



# Faglig sluttrapport for prosjektet «Safe use of the antioxidant ethoxyquin» (FHF prosjekt nr 901327)

## Sammendrag

Ethoxyquin (EQ) er et førtilsetningsstoff som brukes som antioksidant i fôr. Alle tilsetningsstoffer må reautoriseres jevnlig i EU. I en risikovurdering fra 2016 uttalte det europeiske mattrykksorganet EFSA at det var mangel på kunnskap for å kunne konkludere sikkert med at EQ var trygt for husdyr, konsumenten og miljøet. Dette prosjektet har bidratt til å fremskaffe kunnskap som kan svare opp på noen av kunnskapshullene som ble avdekket i EFSA's risikovurdering av EQ.

Formålet med prosjektet var å:

- 1) fremskaffe kunnskap om EQ trygt kan brukes som førtilsetningsstoff mhp fiskens helse
- 2) undersøke hvor mye EQ og nedbrytningsprodukter av EQ som overføres fra fôr til filet
- 3) undersøke eventuell risiko for konsumenten ved inntak av nedbrytningsproduktet ethoxyquin dimer; EQDM

Alle stoffer er giftige ved høye nok doser. For at et tilsetningsstoff skal være tillatt å bruke må man vite hvor mye man kan tilsette uten at det er fare for helseskader. For å undersøke toleransen for EQ hos laks ble det gjort et 90 dagers fôringsforsøk der fisken fikk ulike doser av ethoxyquin i fôret. Ved hjelp av avanserte screeningmetoder ble det lett etter effekter hos fisken. Resultatene viste at fôr tilsatt nivåer av ethoxyquin som var innenfor tidligere fastsatte grenseverdier på 150 mg/kg ikke hadde noen negativ effekt på fiskehelsen. Ved veldig høye doser fikk imidlertid fisken tegn på blant annet oksidativt stress.

Ethoxyquin overføres fra fôret til fiskefileten, der den omdannes til andre forbindelser. Ved hjelp av nye analyseverktøy ble det kartlagt hvilke forbindelser EQ kan omdannes til i fiskefileten. Mange ulike EQ forbindelser ble funnet og disse var et resultat av kjemiske endringer slik som oksygenering, kløyving, konjugering og dimerisering. Den viktigste forbindelsen var en ethoxyquindimer (EQDM), som er en forbindelse laget av to EQ-molekyler.

For å kunne forutsi hvor mye EQ og EQDM man kan finne i filet basert på hvor mye som finnes i fôret ble det utviklet en matematisk modell. Denne modellen viste at EQ forsvinner raskt fra fileten fordi den kjemisk omdannes til andre stoffer, mens EQDM har en lang halveringstid og forsvinner langsomt fra laksefileten.

I fiskefileten omdannes EQ altså hovedsakelig til EQDM. For å undersøke om denne forbindelsen kan utgjøre en risiko for konsumenten ble det gjort et forsøk med mus som modell for mennesker, der musene fikk en syntetisk, ren form av EQDM i maten.

Ved hjelp av avanserte screeningmetoder ble det lett etter effekter hos musen. Etter noen måneder ble det funnet effekter som oksidativt stress og forstyrrelser i fettmetabolismen som kan være et forstadium til fettlever. Musen som ble brukt i forsøket er spesielt sensitive mot uønskede stoffer, og er derfor regnet som en god modell for slike studier. Ved høye doser ble

det funnet tegn på oksidativt stress og forstyrrelser i fettmetabolismen som kan være et forstadium til fettlever.

Det er foreløpig ikke etablert et akseptabelt daglig inntak (ADI) for ethoxyquindimer. Akseptabelt daglig inntak beskriver mengden stoff et menneske kan få i seg daglig gjennom livet uten fare for negative helseeffekter. Ved hjelp av matematiske modeller kunne dataene fra denne studien brukes til å beregne den laveste dosen av stoffet der en effekt i mus kan påvises. Disse resultatene kan brukes til å fastsette ADI for mennesker.

