill, kidney and blood samples of *Mystus vittatus* fish exposed for 43 days to 0.2, 0.25 and 0.5 µg/L endosulfan in a commercial formulation containing 35 % of the active substance. Fish were sampled on a weekly basis and analysed for DNA strand breaks in the selected tissues. Statistically significant increases in the level of DNA breaks were observed for the 3 concentrations in all tissues and time points, with the highest effects observed on day 1 of exposure.

The CONTAM Panel noted that the observed increase in DNA strand breaks could be associated to oxidative stress or to cytotoxicity and not be the result of a direct genotoxic action of endosulfan.

Possible effects of endosulfan on reproduction and development were assessed in bluegill fish and zebrafish. Male bluegill fish (*Lepomis macrochirus*) were exposed to 1.0 µg/L endosulfan up to 2 weeks and the effects on testes were studied after 24, 48, 72, 96 hours, and 1 and 2 weeks of exposure (Dutta et al., 2006). Morphological alterations and damage of seminiferous tubules and changes in cell populations were observed with severity increasing with the time of exposure. In particular, a progressive degeneration of seminiferous tubules with complete disruption after 1-2 weeks was observed. The diameter of primary spermatogonia statistically significantly decreased as from 24 hours of exposure. Leydig cell, spermatids and spermatozoa appeared fewer in number during the exposure period, together with signs of damage in the Sertoli cells.

Balasubramani and Pandian (2008) exposed zebrafish (*Danio rerio*) to 0.044, 0.088, 0.175, 0.350, 0.700, 1.050 and 1.400 µg/L endosulfan (commercial formulation containing 35 % of the active substance) for a cumulative period of 14 hours (2 hours/day on days 18 to 25 of the post-hatching period). Mortality at the end of treatments ranged between 10 and 47 % in fish exposed to endosulfan. The surviving fry were counted and then allowed to grow with *ad libitum* feeding. Endosulfan was found to depress growth in all the treatment groups in comparison to controls, with statistical significance achieved on day 240$^{th}$ post-hatching for both sexes in all the treatment groups. The sexual maturity was delayed from the 120$^{th}$ to the 181$^{st}$ and 129$^{th}$ days post-hatching in females and males exposed to endosulfan, respectively. The inter-spawning and inter-milting periods were prolonged from 7 to 24 and from 4 to 24 days, respectively. The process of egg maturation, vitellogenesis, sperm motility, fecundity and fertizability were also impaired by endosulfan. In view of the observed high mortality, the CONTAM Panel noted that the observed developmental effects could have been indirect.

Overall, the new studies on endosulfan toxicity in fish exposed via the water confirmed the information reported in the previous EFSA opinion (2005). In particular, the main observations following acute and repeated exposure to sub-lethal concentrations of endosulfan were adverse changes in gills and liver.

### 3.4. Comparison of study results and applied exposure conditions

The data indicate that liver toxicity is more pronounced in Nile tilapia compared to Atlantic salmon when administered comparable concentrations of endosulfan in feed for similar periods of time. In Atlantic salmon, the main effect appears to be vacuolation and fusion of the posterior intestinal villi observed at 0.005 mg/kg both after 49 days and 16 weeks of exposure in tanks, although no clear dose-response relationship was observed in either study (Glover et al., 2007; Berntssen et al., 2010). No histological changes in the intestine or other adverse effects were observed in Atlantic salmon exposed for 95 days to up to 0.1 mg/kg endosulfan via feed in open sea cages. Whilst the hypothesis that different sensitivities exist between fish species, e.g. between Atlantic salmon and other fish species such as Nile tilapia or common carp, cannot be discounted, the different experimental designs applied could also explain the differences compiled in Table 3.

It is worthwhile noting the large flow rate differences between the Coimbra et al. (2007) study and the Petri et al. (2006) study and subsequent studies in Atlantic salmon. Furthermore, the comparison of the estimated external doses from the Petri et al. (2006) and Lundebye et al. (2010) studies, as reported in Table 4, clearly shows that, beside the administered concentrations, water flow and feeding conditions can heavily influence the total exposure doses (i.e. through feed and water). As no exposure dose estimation is reported in recent publications on Nile tilapia (Coimbra et al., 2005, 2007), no direct comparison can be made with the studies in Atlantic salmon. On the other hand, Braunbeck and Appelbaum (1999) reported an estimated dose of 15 ng/kg b.w./day for common carp fed a diet

