# SCIENTIFIC OPINION

# Safety and efficacy of a feed additive consisting of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) for all animal species (FEFANA asbl)

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## Abstract

Ethoxyquin is synthetised from p-phenetidine, a possible mutagen, which remains in the additive as an impurity at concentrations of < 2.5 mg/kg additive. Ethoxyquin is considered safe for all animal species at the proposed inclusion level of 50 mg/kg complete feed. However, owing the presence of p-phenetidine, no safe level of the additive in feed for long-living and reproductive animals could be identified. The FEEDAP Panel derived a health-based guidance value of 0.006 mg ethoxyquin dimer (EQDM)/kg bw per day and applied it to the sum of ethoxyquin and its transformation products. A maximum total concentration of 50 mg ethoxyquin/kg complete feed for all animal species, except dairy ruminants, would not pose a risk for the consumer. However, in the absence of data on p-phenetidine residues in tissues and products of animal origin, no conclusion on the safety for the consumer could be drawn. The conclusions on consumer safety assume that the maximum total concentration of 50 mg EQ/kg feed is expressed as the sum of EQ, EQDM, EQI and DHEQ. Exposure of the unprotected user to p-phenetidine via inhalation should be minimised. No safety concerns for groundwater are expected. It is not possible to conclude on the safety of EQ for the terrestrial compartment. A risk for the aquatic compartment cannot be excluded when ethoxyquin is used in terrestrial animals. Unacceptable risk is not expected for freshwater sediment-dwelling organisms. A risk of secondary poisoning via the terrestrial food chain is not expected, whereas a risk via the aquatic food chain cannot be excluded. No concerns for aquatic organisms are expected for ethoxyquin used in fish farmed in land-based system, a risk cannot be excluded for marine sediment dwelling organisms when ethoxyquin is used in sea-cages. Ethoxyquin is considered efficacious in the range 25–50 mg/kg complete feed.

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## Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on ethoxyquin for all animal species.

Ethoxyquin (EQ) is synthetised from p-phenetidine, a recognised possible mutagen, which remains in the additive as an impurity, at concentrations of $< 2.5$ mg/kg additive.

As a conclusion from tolerance studies, EQ at the proposed inclusion level of 50 mg/kg complete feed could be considered safe for chickens for fattening, laying hens, piglets, cattle for fattening and salmon. This conclusion could be extrapolated to all animal species and categories with the exception of cats, for which the Panel cannot conclude on a safe level. However, considering that the additive contains p-phenetidine, a possible mutagen, the Panel cannot conclude on the safety of EQ at any level for long-living and reproductive animals.

The FEEDAP Panel derived a health-based guidance value (HBGV) of 0.006 mg ethoxyquin dimer (EQDM)/kg body weight (bw) per day applying an uncertainty factor of 200 (taking into account the additional UF of 2 for extrapolation from subchronic to chronic study duration in rodents) to the benchmark dose (BMD) lower confidence limit for a benchmark response of 10% ($BMDL_{10}$) of 1.1 mg EQDM/kg bw per day for microvesicular steatosis in liver of mice, and applied this HBGV for EQDM for the sum of EQ and its transformation products TPs (i.e. ethoxyquin dimer (EQDM), ethoxyquin quinone imine (EQI) and dihydro ethoxyquin (DHEQ) and metabolite A).

Based on the European consumption data and residues in tissues and eggs (milk data not available) of food-producing animals, the exposure of the consumer to EQ, EQDM, EQI, DHEQ and metabolite A would not exceed 80% of the HBGV. Therefore, the use of EQ at a maximum total concentration of 50 mg/kg in complete feed for all animal species, except dairy animals, would not result in residues of EQ or its metabolites/transformation products which would pose a risk for the consumer. In the absence of residue data in milk, the Panel cannot conclude on the safety of EQ when used in feed for milk-producing animals. Owing the presence in the additive of p-phenetidine, and in the absence of data on the residues of p-phenetidine in tissues and products of animal origin, no conclusion on the safety of the consumer could be drawn. The conclusions on consumer safety are based on the assumption that the maximum total concentration of 50 mg EQ/kg feed is expressed as the sum of EQ, EQDM, EQI and DHEQ.

Exposure of the unprotected user to p-phenetidine via inhalation cannot be excluded. The Panel concludes that, to reduce the risk, the exposure of the users should be minimised.

Regarding the safety for the environment, no safety concerns for groundwater are expected. Ecotoxicity data on three additional terrestrial plants would be needed to conclude on the safety of EQ for the terrestrial compartment. A risk for the aquatic compartment cannot be excluded when the additive is used in terrestrial animals. Unacceptable risk is not expected for freshwater sediment-dwelling organisms. Ethoxyquin is considered not to pose a risk of secondary poisoning via the terrestrial food chain, whereas a risk for secondary poisoning via the aquatic food chain cannot be excluded. Ethoxyquin, when used in fish farmed in land-based system, is considered not to raise safety concerns for aquatic organisms. A risk cannot be excluded for ethoxyquin used in sea-cages for marine sediment-dwelling organisms.

Ethoxyquin is considered an efficacious antioxidant in the range of 25–50 mg/kg complete feed.

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# 1. Introduction

## 1.1. Background and Terms of Reference

Ethoxyquin is a technological additive belonging to the functional group of antioxidants.

In 2015 the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) adopted an opinion on the safety and efficacy of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) for all animal species (EFSA FEEDAP Panel, 2015) in which it was concluded that:

"Ethoxyquin itself is not genotoxic or carcinogenic and does not cause developmental toxicity. The lowest NOAEL (based on studies in rats and dogs) is 2 mg/kg body weight per day. The genotoxic profile of the dimer reflects that of ethoxyquin. Ethoxyquin quinone imine shows structural alerts for mutagenicity, carcinogenicity and DNA binding; no conclusion on the absence of genotoxicity of ethoxyquin quinone imine is possible. p-Phenetidine is a recognised possible mutagen. Concentrations of 50 mg ethoxyquin/kg and 11 mg ethoxyquin/kg complete feed might be considered as potentially safe for chickens and breeders and for dogs, respectively. No conclusion on potential safe levels for other poultry, pigs, ruminants, fish and cats is possible. Overall, when considering the presence of p-phenetidine in the additive, no conclusion on any safe level of the additive for target animals can be drawn. An assessment of safety for the consumer is prevented by the lack of exposure data, the absence of a safe level of exposure and the presence of p-phenetidine in ethoxyquin. [...] No conclusion on the safety for the environment can be made. Ethoxyquin is a potent antioxidant; however, no data confirm its efficacy at the proposed use level."

The European Food Safety Authority (EFSA) received four mandates from the European Commission to complete the assessment of the safety and efficacy of ethoxyquin, based on the additional data provided by the applicant in four different submissions. The four mandates are reported below.

**First mandate (EFSA-Q-2016-00267)**

Regulation (EC) No 1831/2003[1] establishes rules governing the authorisation of additives for use in animal nutrition and, in particular, Article 9 defines the terms of such authorisation by the Commission.

The applicant, ANTOXIAC EEIG/FEFANA asbl, is seeking an authorisation of ethoxyquin to be used as a feed additive (Table 1).

**Table 1:** Description of the substance

| Category of additive | Technological additives |
|---|---|
| Functional group of additive | Antioxidants |
| Description | Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) |
| Target animal category | All animal species |
| Applicant | ANTOXIAC EEIG, FEFANA asbl (EU Feed Additives & Premixture Association) |
| Type of request | New opinion |

On 11 March 2016, the Commission and the European Food Safety Authority ("Authority") have received from the applicant the "final report of the *Micronucleus test in Bone Marrow cells of the mouse with 2,2,4-trimethylquinolin-6-one*".

In the view of the above, the Commission requests the authority to assess the additional data submitted by the applicant in order to deliver a new opinion on the safety and efficacy of ethoxyquin as a feed additive under the conditions of Regulation (EC) No 1831/2003.

**Second mandate (EFSA-Q-2018-00013)**

Regulation (EC) No 1831/2003 establishes rules governing the authorisation of additives for use in animal nutrition and, in particular, Article 9 defines the terms of such authorisation by the Commission.

In accordance with article 10(2) of Regulation (EC) No 1831/2003 in conjunction with article 7 thereof, an application was submitted on 21 September 2010 for the authorisation of ethoxyquin as a

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[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

feed additive for all animal species, requesting the additive to be classified in the category "technological additives". That application was accompanied by the particulars and documents required under Article 7(3) of Regulation (EC) No 1831/2003 (Table 2).