## Summary

Ethoxyquin (EQ) is a synthetic antioxidant added to fishmeal in order to prevent oxidation during transport. In the current reauthorization process of all feed additives in Europe, EFSA's evaluation on EQ was inconclusive due to lack of data on the safety of ethoxyquin as a feed additive for any target animals, its safety for consumers or the environment.

The current project has contributed to cover some of the knowledge gaps on salmon identified by EFSA in their recent risk assessment on EQ by studying:

- 1) the safety of use of the additive in Atlantic salmon
- 2) residue studies on the presence and permanence of EQ and its metabolites in edible tissues
- 3) the risks to the consumer of food derived from animals given feedingstuffs containing the additive or its metabolites

A well known concept in toxicology is that the “dose makes the poison”. For authorization of a feed additive, knowledge on how much can be added without risk of health consequences is of central importance. To investigate the tolerance of EQ for salmon, a sub-chronic oral toxicity study was performed where Atlantic salmon received graded levels of EQ in fish feed for 90 days. The fish were screened for markers of effect with subsequent targeted analyses of relevant biomarkers of toxicity. The results showed that doses within the previous legal limit of 150 mg/kg did not cause any negative effects on fish health. However, with high doses, the fish showed sign of oxidative stress.

EQ is transferred from feed to the fish fillet where it is transformed into other compounds. An investigation of which transformation products that were found in salmon fillet was performed with state-of-the-art analytical tools. Many different compounds were found as a result of oxygenation, cleavage, conjugation and dimerization. The most significant compound was an ethoxyquin dimer (EQDM), which is a compound made by two EQ molecules.

In order to predict the permanence of the EQ residues in the tissues, a physiologically based kinetic model was established. The model showed that EQ was quickly eliminated from the fillet through transformation into other compounds while EQDM had a long half-life and disappeared slowly from the fillet.

The use of EQ in fish feed results in residues of this compound and its metabolites in fish muscle, mainly the EQDM. In order to investigate whether EQDM can pose a risk to the consumer, a sub-chronic repeated dose oral toxicity study on EQDM using the BalbC mouse strain was performed. BalbC is an inbred mouse strain sensitive to contaminants and frequently used in toxicological evaluation. The mice were screened for markers of effect with subsequent targeted analyses of relevant biomarkers of toxicity. The results showed that at high doses, signs of oxidative stress and disturbances in the lipid metabolism indicative of an early stage of fatty liver could be seen. So far, no acceptable daily intake (ADI) has been established for EQDM.

The acceptable daily intake describes the amount of a compound a human can ingest throughout a lifetime without risk of negative health effects. By using mathematical models the data from this study were used to calculate the lowest dose at which an effect in mice could be seen. These results can be used to establish an ADI for humans.

## Introduction

Storage of animal feed may lead to lipid peroxidation through auto-oxidation resulting in feed rancidity and reduced shelf life of the product. EQ is one of a number of authorised technological feed additives that are added to fishmeal in order to prevent it from becoming rancid and in order to prevent it from igniting during sea transport and storage. Other areas of use is fish silage, and in vitamin and pigment premixes. However, the use of EQ in feed results in residues of this compound and its metabolites in e.g. fish muscle. EQ has therefore received media attention and remains a controversial issue in the public debate. EQ is currently under reauthorisation in the European Union and faces a possibility of not being allowed for use in animal feed. The consequences of a potential withdrawal of the authorisation have been outlined by the EU Association of Specialty Feed Ingredients and their Mixtures (FEFANA) in a recent position paper<sup>1</sup>:

- *Lack of availability of important micronutrients currently being supplied via compound feed to food-producing and companion animals, thereby affecting animal health and welfare;*
- *Financial risk and uncertainty due to the loss of a cost-effective supply of safe quality feed additives to aquaculture and livestock production systems;*
- *Reduced sensory quality (colour) of food products (e.g. eggs and salmon), thereby decreasing market value of the products, and potentially diminishing the economic viability of poultry and fish farms; and*
- *Negative impact on the overall trade of food and feed products from and to the EU*