**Table 3:** Summary of statistically significant changes ($p<0.05$) observed in fish studies with dietary exposure to endosulfan.[a]

| Species | Days of exposure | Concentration in feed (mg/kg) | | | | | | References |
|---|---|---|---|---|---|---|---|---|
| | | **0.001** | **0.005** | **0.05** | **0.1** | **0.5** | **1** | |
| Nile tilapia | 21 | n.a. | n.a. | n.a. | n.a. | Severe liver damage (hepatic damage and vacuolisation, blood vessel rupture, inflammation). | Severe liver damage (hepatic damage and vacuolisation, blood vessel rupture, inflammation). | Coimbra et al., 2007 |
| | 35 | Liver morphological changes.[b] ↑ hepatic EROD activity. | n.a | n.a. | Liver morphological changes.[b] ↓ T4 plasma levels. | n.a. | n.a. | Coimbra et al., 2007 |
| Atlantic salmon | 35 | n.a. | - | - | n.a. | Morphological alterations of lymphocytes in blood. ↑ hepatic EROD activity after 35 days of exposure. ↓ $Na^+$, $K^+$-ATPase activity in the intestine. | n.a. | Petri et al., 2006 Glover et al., 2007 |

**Table 3:** Continued.

| Species | Days of exposure | Concentration in feed (mg/kg) | | | | | | References |
|---|---|---|---|---|---|---|---|---|
| | | 0.001 | 0.005 | 0.05 | 0.1 | 0.5 | 1 | |
| Atalntic salmon | 49 | n.a. | Glycogen store depletion coupled with lipidosis. Vacuolation and fusion of intestinal villi in hind gut. | Glycogen store depletion coupled with lipidosis. Signs of atrophy and necrosis in liver cells. Vacuolation and fusion of intestinal villi in hind gut. | n.a. | Slightly decreased condition factor. Glycogen store depletion coupled with lipidosis. Vacuolation and fusion of intestinal villi in the hind gut (higher severity). | n.a. | Petri et al., 2006<br>Glover et al., 2007 |
| | 95[(c)] | n.a. | - | - | ↓ lipid digestibility compared to 0.005 mg/kg but not to control group. | n.a. | n.a. | Lundebye et al., 2010 |
| | 112 (16 weeks) | n.a. | ↑ hepatic EROD. Hind gut villi vacuolisation and fusion. ↑ red pulp in spleen | ↑ hepatic EROD. Hind gut villi vacuolisation and fusion. ↑ red pulp in spleen | n.a. | n.a. | ↓ HSI Hind gut villi vacuolisation and fusion. | Berntssen et al., 2010 |

[(a)] Transient effects not reported in the table.
[(b)] Lower severity compared to fish exposed to 0.5 and 1 mg/kg.
[(c)] Study carried out in sea cages.
n.a.: not available. Concentration not tested at the specified exposure duration.

**Table 4:** Exposure doses (expressed as ng/kg fish/day) as estimated by Petri et al. (2006) and Lundebye et al. (2010) in Atlantic salmon and by Braunbeck and Appelbaum (1999) in common carps.

| Concentration in feed (mg/kg) | 0.0005 | 0.005 | 0.05 | 0.1 | 0.5 | 0.033[(a)] | 0.017[(b)] |
|---|---|---|---|---|---|---|---|
| Petri et al. (2006) | n.a. | 60 | 758 | n.a. | 10,621 | 279 | 441 |
| Lundebye et al. (2010) | n.a. | 28.8 | 239.3 | 528.6 | n.a. | n.a. | n.a. |
| Braunbeck and Appelbaum (1999) | 15 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |

[(a)] α-endosulfan
[(b)] β-endosulfan
n.a.: not available

containing a nominal concentration of 0.0005 mg/kg endosulfan. In addition, to the high water flow rates, several precautions were undertaken in the studies in Atlantic salmon to limit the leakage of endosulfan into the water. Namely, a second coat of oil was sprayed on the feed pellets and uneaten feed and faeces were routinely removed from the tanks. On the other hand, no precautions to limit the exposure to endosulfan through the water were taken in the Nile tilapia studies (Coimbra et al., 2005, 2007), suggesting that this pathway could be responsible for the observed effects. However, this hypothesis can neither be confirmed nor ruled out since endosulfan concentrations in water were not monitored in the studies in Nile tilapia. An overview of the different experimental conditions that could have influenced the study results is reported in Table 5.

The studies on endosulfan exposure through the water confirmed the high toxicity of the substance in fish via this route of exposure. Due to differences in toxicity related to route of exposure, data from exposure via water cannot be used directly in the evaluation of dietary exposure to endosulfan.