**Table 2:** Description of the substance

| Category of additive | Technological additives |
|---|---|
| Functional group of additive | Antioxidants |
| Description | Ethoxyquin |
| Target animal category | All animal species |
| Applicant | FEFANA asbl |
| Type of request | New opinion |

On 21 October 2015, the Panel on Additives and Products or Substances used in Animal Feed of the European Food Safety Authority ("Authority"), stated that the assessment of the particulars and documents submitted by the applicant makes it impossible to conclude on the safety of the additive ethoxyquin for any target animals, for consumers and for the environment. This is due to an overall lack of data submitted to assess the exposure and the safety of ethoxyquin for the animals, consumers and the environment. In particular, no conclusion is possible on the absence of genotoxicity of one of the metabolites of the additive ethoxyquin, ethoxyquin quinone imine. In addition, p-phenetidine, an impurity of the additive ethoxyquin, is recognised as a possible mutagen. The Authority considered the additive ethoxyquin as a potent antioxidant in feed but efficacy at the proposed use level, which has been reduced as compared with the currently authorised maximum content in feed, could not be confirmed by the submitted data. The authority also verified the report of the method of analysis of the feed additive in feed submitted by the reference Laboratory set up by Regulation (EC) No 1831/2003.

According to the Commission Implementing Regulation suspending the authorisation of ethoxyquin as a feed additive for all animal species and categories the applicant committed itself to produce supplementary data according to a time schedule listing in order of priority the studies to be carried out successively and planning that the outcome of the last of them would be available by July 2018. The set of new data have been sent to the Commission and EFSA on 15 December 2017.

In view of the above, the Commission asks the Authority to deliver a new opinion for ethoxyquin to be used as a technological additive for all animal species based on the additional data submitted by the applicant.

**Third mandate (EFSA-Q-2018-00372)**

Regulation (EC) No 1831/2003 establishes rules governing the authorisation of additives for use in animal nutrition and, in particular, Article 9 defines the terms of such authorisation by the Commission.

The applicant, FEFANA asbl, is seeking a Community authorisation of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) as a feed additive to be used as an antioxidant for all animal species (Table 3).

**Table 3:** Description of the substance

| Category of additive | Technological additives |
|---|---|
| Functional group of additive | Antioxidants |
| Description | Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) |
| Target animal category | All animal species |
| Applicant | FEFANA ASBL |
| Type of request | New opinion |

On 21 October 2015, the Panel on Additives and Products or Substances used in Animal Feed of the European Food safety Authority ("Authority"), stated in its opinion that the assessment of the particulars and documents submitted by the applicant makes it impossible to conclude on the safety of the additive ethoxyquin for any target animals, for consumers and for the environment. This is due to an overall lack of data submitted to assess the exposure and the safety of ethoxyquin for the animals, consumers and the environment. In particular, no conclusion is possible on the absence of genotoxicity

of one of the metabolites of the additive ethoxyquin, ethoxyquin quinone imine. In addition, p-phenetidine, an impurity of the additive ethoxyquin, is recognised as a possible mutagen.

The Commission gave the possibility to the applicant to submit complementary information in order to complete the assessment and to allow a revision of the Authority's opinion. The new data have been received on 20 April 2018.

In view of the above, the Commission asks the Authority to deliver a new opinion on ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) as a feed additive for all animal species based on the additional data submitted by the applicant.

**Fourth mandate (EFSA-Q-2021-00523)**

Regulation (EC) No 1831/2003 establishes rules governing the Community authorisation of additives for animal nutrition and, in particular, Article 9 defines the terms of the authorisation by the Commission.

In accordance with Article 10(2) of Regulation (EC) No 1831/2003 in conjunction with Article 7 thereof, an application was submitted on 21 September 2010 for the authorisation of ethoxyquin as a feed additive for all animal species, requesting the additive to be classified in the category "technological additives". That application was accompanied by the particulars and documents required under Article 7(3) of Regulation (EC) No 1831/2003. (Table 4).

**Table 4:** Description of the substance

| Category of additive | Technological additives |
|---|---|
| Functional group of additive | Antioxidants |
| Description | Ethoxyquin |
| Target animal category | all animal species |
| Applicant | FEFANA ASBL |
| Type of request | New opinion |

On 21 October 2015, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA), in its opinion on the safety and efficacy of the product could not conclude on the safety and efficacy of the additive.

Under Regulations (EU) 2017/962 and 2021/412, the Commission gave the possibility to the applicant to submit supplementary information and data in order to complete the assessment and to allow a revision of the EFSA's opinion. The new data have been received on 23 June 2021 and sent directly to EFSA by the applicant.

In view of the above, the Commission asks EFSA to deliver a new opinion on ethoxyquin as a feed additive for all animal species based on the supplementary data submitted by the applicant, in accordance with Article 29(1)(a) of Regulation (EC) No 178/2002.

## 1.2. Additional information

The additive ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) was authorised in the EU for use as a technological additive (functional group: antioxidants) in all animal species and categories until 7 June 2017.[2] Ethoxyquin was also authorised in the EU as pesticide until 2009, when the authorisation for its use was withdrawn.[3]

It is a legal requirement of the United Nations International Maritime Organisation (IMO) that 'Stabilization of fishmeal shall be achieved to prevent spontaneous combustion by effective application: of between 400 and 1,000 mg/kg ethoxyquin or liquid BHT (butylated hydroxy toluene); or between 1,000 and 4,000 mg/kg BHT in powder form at the time of production' (IMO, 2014) and that: 'fish scrap of fish meal shall contain at least 100 ppm of antioxidant (ethoxyquin) at the time of consignment' (UN, 2014).

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[2] Commission Implementing Regulation (Eu) 2017/962 of 7 June 2017 suspending the authorisation of ethoxyquin as a feed additive for all animal species and categories OJ L 145, 8.6.2017, p. 13.

[3] Commission Decision 2008/941/EC of 8 December 2008 concerning the non-inclusion of certain active substances in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing these substances. OJ L 335, 13.12.2008, p. 91.

The Scientific Committee on Animal Nutrition (SCAN) issued an opinion on the safety of ethoxyquin for dogs (EC, 1993). In 2015, the FEEDAP Panel adopted an opinion on the safety and efficacy of ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) for all animal species (EFSA FEEDAP Panel, 2015).

The Scientific Committee for Food (SCF) issued an opinion on the safety of ethoxyquin used for the treatment of scald in apples and pears (EC, 1975). The Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment (JMPR) has delivered several opinions on the safety of ethoxyquin (FAO, 1969, 1998, 2005). EFSA issued a conclusion on the peer review of the pesticide risk assessment of ethoxyquin (EFSA, 2010) and a reasoned opinion on the review of the existing maximum residue levels (MRLs) for ethoxyquin (EFSA, 2013).

## 2. Data and methodologies

### 2.1. Data

The present assessment is based on data submitted by the applicant in the form of additional information[4] to a previous application of the same product.[5]

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers and other scientific reports to deliver the present output.

### 2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of ethoxyquin is in line with the principles laid down in Regulation (EC) No 429/2008[6] and the relevant guidance documents: Guidance on technological additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a, revised in 2009), Guidance for the preparation of dossiers for the re-evaluation of certain additives already authorised under Directive 70/524/EEC (EFSA, 2008b, revised in 2009), Guidance for the preparation of dossiers for additives already authorised for use in food (EFSA FEEDAP Panel, 2012b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012c), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012d), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

## 3. Assessment

Ethoxyquin (EQ) is intended to be used as a technological additive (functional group: antioxidants). In its previous opinion (EFSA FEEDAP Panel, 2015), the FEEDAP Panel could not conclude on the safety of the additive for the target animals, the consumer and the environment and on its efficacy. The applicant submitted additional information in order to allow a conclusion on the safety and efficacy of EQ to be reached. In this context, the applicant modified the specifications of the additive, in particular by reducing the content of p-phenetidine.

### 3.1. Characterisation

The additive ethoxyquin is synthetised by the reaction of p-phenetidine and acetone. The starting material p-phenetidine, a recognised possible mutagen, remains in the additive as an impurity.

In the previous applications, the additive was specified as '> 91% EQ, not more 8% of ethoxyquin polymers, ≤ 3% p-phenetidine and ≤ 0.02% acetone'. In the current submission, the applicant has modified the specifications to reduce the content of p-phenetidine.