## Aims

In the current reauthorization process of all feed additives in Europe, EFSA's evaluation on EQ<sup>2</sup> was inconclusive due to lack of data on the safety of ethoxyquin as a feed additive for any target animals, its safety for consumers or the environment. The documentation needed when preparing technical dossiers for authorisation of a feed additive are specified in regulation EC 429/2008<sup>3</sup>. Our project aimed to cover some of the knowledge gaps on salmon identified by EFSA in their recent risk assessment on EQ by studying:

- 1) the safety of use of the additive in Atlantic salmon
- 2) residue studies on the presence and permanence of EQ and its metabolites in edible tissues

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<sup>1</sup> Position Paper: Ethoxyquin and the potential impacts of its withdrawal on availability of nutritional, sensory and other feed additives used in animal feed in the European Union. FEFANA

<sup>2</sup> <http://www.efsa.europa.eu/en/press/news/151118>

<sup>3</sup> <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:133:0001:0065:en:PDF>

3) the risks to the consumer of food derived from animals given feedingstuffs containing the additive or its metabolites

## **Project organization**

The project was organized as a project group consisting of researchers from NIFES and a project reference group consisting of representatives from the Norwegian Seafood Federation, the salmon feed companies (EWOS, Biomar, Skretting, Europharma and Marine Harvest) and the Marine Ingredients Organisation (IFFO). Meetings were held with the project reference group at NIFES for the start-up meeting, and as telephone conferences every six months and as a final meeting discussing the main findings.

## **Results and discussion**

In order to investigate possible risks of EQ for fish health, the present study investigated the toxic mode of action of EQ in a 90 day subchronic dietary exposure study at doses ranging from 0.47- 9666 mg EQ/kg feed in Atlantic salmon (*Salmo salar* L.) as a target species. Feed at concentrations higher than 1173 mg EQ/ kg were rejected by the fish, resulting in reduced feed intake and growth performance. However, no overt toxicity was observed in fish exposed to any of the doses. A multi-omic screening of metabolome and proteome in salmon liver indicated an effect of dietary EQ on bioenergetics pathways and hepatic redox homeostasis in fish fed concentrations above 118.8 mg EQ/kg feed. Increased energy expenditure associated with an activation of hepatic fatty acid  $\beta$ -oxidation and induction of carbohydrate catabolic pathways, resulted in a dose-dependent depletion of intracytoplasmic lipid vacuoles in liver histological sections, decreasing whole body lipid levels and altered purine and pyrimidine metabolism. Increased GSH and TBARS in the liver indicated a state of oxidative stress, which was associated with activation of the Nrf2-mediated oxidative stress response and glutathione-mediated detoxification processes. However, no oxidative DNA damage was observed. As manifestation of altered energy metabolism, the depletion of liver intracytoplasmic lipid vacuoles was considered the critical endpoint for benchmark dose assessment and a BMDL10 of 243 mg EQ/kg feed was established for derivation of a safe upper limit of EQ exposure in Atlantic salmon.

Only few studies have reported on the EQ uptake and elimination kinetics in farmed fish, and kinetic information on EQDM is lacking or incomplete. A study aiming to assess the uptake and elimination of EQ and EQDM from the muscle of Atlantic salmon fed EQ in a feeding experiment followed by an extended (~ 3months) depuration period was therefore performed. The estimated half-life for EQ was approximately 8 days, while the estimated half-life of EQDM was 71-96 days. Thus, EQ will be rapidly eliminated from the fillet through a combination of elimination and conversion to other transformation products, while EQDM will be slowly eliminated and transformed requiring a period exceeding 3-6 months to obtain EQDM free fish. Furthermore, a simple one-compartment fish biomagnification model was established to predict levels of EQ and EQDM in fish fillets based on feed levels. The model was validated and showed a good predictive ability. However, more work is required to elaborate on this model and future feed-to-fillet transfer assessment will be based on physiological based toxicokinetic (PBK) multi-compartmental models.