**Table 5:** Experimental conditions applied in fish studies with dietary exposure to endosulfan.

| Parameter | Petri et al. (2006) Glover et al. (2007) | Berntssen et al. (2010) | Lundebye et al. (2010) | Coimbra et al. (2007) |
|---|---|---|---|---|
| Species | Atlantic salmon, pre smolt | Atlantic salmon, post smolt | Atlantic salmon, post smolt | Nile tilapia |
| Weight at the start of the study (g) | 46.6 ± 7.9 | 255 ± 54 | 245 ± 22 | 1st experiment: 97.6 ± 37.0<br>2nd experiment: 134.8 ± 43.8 |
| Exposure conditions | Glass fibre tanks (510 L), ~150 fish/tank | Glass fibre tanks (500 L), 60 fish/tank | Open sea-cages (125 $m^3$), 125 fish/cage | Tanks (70 L). 6 fish/tank |
| Feeding method and rate | Automatic feeders, | Automatic feeders, | Hand feeding till satiation | n.a. |
| Feeding rate | 1.5 % of b.w./day | 0.7 % of b.w./day | n.a. | 2 % of b.w./day |
| Water | n.a. (likely freshwater) | Saline | Sea water | Dechlorinated tap water |
| Salinity (g/L) | n.a. | 33 – 35 | 30.0-34.2 | n.a. |
| Water temperature (°C) | 10-12 | 7-10 | 4.6-8.4 | 20 |
| Dissolved oxygen (mg/L) | ≥ 8 mg/L | 8 – 10[(a)] | 8.7-12.2 mg/L | n.a. |
| Water flow (L/min) | 12 | ~15 | - | 3 |
| Substance purity (%) | 99.8 | 99.8 | 99.8 | 95 |

[(a)] Estimated from oxygen saturation values of 70-84 % reported by Berntssen et al. (2010) by assuming a standard atmospheric pressure of 760 mm Hg.
n.a: not available; b.w.: body weight.

## CONCLUSIONS AND RECOMMENDATION

### CONCLUSIONS

- The Panel on Contaminants in the Food Chain of the European Food Safety Authority (CONTAM Panel) examined the studies on oral toxicity of endosulfan in fish published after the adoption of its Opinion on Endosulfan as Undesirable Substance in Animal Feed in 2005 and 2006.

- Endosulfan is highly toxic to fish when exposed via water.

- Endosulfan toxicity in fish exposed via feed was mainly studied in Atlantic salmon. In this species, endosulfan caused minor adverse effects in the intestine following 49 or 112 days of exposure to ≥ 0.005 mg/kg endosulfan via feed in tanks, although no clear dose-effect relationship was observed in either study. In open sea-cages, no toxicity was observed up to 0.1 mg/kg endosulfan in Atlantic salmon.

- From a limited study, there are some indications that exposure of Nile tilapia to endosulfan via feed in tanks caused morphological changes in liver tissue already at 0.001 mg/kg, increasing in severity at higher doses.

- The CONTAM Panel concluded that it is not possible to establish whether the apparent differences in toxicity between Atlantic salmon and Nile tilapia are the result of a difference in sensitivity between fish species or are due to the different experimental conditions.

- Although the new available data give further evidence on the toxicity of endosulfan in fish, no firm conclusion can be drawn in view of the limitations of the available studies. The CONTAM Panel concluded that the recent information on toxicity of endosulfan in fish does not change the conclusions on toxicity in fish drawn in its previous opinion.

### RECOMMENDATION

- The CONTAM Panel recommended to perform further studies on oral toxicity in fish, in order to clarify the possible existence of fish species with higher sensitivity to endosulfan. The studies should be designed to assure that the experimental conditions are as similar as possible to the farming conditions and to include the evaluation of the possible exposure of fish to endosulfan via the water caused by the leakage from the feed. In addition, dose response data are needed at low doses (i.e. in the region of the maximum level in feed) for fish species different from Atlantic salmon.

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## ABBREVIATIONS

| | |
|---|---|
| AchE | acetylcholinesterase |
| ALT | alanine aminotranferase |
| AST | aspartate aminotransferase |
| BMF | biomagnification factor |
| CONTAM Panel | EFSA Panel on Contaminants in the Food Chain |
| EFSA | European Food Safety Authority |
| EROD | 7-ethoxyresorufin-O-deethylase |
| GPx | glutathione peroxidase |
| GR | glutathione reductase |
| GST | glutathione-S-transferase |
| HSI | hepatic somatic index |
| $Na^+$, $K^+$-ATPase | Sodium/potassium adenosine triphosphatase |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NCCR | NADPH cytochrome c reductase |
| SSI | spleen somatic index |
| T3 | triiodothyronine |
| T4 | thyroxine |
| TSH | thyroid stimulating hormone |