The applicant, with the aim to better characterise the additive, performed a ring trial comparing different methods of analysis (titration, gas chromatography with flame ionisation detection (GC-FID) or high-performance liquid chromatography with ultraviolet detection (HPLC-UV)) and analysing

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[4] FEED dossier references: FAD-2016-0018, FAD-2017-0073, FAD-2018-0029, EFSA-Q-2021-00527.

[5] FEED dossier reference: FAD-2010-0141.

[6] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

different batches of ethoxyquin from three producers (three batches each).[7] Based on the results of the ring trial, the applicant proposed the following specifications for the additive ethoxyquin:

Ethoxyquin > 96% w/w (determined by titration); ethoxyquin monomer > 92% determined by (GC-FID); ethoxyquin-related substances < 7% (GC-FID)[8]; p-phenetidine < 2.5 mg/kg (GC-FID, corresponding to 0.00025%); ethoxyquin quinone imine < 0.09% (GC-FID).

The analysis of six pilot batches of the additive prepared by two producers (three batches each) was analysed using the above-described methods.[9] The results showed that the pilot batches of the additive comply with the newly proposed specifications; in particular, the content of p-phenetidine was on average 1.7 mg/kg (range: 0.5–2.3 mg/kg).

### 3.1.1. Stability

Antioxidants are by essence unstable chemicals degraded progressively during feed protection and converted by oxidation to other chemical structures (transformation products (TPs)), sometimes endowed of antioxidative properties also (e.g. 1,8'-ethoxyquin dimer (1,8'-EQDM, further referred as EQDM), ethoxyquin quinone imine (EQI) and dihydro ethoxyquin (DHEQ)). The genesis of TPs in feeds is highly variable, depending on many physical and chemical parameters associated with the composition and to the technological treatments of the feed. The more sensitive analytical methods used in the newly submitted studies, with an increased capacity of structural elucidation, enlarge to a considerable extent the spectrum of EQ TPs formerly described (EFSA FEEDAP Panel, 2015). The structural formulas of EQ and its main TPs described in the stability studies is reported in Figure 1. The applicant provided two stability studies, also aiming to identify EQ TPs in premixtures and compound feed.
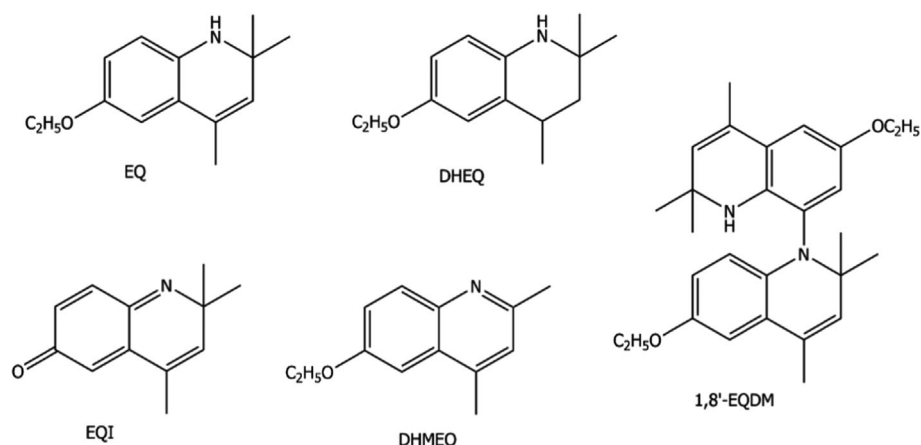


**Figure 1:** Structural formula of ethoxyquin (EQ) and the main transformation products (TPs) identified in in feed and tissues of target species: dihydro ethoxyquin (DHEQ), ethoxyquin quinone imine (EQI), (DHMEQ) and 1,8'-ethoxyquine dimer (1,8'-EQDM)

**EQ persistence in premixtures**

One batch of a vitamin/mineral premixture was supplemented with 5% EQ, one from each of the three producers. The samples were kept at 25°C, 60% relative humidity (RH) or at 45°C, 75% RH for 90 days.[10] EQ and related degradation products EQI, DHMEQ and EQDM were analysed.[11] About 50% EQ disappeared after 90 days at 25°C. A similar decline occurred already after 8 days at 40°C. The sum of EQ and related degradation products amounted to about 85% and 25% of the initial EQ content after 90 days at 25°C and 40°C, respectively. The results were qualitatively and quantitatively similar for the three sources of EQ.

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[7] FAD-2017-0073/Technical dossier/Annex 01.
[8] The sum of eight ethoxyquin related impurities (including EQDM).
[9] FAD-2017-0073/Technical dossier/Annex 06.
[10] FAD-2017-0073/Technical dossier/Annex 07.
[11] By HPLC-UV/Vis with detector set at 220, 249 and 375 nm, using their corresponding relative response factors.

**EQ persistence in feed for chickens for fattening**

A study of the stability of EQ and TPs was performed with a complete feed for chickens for fattening (based on corn and soybean meal) supplemented with 50, 150 or 500 mg EQ/kg, kept under ambient temperature and humidity (not specified) or at 40°C, 75% RH for 3 months.[12] EQ degradation rates were similar for the three supplementation levels. EQ losses after 90 days were about 30–50% under ambient conditions and about 80% at 40°C.

**Characterisation of EQ transformation products**

In the two stability studies described above, EQ TPs were identified after forced degradation (under basic, acidic or oxidative ($H_2O_2$) conditions). In the vitamin mineral premixture, EQI was the major TP in basic conditions, EQDM being also detected; chemical oxidation accelerated the degradation process with the genesis of the oxygenated species EQI-oxide(1) and EQI-oxide(2) plus EQDM and another dimer. In the compound feed (oxidative ($H_2O_2$) conditions only), EQ was rapidly oxidised (75% spontaneously, 83% after 4 h) but only around 56% of the lost EQ was identified as DMEQ, EQI and EQDM. These primary TPs were further oxidised to minor and numerous compounds (89 chromatographic peaks partly separated). The forced degradation of the same amount of ^{14}C-EQ correlated well with the above analysis.

Negreira et al. (2017) studied the fate of EQ during the storage of fish feed. EQ oxidation under forced oxidations (by $H_2O_2$ or 2,2-diphenyl-1-picrylhydrazyl (DPPH)) resulted in 37 EQ TPs, which could be grouped into six classes of compounds according to their formation mechanism: dimerisation (12 compounds), dimerisation or other alteration (four compounds), oxygenation (six compounds), cleavage and oxygenation (nine compounds), cleavage (three compounds) and other alterations (three compounds). In the same publication, 18 samples of Atlantic salmon complete feed were collected from different feed factories in Norway in 2015. EQ was found in 17 samples, and EQDM was present in all analysed samples; DHMEQ and two other TPs were present in at least 89% of the samples and three other TPs were present in more than half of the samples. EQI, DEQ and other eight dimeric TPs could also be identified in several samples. A great variability of EQ and TPs distribution was observed amongst the fish feed samples tested. In particular, EQDM was the predominant TP in 10 samples, and EQ in four.

The FEEDAP Panel noted a recent publication by Rasinger et al. (2022) in which additional degradation products from EQ identified in fish feed (Negreira et al., 2017) and fillet (Merel et al., 2019) were ranked according to occurrence data and to the mutagenicity screening analysis done using different *in silico* models. Positive predictions have been obtained for some compounds; however, the results among the individual models do not seem to be consistent, are deemed to be preliminary and not further considered in the present opinion.

### 3.1.2. Conditions of use

Ethoxyquin is intended to be used in feed for all animal species with a maximum content of 50 mg/kg complete feed (in combination with BHA and/or BHT, the content of the total mixture should not exceed 150 mg/kg complete feedingstuffs). No withdrawal period is proposed.

## 3.2. Safety

### 3.2.1. Absorption, distribution, metabolism and excretion (ADME)

In its previous opinion (EFSA FEEDAP Panel, 2015), the Panel highlighted the lack of data on the comparative metabolic fate of EQ in different animal species. The applicant has now provided a study of the metabolism of EQ in liver slices and liver microsomes from the rat, laying hen and goat.[13]

Liver slices were prepared from rat (Han-Wistar), hen (Isa-Warren) and goat (Deutsche Edelziege/Burenziege). Liver microsomes were prepared from the same livers of hen and goat but were of commercial origin for rat. These biological samples were incubated at 37°C for 2 h with [14]C- EQ (ring-[14]C(U)) at concentrations of 10 and 100 μM. The corresponding heat-treated biological samples were used as control. Testosterone was used as a positive control for metabolic activity. EQ and metabolites were then extracted, separated and determined by HPLC-LC/MS. The comparison of

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[12] FAD-2017-0073/Technical dossier/Annex 08.
[13] FAD-2018-0029/ Technical dossier/Annex 03.

transformation/metabolic profiles obtained from the test substance (at the beginning and the end of the experiments), active incubates and heat-deactivated incubates allowed to distinguish between EQ-TPs originating from abiotic oxidation or metabolism.