The metabolism of EQ in salmon was investigated using UHPLC-TWIMS-QTOFMS in the salmon described in the tolerance study along with some samples of commercially available salmon. This revealed the occurrence of a range of EQ transformation products (TPs). The most abundant of them was the dimer 1,8'-EQDM identified along with another dimer, TP-435C. The overall relative abundances of dimers were in the range 57-97 %. In salmon from the feeding trial, the 1,8-EQDM contributed with 99% while in farmed salmon the 1,8-EQDM contributed with 80-95% of all dimers. The remaining TPs resulted from oxygenation, cleavage combined with oxygenation, or other alterations of EQ. In some of the samples of commercial salmon, oxygenation in addition to cleavage combined with oxygenation, constituted a relatively larger signal than the dimers. Although ethoxyquin dimers were the most predominant TPs as measured by relative abundance, the data should be treated with caution until standards for the other TPs become available in order to measure their occurrence quantitatively.

To investigate the toxicity and dose-response of dietary exposure to EQDM, male BALB/c mice were exposed to one of six dietary doses of EQDM, ranging from 0.1-500 mg/kg body weight/day for 90 days. Doses above 10 mg/kg body weight/day affected whole body lipid metabolism resulting in increased liver weights and decreased adipose tissue mass. Metabolic screening of livers revealed alterations in hepatic lipid profiles and hepatic redox homeostasis, indicating incomplete fatty acid  $\beta$ -oxidation and hepatic oxidative stress. Histopathological evaluation of the liver and biochemical analyses confirmed the development of microvesicular steatosis and activation of the glutathione system. Hepatic protein profiling and pathway analyses indicated a state of mitochondrial dysfunction and disruption of mitochondrial respiration, and indicated further that EQDM-induced responses are likely mediated through activation of CAR/PXR nuclear receptors and induction of a Nrf2-mediated oxidative stress response. The lowest BMDL10 (1.1 mg EQDM/kg BW/day) was established for the development of microvesicular steatosis as the critical endpoint of the study, from which an ADI of 0.011 mg/kg BW was suggested for dietary exposure to EQDM.

## Main findings

- The mode of action for EQ and a dose for the tolerance of EQ to Atlantic salmon was suggested
- The conversion of EQ into other transformation products in salmon fillet was described
- The permanence of EQ and its main metabolite EQDM in salmon fillet was described
- The mode of action for EQDM and an acceptable daily intake for EQDM in humans was suggested using BalbC mice as a surrogate model

## Deliverables

- Minutes from meetings in the project reference group.
- Administrative final report
- Scientific summary report (this report)
- Final report consisting of several manuscripts

Manuscripts delivered as part of the final report:

Tolerance of dietary ethoxyquin in Atlantic salmon (*Salmo salar* L.)

Annette Bernhard, Josef D. Rasinger, Monica B. Betancor, Maria Jose Caballero, Marc H.G. 4 Berntssen, Anne-Katrine Lundebye and Robin Ørnsrud

Uptake and elimination of dietary ethoxyquin and its main metabolite, ethoxyquin dimer, in fillet of Atlantic salmon (*Salmon salar* L.)

Berntssen, M.H.G., Bernhard, A., Lundebye, A.K., Ørnsrud, R.

Identification of ethoxyquin metabolites in salmon exposed via fish feed

Sylvain Merel, Jorge Regueiro, Marc H.G. Berntssen, Rita Hannisdal, Robin Ørnsrud and Noelia Negreira

Effects of subchronic dietary exposure to ethoxyquin dimer on hepatic lipid metabolism in male BALB/c mice

Annette Bernhard, Josef D. Rasinger, Helene Wisløff, Lene S. Myrmel, Marc H.G. Berntssen, Anne-Katrine Lundebye, Robin Ørnsrud and Lise Madsen

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Robin Ørnsrud, project leader