In liver slices and liver microsomal incubates from rats, goats and hens, EQ was metabolised extensively; in liver slices, 17 metabolites were identified, while in liver microsomes, 18 metabolites were identified, irrespective of their origin. The two experimental approaches allowed establishing the metabolic pathways of EQ which appeared qualitatively to be essentially similar in the three species. Major common metabolites were EQI and EQDM as well as conjugates of de-ethylated EQ (DEQ). The DEQ sulfate conjugate was present in all investigated species, whereas its glucuronic acid conjugate was formed in goat liver slices. The metabolites formed in lesser and variable amounts among the species were the oxidative products of EQ and DEQ, free or conjugates of glucuronic acid and glutathione. Lower amounts of EQI, EQDM and derived oxidation reaction products were detected in heat-deactivated incubates demonstrating the concomitant abiotic origin of these structures.

The results of this study showed (i) the similarity between the *in vitro* and the known *in vivo* metabolic pathways in the rat (EFSA FEEDAP Panel, 2015) and (ii) that the metabolic pathways of EQ *in vitro* show high similarities between the species.

An additional study on the metabolic fate of EQ in fish was submitted, aiming at the identification of EQ metabolites/degradation products.[14] Atlantic salmon (nine tanks with 70 fish each) received for 90 days a diet either not supplemented or supplemented with EQ at approximately 120 mg EQ/kg feed or 1175 mg EQ/kg feed. The animals were slaughtered and fillets (five per tank) analysed for EQ-derived metabolites/TPs, using a combination of highly specific and sensitive analytical methods. Sixteen compounds distributed into five classes were identified. The most predominant one (in the range of 57–97%) consisted of EQDM with minor amounts of another dimer. A second class corresponded to four oxidised products, with a relative abundance of 10%. A third class corresponded to those formed by other reactions (three products), with a relative abundance of 11%. Two other classes with relative abundances not exceeding 3% corresponded to EQ cleavage and conjugation (including two glucurono-, one sulfo- and one glutathione conjugates). These data confirm and complete those already assessed (EFSA FEEDAP Panel, 2015) and establish that EQ metabolic pathways in the fish are essentially similar to those in the rat. In the same study, EQ and EQ-TPs were identified in commercial fish (12 samples). Ten out of 12 EQ-TPs detected in the feed trial above were found in commercial fish, but 11 TPs identified in the latter were not present in the former. This indicates that EQ may have already been degraded in fish feed, residual EQ and TPs being transferred to fish and further metabolised/transformed. Forty-seven TPs were isolated, which abundance showed high variability; 21 belonging to six classes of TPs were observed at least once. As in the feeding trial, dimers were the main EQ-TPs in commercial fish, EQDM being by far the most abundant one (about 63%). A scheme of EQ transformation pathways is proposed in the publication of Merel at al. (2019), based on the results of this study. The tentative transformation pathways are summarised in Appendix A.

## 3.3. Toxicological profile

In the previous opinion (EFSA FEEDAP Panel, 2015), the Panel extensively described and assessed the toxicological profile of ethoxyquin. The Panel concluded that: 'Ethoxyquin itself is not genotoxic, carcinogenic, and does not cause developmental toxicity in the offspring. The toxicological profile of the ethoxyquin dimers, present in feed and animal tissues, is considered to reflect that of the precursor monomer'.

In 2015, the Panel could not draw conclusions on the absence of genotoxicity of EQI, and on the safety of p-phenetidine, a possible mutagen: 'substance which causes concern for humans owing to the possibility that it may induce heritable mutations in the germ cells of humans classified as (Germ cell mutagenicity: Muta. 2)' according to Regulation (EC) No 1272/2008.

In order to complete the data set on the toxicological profile of the additive EQ, the applicant provided genotoxicity studies with EQI and a subchronic study in mice with EQDM. No additional information on the safety of p-phenetidine was provided.

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[14] FAD-2018-0029/Technical dossier/Annex 21.

### 3.3.1. Ethoxyquin

No additional information on the toxicological profile of ethoxyquin is available. However, in the context of the present assessment, and in order to complete the assessment of the safety of ethoxyquin, the FEEDAP Panel re-evaluated the toxicological data set assessed in its previous opinion (EFSA FEEDAP Panel, 2015), and confirmed the previous conclusions.

### 3.3.2. Ethoxyquin quinone imine

The structure–activity analysis performed by the FEEDAP Panel on EQI by the OECD quantitative structure–activity relationship (QSAR) toolbox[15] revealed structural alerts for the formation of reactive oxygen species and for mutagenicity, carcinogenicity and DNA bindings.

In order to clarify the biological relevance of these structural alerts, the applicant submitted two *in vitro* mutagenicity studies with EQI, which were evaluated in a previous opinion (EFSA FEEDAP Panel, 2015). A bacterial reverse mutation assay gave negative results. In an *in vitro* micronucleus assay with cytokinesis block, EQI induced a dose-dependent, statistically significant increase in the number of mono- and binucleated cells with micronuclei, both in the absence and in the presence of metabolic activation. The FEEDAP Panel concluded that, in the absence of the analysis of the micronuclei by fluorescence *in situ* hybridisation (FISH), it was not possible to exclude a clastogenic activity of EQI. Consequently, the FEEDAP Panel was not in a position to conclude on the absence of genotoxicity of EQI (EFSA FEEDAP Panel, 2015).

**Micronucleus test in mouse bone marrow**

The applicant submitted an *in vivo* micronucleus assay in mouse bone marrow.[16] EQI did not induce statistically significant increases in the frequency of micronucleated polychromatic erythrocytes in the bone marrow of mice treated up to the maximum tolerated dose of 425 mg/kg body weight (bw). The test item was detected in the plasma of the animals treated with the top dosage at a maximum level of 2.15 $\mu$g/mL; however, no sign of local cytotoxicity (decrease in the ratio PCE/NCE) was reported. Therefore, the FEEDAP Panel concluded that, while the *in vivo* micronucleus study demonstrates that the substance, administered orally at the maximum tolerated dose, does not reach in the bone marrow a concentration sufficient to induce micronuclei, it does not rule out the possibility that the substance is mutagenic in other tissue/organs, in particular at the site of contact.

***In vitro* micronucleus assay with fluorescence *in situ* hybridisation (FISH)**

In order to clarify the mechanism underlying the induction of micronuclei previously observed, an *in vitro* micronucleus assay with FISH analysis was carried out in isolated human peripheral blood lymphocytes.[17] This study was in general accordance with the Organisation for Economic Cooperation and Development (OECD) Guideline 487, with the exception that only a single treatment condition (24-h incubation without metabolic activation) was used, as this assay was a follow-up to a previous study. The following concentrations were selected for micronuclei analysis based on cytotoxicity: 8.5, 9.0, 9.5 and 10 $\mu$g/mL. At the top concentration the cytotoxicity was 57%, evaluated as cytokinesis-block proliferation index.

A statistically significant and dose-dependent increase in the percent of micronucleated cells was observed in all four concentrations scored. Additionally, the percent of micronucleated cells was higher than the historical control range of the vehicle control at all four concentrations scored. The vehicle and positive controls and all four test article concentrations were further analysed by FISH for the presence of centromere positive micronuclei from 100 micronucleated cells per culture, when available. A clear dose-response relationship could not be observed with regard to centromere positive micronuclei; therefore, the observed induction of micronuclei was attributed to both aneugenicity and clastogenicity. Therefore, the applicant decided to perform, as *in vivo* follow-up, a micronucleus assay in bone marrow and a comet assay in liver and digestive tract.

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[15] http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm
[16] FAD-2016-0018/Technical dossier/2016-03-10_ANTOXIAC-EQ_Micronucleus vivo OECD 474_QI.
[17] FAD-2016-0018/Supplementary Information August 2018/ Annex SIn 02.

### *In vivo* micronucleus assay

Based on the results of a previous dose range-finding study, the oral dose level of 350 mg/kg body weight was selected as appropriate dose for the *in vivo* micronucleus assay.[18]

Since no sex differences were seen in the range-finding study using oral dosing, female rats only were used in the main study. They were dosed twice by gavage with vehicle or with 87.5, 175 or 350 mg EQI/kg bw. Five animals per treatment group were dosed twice with a 24-h interval. A positive control group was dosed once by oral gavage with 20 mg cyclophosphamide/kg bw. In total, five treatment groups were used, each consisting of five animals. The animals treated with 350 mg EQI/kg bw showed the following toxic signs after dosing: lethargy, rough coat and a hunched posture. No treatment-related clinical signs or mortality were noted in animals treated with 87.5 or 175 mg EQI/kg bw or control animals receiving vehicle or cyclophosphamide. Bone marrow was sampled 48 h after the first dosing.

Cyclophosphamide, used as positive control item, induced a statistically significant increase in the number of micronuclei.

A slight increase in micronuclei frequency was seen in the lowest EQI dose group, and this frequency fell above the upper 95% limit of the historical negative control reference range. This was due to a single animal that exhibited a very high incidence of micronuclei. Moreover, the increase in the mean micronuclei frequency in this group was not significantly different from concurrent control and it was not observed at higher treatment doses; therefore, it was considered not biologically relevant. The micronuclei frequencies in the other EQI groups were similar to or lower than in concurrent controls.

In the group treated with the highest dosage of EQI, three out of five animals showed a 54% reduction in the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) compared to the concurrent vehicle control group. Overall, the mean group ratio was reduced by 30%. This reduction is indicative of toxic effects on erythropoiesis and can be considered a proof of bone marrow exposure to the test item EQI.

The FEEDAP Panel concluded that the test item did not induce a biologically relevant increase in micronuclei.

### *In vivo* alkaline comet assay in the rat

An *in vivo* comet assay was conducted to investigate the potential of EQI to induce DNA strand breaks in liver, duodenum/jejunum and stomach when administered to rats at the maximum tolerated acute dose.[19]

Based on the results of the dose range-finding study performed for the *in vivo* micronucleus study (see above), the maximum tolerated dose (MTD) was established at 350 mg/kg bw and only female rats were used. The study was conducted in line with the OECD Guideline 489 and in accordance with the OECD principles of Good Laboratory Practice (GLP). Wistar Han rats were treated twice via oral gavage with vehicle or with 350, 175, 87.5 or 43.75 mg EQI per kg bw for two consecutive days at an interval of 21 h. Additional animals were dosed twice with the positive control ethyl methanesulfonate (EMS) by oral gavage.

The following treatment-related clinical signs were observed in the group treated with 350 mg EQI/kg bw: lethargy, rough coat, slow breathing, hunched posture and uncoordinated movements. Two out of eight animals treated with 350 mg EQI/kg bw died. These animals were replaced by satellite animals. In the other treatment groups, the animals showed no clinical sign related to the treatment. Approximately after 3–4 h of the second dose, liver, duodenum and stomach were collected and examined for DNA damage with the alkaline comet assay. Three slides per tissue per animal were prepared and treated by means of electrophoresis. One hundred and fifty comets per tissue per animal were analysed in total with a fluorescence microscope connected to an image analysis system. The DNA damage was quantified as percentage tail intensity (percentage of DNA migrating into the tail).

Although a statistically significant trend was present in the liver, all values were in the historical control range of the negative control and no individual statistically significant increases in the mean tail intensity (%) were observed in liver cells. No statistically significant increase in the mean tail intensity was observed in the cells of duodenum or of stomach isolated from EQI-treated animals.

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[18] FAD-2016-0018/Supplementary Information August 2018/Annex SIn 04.
[19] FAD-2016-0018/Supplementary Information August 2018/Annex SIn 05.

Test item-related morphologic alterations following the administration of EQI were present in the liver at 87.5 mg/kg bw per day and above and in the stomach at 175 and 350 mg/kg bw per day.

In conclusion, EQI was not genotoxic in the comet assay in liver, duodenum and stomach cells when sampled approximately 3–4 h post dosing in female rats dosed by oral gavage for two consecutive days up to a dose of 350 mg/kg bw per day (i.e. the maximum tolerated dose according to current regulatory guidelines) in conditions in which histopathological alterations were reported.

### Conclusions on the genotoxicity of EQI

EQI was positive for inducing micronuclei *in vitro* in isolated human peripheral blood lymphocytes in the 24-h treatment without metabolic activation. Based on the detection of centromeres by FISH analysis, the observed induction of micronuclei was attributed to both aneugenicity and clastogenicity.

In the bone marrow micronucleus test, female rats exposed to up to a dose of 350 mg/kg (the maximum tolerated dose) showed no induction of micronuclei over the concurrent controls; the clear toxic effects on erythropoiesis observed in the animals treated with the highest dosage are considered a proof of bone marrow exposure to the test item EQI.

Moreover, EQI was not genotoxic in the comet assay in liver, duodenum and stomach cells in conditions in which histopathological alterations were reported in the analysed tissues.

The FEEDAP Panel observed that the genotoxic activity of EQI reported *in vitro* is not expressed *in vivo* and concluded that EQI does not pose a concern for genotoxicity.

### 3.3.3.  Ethoxyquin dimer

A subchronic toxicity study was performed using 80 42-day-old male BALB/c mice.[15] The FEEDAP Panel notes that the experiment did not conform to OECD Guideline No 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents) for the use of only male animals and the absence of microscopic examination of most organs. Groups of 10 mice were given EQDM in their diet for 90 days at nominal concentrations of 0 (unspiked control), 0.1, 1, 100, 1,000, 3,000 or 5,000 mg/kg feed, which was calculated to be equal to mean dosages of 0, 0.015, 0.081, 10, 99, 286 and 518 mg EQDM/kg bw per day, respectively. A further group was first given EQ at a nominal concentration corresponding to the highest EQDM dosage (5,000 mg/kg feed). However, since mice refused their feed and lost 11% of their initial body weight after 10 days, the EQ dosage was reduced to 1,000 mg/kg feed. The animals tolerated this lower EQ concentration and started gaining weight. However, the initial deviations in feed intake and in body weight led the authors to exclude EQ-treated mice from the study.

All the results were subjected to statistical analysis using ANOVA with group comparison with Tukey test for normally distributed data, and with Kruskal–Wallis' test and Wilcoxon's test for multiple pairwise comparison for not normally distributed data. All the results were also analysed with benchmark dose assessment. Feed intake and body weight were not affected by the treatments. All animals were killed at the end of the 90-day feeding period and subjected to necropsy. Samples of the organs were fixed in 4% paraformaldehyde or frozen in liquid nitrogen. Crude body composition was assessed using an NMR analyser, which showed no effects on total body fat mass or total body lean mass. White adipose tissue mass and white adipose tissue mass relative to body weight were significantly decreased in mice fed EQDM at the level of 286 mg/kg bw per day and above.

There were no effects on the absolute and relative weights of heart, brain or kidneys. Both liver weight and hepatosomatic index were increased in a dose-related manner at levels of 99 mg/kg bw per day and greater. Splenosomatic index was increased in groups given 286 mg EQDM/kg bw per day or greater.

Terminal blood samples were taken for haematology[20] and clinical chemistry.[21] There were significant reductions in packed cell volume (haematocrit) in groups fed EQDM concentrations of 286 mg EQDM/kg bw per day or greater and in mean corpuscular volume (MCV) at 99 mg EQDM/kg bw per day or greater. Among the tested parameters, only plasma ALT activity was significantly elevated in groups given 99 mg EQDM/kg bw per day or greater.

EQDM affected liver lipid content and composition. Treatment-related increases in concentrations of hepatic triacylglycerol and total neutral lipids were recorded at 286 mg EQDM/kg bw per day or

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[20] rbc, Hb, pcv, MCV, MCH, MCHC, RDWc, RDWs, platelet count, MPV, PCT, PDWc, PDWs, wbc, lymphocyte count, monocyte count, neutrophil count, lymphocyte %, monocyte %, neutrophil %.

[21] ALT, AST, AP, bile acids, bilirubin, total protein, albumin, urea, creatinine, urea/creatinine ratio, glucose, cholesterol, free fatty acids, triglycerides, sodium ($Na^+$), chlorine ($Cl^-$).

greater; an increase in total lipids as well as in the relative content of triacylglycerol, phospholipids and neutral lipids was detected at EQDM levels of 99 mg EQDM/kg bw per day.

No gross pathological alteration was reported for any animal, although no evidence was provided.

Liver, spleen and kidneys were examined microscopically, and the results expressed as quantal data. While spleen and kidney did not exhibit appreciable changes,[22] dose-related increases in steatosis (particularly microvesicular), single-cell necrosis and necrotic foci were in general seen in liver sections from mice exposed to EQDM doses higher than 10 mg/kg bw per day. Benchmark dose assessment was performed on the results by the study author.[23] The increase in single cell necrosis showed a BMD lower confidence limit for a benchmark response of 10% ($BMDL_{10}$) of 0.01 mg EQDM/kg bw per day: however, this value was not taken into account due to the great uncertainty arising from the large BMDU/BMDL ratio (10,156). Benchmark procedure applied to severe necrosis ($> 2$ foci) resulted in a $BMDL_{10}$ value of 10.3 mg/kg bw per day, for which a BMDU/BMDL ratio of 18.3 was observed. Microvesicular steatosis showed the lowest $BMDL_{10}$ (1.1 mg/kg bw per day) with a BMDU/BMDL ratio of 227.

Metabolomic screening of livers was performed investigating the main pathways involving carbohydrates, lipids and the redox status. The results were expressed as scaled and mean centred intensities using data from three animals/group. Taken together, the EQDM-induced alterations of liver metabolite profiles indicated mobilisation of energy stores, and a disruption of fatty acid β-oxidation in liver mitochondria. As regards redox status, the observed trends in the increase in reduced glutathione (GSH) levels and of the intermediates required for GSH synthesis, notably γ-glutamyl-cysteine, coupled with a decrease in α-tocopherol and its metabolite γ-CEHC glucuronide pointed to a pro-oxidant activity of EQDM. Most of the observed changes occurred at EQDM dosed higher than 10 mg/kg bw per day.

The EQDM effects on the redox status of the liver were therefore examined by measuring GSH, oxidised glutathione (GSSG), α-tocopherol and thiobarbituric acid reactive substances (TBARS) in liver samples. Results showed a dose-related decrease in GSH:GSSG ratio, with significant decreases in GSH ($BMDL_{10}$ = 23.5 mg/kg bw per day) and decreases in GSSG ($BMDL_{20}$ = 10.3 mg/kg bw per day). TBARS levels were unaffected. There was a dose-related decrease in liver concentrations of alpha-tocopherol ($BMDL_{20}$ = 0.195 mg/kg bw per day), but it was considered by the authors a biomarker of effect rather than an adverse effect.

Proteomic investigations were also performed on liver samples. The combination of liver metabolomic and proteomic profiling suggested that EQDM-induced hepatotoxicity could be mediated by the activation of the orphan nuclear receptors PXR and/or CAR and by the induction of a oxidative stress response NRF2-mediated.

The authors of the study concluded that subchronic dietary exposure to EQDM at doses higher than 10 mg/kg bw per day impairs fatty acid β-oxidation, disrupting hepatic lipid metabolism and leading to an increased deposition of lipid and to steatosis in mice. They considered hepatic lesions as the endpoint for risk assessment and identified the $BMDL_{10}$ of 1.1 mg EQDM/kg bw per day for microvesicular steatosis as the critical Reference Point.

The FEEDAP Panel has verified the BMD analysis and agrees with the results; the Panel also agrees with the authors that the liver toxicity is the principal adverse effect of EQDM in BALB/c mice. Liver cell necrosis and microvesicular steatosis are considered as indicators of liver toxicity, showing the lowest calculated BMDL values. Only the $BMDL_{10}$ for microvesicular steatosis (1.1 mg EQDM/kg bw per day) could be considered, since the one for liver cell necrosis ($BMDL_{10}$ 0.01 mg EQDM/kg bw per day) was not applicable due to methodological reasons. Theoretically, the decrease in liver concentrations of alpha-tocopherol ($BMDL_{20}$ 0.195 EQDM/kg bw per day) could also be considered as an endpoint; however, considering the pro-oxidant action of EQDM, the Panel considered it a compensatory mechanism rather than an indicator of liver toxicity.

The FEEDAP Panel considers that, even in the presence of the described deviations from the relevant EOCD guideline, the study could be considered as a reliable indicator of the toxicological profile of EQDM. In its previous opinion (EFSA FEEDAP Panel, 2015), the FEEDAP Panel identified an NOAEL of 2 mg EQ/kg bw per day in a study in dogs based on hepatocellular necrosis. Considering

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[22] Data not shown.

[23] Bench mark dose (BMD) analysis was performed on all measured endpoints according to the EFSA benchmark dose technical guidance (EFSA Scientific Committee, 2017). Results were obtained using the EFSA BMD online application (https://shiny-efsa.openanalytics.eu/app/bmd). Fitting benchmark dose models was based on the R-package PROAST (http://www.rivm.nl/en/Documents_and_publications/Scientific/Models/PROAST), versions 64.16 (continuous data) and 61.3 (quantal data). Model performance was evaluated based on the Akaike information criterion (AIC).

that (i) the main toxicological effects of EQ and EQDM were observed in the same target organ and (ii) the doses at which such effects were observed are similar, the FEEDAP Panel considers that the $BMDL_{10}$ identified for EQDM in the present study could be used to derive a health-based guidance value (HBGV).

## 3.4. Safety for target species

In the previous opinion, the FEEDAP Panel concluded that: 'The proposed maximum concentration of 50 mg ethoxyquin/kg might be considered as potentially safe for chickens and breeders, but extrapolation to other poultry (including laying hens) is not possible. No conclusion on the safety of ethoxyquin for pigs, ruminants and fish can be drawn. The maximum potentially safe ethoxyquin concentration in feeds for dogs is 11 mg/kg complete feed. The FEEDAP Panel cannot conclude on safe concentrations for cats and other pets'.

To complete the data set of tolerance studies needed to demonstrate the safety of the additive for all animal species, four tolerance studies, one with weaned piglets, one with salmon, one with cattle for fattening and one with laying hens were made available. In addition, a supportive study in dairy cows was also submitted.

### 3.4.1. Weaned piglets

A total of 128 male and female piglets (Pietrain × (Duroc × Landrace and Large White × Landrace), 28 days of age, initial body weight 8.1 kg) were distributed in groups of four piglets (two males and two females) to 32 pens, acclimatised for 6 days in their pens on control feed and then allocated to four dietary treatments (representing eight replicate pens per treatment).[24] A basal EQ-free diet (mash form) was either not supplemented (control) or supplemented with EQ at 50 mg/kg feed (1× the maximum use level ), 150 mg/kg feed (3×) or 300 mg/kg feed (6×). The diets (pre-starter, from day 1 to day 14; starter, from day 15 to day 42), consisting mainly of maize and soybean meal, were isonitrogenous (pre-starter: 209 g crude protein – CP/kg feed; starter: 182 g CP/kg feed) and isoenergetic (pre-starter: 14.02 MJ metabolisable energy – ME/kg feed; starter: 13.81 MJ ME/kg feed). The concentrations of EQ were analytically confirmed. Feed and water were offered ad libitum for 42 days. Health status and mortality were monitored daily and most probable cause of death determined by necropsy. Feed intake and body weight were recorded at the beginning and at days 14 and 42 of the experiment, and average daily weight gain and feed to gain ratio were calculated. At the end of the experiment, one piglet per pen having body weight closest to the average pen weight (overall 19 male and 13 female piglets) was selected, blood sampled for routine blood haematology[25] and biochemistry[26] collected and killed to perform necropsy for macropathological examination of various organs/tissues, including weight determination of liver, spleen and kidneys. In case abnormalities or weight differences were detected in organs/tissues, these were submitted to histological examination. A one-way ANOVA was performed with the data, considering the pen as the experimental unit, with blocks included in the model as a random effect and treatment as a fixed effect. When required, treatment means were compared using Tukey's test. Differences were considered significant at $p < 0.05$.

No piglet died or was culled during the study. The zootechnical parameters of the three groups given ethoxyquin were not different from those of the control group (average daily gain 460 g; average daily feed intake 730 g; feed to gain ratio 1.58), as well as the blood haematological and biochemical parameters, except for alkaline phosphatase. Alkaline phosphatase showed significant differences between the groups; however, the change was not treatment-related. No macroscopic and histopathological findings of the examined organs/tissues, as well as no significant differences in absolute and relative weights of liver, spleen and kidney, were observed between treatments.

No negative effect up to 6× the maximum EQ recommended level were observed. Therefore, the additive is considered safe for weaned piglets at the maximum use level (50 mg ethoxyquin/kg feed) with a margin of safety of at least six.

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[24] FAD-2017-0073/Technical dossier/Annex 15 and FAD-2017-0073/Supplemental information August 18/Annexes 04 and 05.

[25] Haematocrit (HT), haemoglobin (HB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), erythrocytes count, white blood cell count (WBC) and differential leukocyte count (eosinophils, basophils, lymphocytes, monocytes, neutrophils).

[26] Alanine aminotransferase (ALT), alkaline phosphatase (APH), aspartate amino transferase (AST), gamma-glutamine transpeptidase (GGT), creatinine kinase (CK), lactate dehydrogenase (LDH), total bilirubin, uric acid, total protein, albumin, cholesterol, creatinine, glucose, potassium and sodium.

### 3.4.2. Salmon

A total of 1,260 Atlantic salmons (*Salmo salar* L.; initial bw 150–200 g; 6-month-old) were distributed in groups of 70 fish to 18 tanks, acclimatised for 2 weeks in their tanks on control feed and then allocated to six dietary treatments (representing three replicate tanks per treatment).[27] A basal EQ-free diet (commercial feed as uncoated 5 mm extruded pellets) was either not supplemented (EQ0; control) or supplemented with EQ at 50 (EQ1; 1× the maximum use level), 150 (EQ2; 3×), 1,500 (EQ3; 30×), 5,000 (EQ4; 100×) or 10,000 (EQ5; 200×) mg/kg feed. For the preparation of treatment diets, EQ was dissolved directly into fish oil and uncoated feed pellets were mixed with EQ-containing fish oil and exposed to a reduced pressure of 0.1 bar for 10 min. The resulting oil-coated pellets were stored at −20°C throughout the experimental period. Concentrations of EQ and EQDM were measured in samples from each feed batch taken immediately after production and again after the 90-day feeding experiment to ensure there was no degradation of EQ during the trial period. Ethoxyquin concentrations measured in feed were $0.47 \pm 0$ mg (EQ0), $40.6 \pm 2.3$ mg (EQ1), $118.8 \pm 7.3$ mg (EQ2), $1,173 \pm 113$ mg (EQ3), $3,985 \pm 228$ mg (EQ4) and $9,666 \pm 979$ mg (EQ5)/kg feed at trial start, and did not show any degradation during the experimental period. Concentrations of EQDM were below the limit of quantification (LOQ < 0.07 mg EQDM/kg feed) at both time points. Using automatic feeders, feed in pelleted form (diameter 5 mm) was provided ad libitum in six daily meals for 90 days.

Unconsumed feed pellets were collected and weighted once per day, and feed intake, feed conversion and EQ exposure calculated. Survival and health status of fish were monitored. At day 90 (end) of the experiment, 10 fish per tank (30 fish per treatment) were killed, individually weighed and length recorded. In order to assess growth performance and EQ exposure of the Atlantic salmon during the 90-day feeding trial, body weight gain, condition factor, specific growth rate, daily feed intake, total feed intake, feed to gain ratio and estimated daily EQ exposure were calculated. In addition, at day 90 of the experiment, five (out of 10) fish per tank (15 fish per treatment) were homogenised to get one pooled sample per tank (three samples per treatment) for analysis of proximate composition. In the other five fish, blood samples were obtained to determine haematological[28] and biochemical[29] parameters, and liver, heart and spleen were weighed and the hepatosomatic index, cardiosomatic index and splenosomatic index were determined. Moreover, liver, kidney and spleen samples were obtained for histopathological evaluation. Measurements performed on tank-pooled samples or tank means (n = 3/group) were analysed with one-way ANOVA and Tukey's test for multiple comparison of group means, using the tank as the experimental unit. Data not complying with the criteria of the ANOVA were analysed using Kruskal–Wallis test, and a Wilcoxon's test for multiple pairwise comparison of the groups. Differences were considered significant at p < 0.05.

No fish died during the study and no overt signs of toxic responses were observed in any of the experimental groups throughout the experiment. However, the supplementation with EQ of 5,000 mg/kg feed and above decreased the final bw (EQ4 537 g and EQ5 301 g vs. EQ0 716 g), bw gain (EQ4 276 g and EQ5 38 g vs. EQ0 454 g), specific growth rate (EQ4 0.8% bw per day and EQ5 0.1% bw per day vs. EQ0 1.1% bw per day), condition factor (EQ4 1.3 $g/cm^3$ and EQ5 1.1 $g/cm^3$ vs. EQ0 1.5 $g/cm^3$), daily feed intake (EQ4 2.9 g/fish per day and EQ5 1.1 g/fish per day vs. EQ0 4.9 g/fish/day) and total feed intake (EQ4 255 g/fish and EQ5 100 g/fish vs. EQ0 436 g/fish) in comparison with the control; and the inclusion of the highest level (10,000 mg/kg) also reduced final salmon length (EQ5 29 cm vs. EQ0 36 cm).

EQ supplementation of fish diets at all inclusion levels did not affect crude protein and ash content of fish and relative liver, heart and spleen weight, but treatment EQ5 dry matter, crude lipid and crude lipid muscle content of fish, as well as the absolute weight of liver, heart and spleen. Treatment EQ4 also decreased dry matter, crude lipid and crude lipid muscle content of fish and treatment EQ3 decreased crude lipid content of fish. Moreover, EQ supplementation of fish diets at all inclusion levels did not affect any blood haematological and most of the biochemical parameters. However, creatinine and total protein blood levels decreased at inclusion levels above 1,500 (for creatinine) and 10,000 (for total protein) mg EQ/kg feed in comparison with the control diet.

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[27] FAD-2017-0073/Technical dossier/Annex 21 and FAD-2017-0073/Supplemental information August 2018/Annexes 01 and 02.

[28] Haemoglobin (Hb), red blood count (RBC), haematocrit, mean corspuscular volume (MCV), mean corspuscular haemoglobin (MCH), mean corspuscular haemoglobin concentration (MCHC), white blood cell differentials (neutrophils, lymphocytes, monocytes).

[29] Albumin (Alb), total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, bile acids, creatinine, lysozyme, sodium, potassium, calcium, chloride, osmolality.

Histopathology of liver tissue showed that the increase of dietary EQ concentrations lead to a reduction ($p < 0.05$) in the glycogen and lipid storage. The decreased hepatocellular vacuolation was correlated with the reduced feed intake observed in fish supplemented with the highest EQ concentrations (EQ4 and EQ5 vs. EQ0). However, a significantly decrease in liver cytoplasmic vacuolation was also observed in fish receiving EQ3 compared to control animals, although this level did not negatively affect feed consumption. In kidney, the presence of higher number and intensity of pigmented macrophage aggregates (PMA) was observed in fish exposed to the highest level of EQ ($p < 0.05$; EQ5 vs. EQ0). The main histopathological finding in the splenic tissue was red pulp congestion and subcapsular haemorrhage in fish fed the highest dose of ethoxyquin ($p < 0.05$; EQ5 vs. EQ0). No other abnormal findings were reported from gross pathology.

The supplementation of the experimental diets with EQ at up to $3\times$ the maximum intended level did not have any negative effect on the health and performance of Atlantic salmon. Therefore, the additive is considered safe for Atlantic salmon at the recommended level (50 mg ethoxyquin/kg feed), with a margin of safety of $> 3$ and $< 30$.

### 3.4.3. Cattle for fattening[30]

A total of 32 ruminating male Holstein calves (110 days of age, average body weight about 157 kg) were allotted to four groups.[31] The control group was fed the basal diet and the other three groups were fed diets with intended levels of 50 ($1\times$ maximum intended use level), 150 ($3\times$) and 500 ($10\times$) mg ethoxyquin/kg feed (administered individually via an oesophageal tube), and for 42 days. Feed and water were offered ad libitum. Feed intake was daily monitored on an individual basis; the EQ dose of each animal was adjusted to the corresponding feed intake and administered via oesophageal tube once daily. The mean daily EQ intake corresponded to 51, 153 and 515 mg/kg complete feed (standardised with 88% DM). Group size was two pens with four calves per pen. The basal diet, consisting mainly of ryegrass hay, soybean meal, barley and corn, was prepared once daily as TMR and contained (analysed values) 87.1% DM, and in the DM 19.9% CP, 27.7% NDF, 3.5% ether extract and 39.2% non-fibre carbohydrate. Endpoints were final body weight and body weight gain, feed intake and feed/gain ratio. At the end of the study, blood samples were taken from five calves per treatment for haematology[32] and routine biochemistry.[33] Four animals per treatment were killed for necropsy,[34] organ and tissue samples (and faeces and urine) were prepared for residue analysis and stored deep frozen. The study was based on a randomised complete block design. The mixed-effects model for repeated measures with treatment, time and their two-way interaction as fixed effects was applied with animal and pen as random effects. Blood data (without repeated measures) were analysed with treatment as main effect.

No adverse symptoms were observed during the course of the study, the health status of the animals was good. There were no differences in average final body weight (average 212.2 kg), average daily gain (average 1.31 kg), daily dry matter intake (average 5.5 kg) and DM-feed to gain ratio (about 4.19) among treatments. Similarly, none of the haematological parameters was affected by treatment with the exception of platelet count, which showed a treatment-related decrease (control group: $547 \times 10^3/\mu L$, $1\times$: $452 \times 10^3/\mu L$, $3\times$: $394 \times 10^3/\mu L$ and $10\times$: $264 \times 10^3/\mu L$ ($p = 0.01$ to the control)). Blood biochemistry values were not affected by treatments except for gamma-glutamyl transpeptidase ($p = 0.04$ for the difference between the control (13.2 $\mu$/L) and the $1\times$ (22.4 $\mu$/L) and the other two groups with intermediate values not different to both). This effect is not considered treatment-related. Post-mortem examination is reported without any signs of anomalies in organs and tissues. Organ weights were not reported.

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[30] The animal category in the study submitted was called calves for fattening. However, Regulation (EC) 429/2008 defines calves for fattening as calves for veal production. Since calves of the study were not fed milk or milk replacer, they cannot be assigned to the category calves for fattening. They were also not calves for rearing since the maximum age of 4 months (see Regulation (EC) 429/2008) was nearly reached by the calves at study start. The animals of the study had already completed the weaning period then will consequently be called in the study description cattle for fattening and the conclusions from the study refer to cattle for fattening.

[31] FAD-2017-0073/Technical dossier/Annex 14 and FAD-2017-0073/Supplemental information August 2018/Annex 03 to Annex 11.

[32] WBC, RBC, Hb, haematocrit, MCV, MCH, MCHC, platelets, MPV, neutrophils, lymphocytes, monocytes, eosinosphils, leukocytes, basophils.

[33] Total protein, albumin, NEFA, cholesterol, glucose, creatinine, urea, AST, ALT, ALP, $\gamma$-GT, LDH, amylase, Ca, P, Mg, Na. K, Cl.

[34] Post-mortem examinations of heart, respiratory system, digestive tract, liver, kidneys, spleen, stomach (the four compartments), reproductive organs, muscles and joints; organ weights.

The supplementation of the experimental diets with EQ at up to 10× the maximum content in feed did not have any negative effect on the health and performance of cattle for fattening. Therefore, the additive is considered safe for cattle for fattening at 50 mg ethoxyquin/kg feed, with a margin of safety of 10.

### 3.4.4. Laying hens

A total of 308 layers (HyLine Brown, about 24 weeks of age, average body weight at start 1.8 kg) were allotted to four groups.[35] The control group was fed the basal diet and the other three groups were fed diets with intended levels of 50 (1×), 150 (3×) or 500 (10×) mg ethoxyquin/kg feed (confirmed by analysis), for 56 days. Feed and water were offered ad libitum. Group size was 25 replicates with three hens per cage. The basal diet consisted mainly of corn and soybean meal, and contained (calculated values) 15% CP, 0.68% Met + Cyst, 3.7% ether extract, 3.7% Ca and 0.58% P and 3,565 kcal gross energy/kg. Clinical observations and mortality were recorded daily. The measured endpoints were body weight, feed intake, laying rate, egg weight and mass, egg quality (incidence of broken, dirty, faulty and soft-shelled eggs, yolk colour and Haugh Units, shell index, egg classification) and feed/egg ratio. At the end of the trial, blood samples were taken from eight birds per treatment for haematology[36] and routine biochemistry.[37] The same birds were killed for necropsy.[38] In case abnormalities or weight differences in organs/tissues, these were submitted to histological examination. For statistical analysis of continuous variables, a randomised complete block design was used, categorical variables were assessed using categorical data analysis or as performance variables if data could be approximated to a normal distribution.

No mortality occurred. Overall performance could be considered good with no differences between the treatments; mean values for all groups were final body weight 1.86 kg, daily feed intake 113 g, laying rate 92.1%, egg weight 60.5 g, egg mass 55.6 g and feed to egg mass ratio 2.03. Also egg quality criteria did not differ between treatments, except egg yolk colour, which increased dose dependently (Roche colour fan: 7.5, 7.8, 7.8 and 7.9 in the first 4 weeks; and 6.7, 7.2, 7.3 and 7.1 in the second 4 weeks for the groups with 0, 50, 150 and 500 mg EQ/kg feed, respectively). No significant differences were found among treatments in haematological parameters. Although blood biochemistry data did not show significant differences between the control and the treated groups, small differences became significant for creatine kinase between 50 and 150 mg EQ/kg feed and for bilirubin for 50 and 500 mg EQ/kg. These were not considered relevant for the assessment. Organ weights were not significantly different between the groups. The distribution of animals with hepatic lesions (yellowish-brownish coloration and friability, congestion or presence of focal reddish areas, mild hepatic steatosis) in all experimental groups was numerically similar and suggested no treatment relation.

The supplementation of the experimental diets with EQ at up to 10× the maximum content dose did not have any negative effect on the health and performance of laying hens. Therefore, the additive is considered safe for laying hens at 50 mg ethoxyquin/kg feed, with a margin of safety of 10 ×.

### 3.4.5. Dairy cows

A 36-day feeding study with lactating cows, aimed to collect milk for the analysis of EQ residues in milk, was submitted.[39] The study did not fulfil the requirements for the tolerance study (short duration, limited endpoints measured)[40]; therefore, data from this study could be taken only as supportive for the safety assessment of EQ in dairy cows.

A total of 20 lactating cows (Ponderosa Holsteins, about 2 years of age, average body weight 685 kg) in one pen were allotted to four groups of which three groups were fed diets with intended

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[35] FAD-2017-0073/Technical dossier/Annex 13.

[36] Haematocrit (HT), haemoglobin (HB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), erythrocytes count (ERY), platelets count (PLA), white blood cell count (WBC) and differentials as well (eosinophil, basophile, lymphocyte, monocyte, heterophyl).

[37] Alanine aminotransferase (ALT), alkaline phosphatase (APH), aspartate amino transferase (AST-SGOT), gamma glutamin transpeptidase (GGT), creatinine kinase (CK), lactate dehydrogenase (LDH), total bilirubin, uric acid (URIC), total proteins (PROT), albumin (ALB), cholesterol, creatinine, glucose, K, Na.

[38] Post-mortem examinations of heart, respiratory system, digestive tract, liver, kidneys, spleen, stomach, reproductive organs, muscles and joints; relative weight of liver, spleen and kidneys.

[39] FAD-2017-0073/Technical dossier/Annex 17.

[40] Study duration (36 days) was too short to be considered as a tolerance study (56 days required). Data on hematology and blood biochemistry were not provided.

levels of 50 (1× maximum intended use level), 150 (3×) or 500 (10×) mg ethoxyquin/kg DM feed, and the control group was fed the basal diet. Feed as TMR and water were offered ad libitum. Feed intake was measured individually per day. The daily dose of EQ was individually adjusted to the corresponding intake of each animal. EQ was incorporated in a concentrate which was fed twice daily during milking in the milking parlour using an electronic delivery system. Group size was 5 cows, with cows characterised by a pre-experimental period milk yields of < 25 kg and > 30 kg, but number of cows in the respective subgroups is not reported. The basal diet consisting mainly of ryegrass hay, soybean meal, barley and corn contained (analysed values) 67.6% DM, and in the DM 16.3% CP, 22.1% acid detergent fibre, 33.5% neutral detergent fibre, 2.2% ether extract and 6.9% ash. Endpoints were final body weight, dry matter intake, daily milk yield, milk percentages of crude protein and fat, lying behaviour and milk yield/DM-intake ratio. A mixed-effects model for repeated measures with treatment, time and their two-way interaction as fixed effects and animals as a random effect was used for statistical evaluation. In none of the measured parameters, differences were observed among the groups (control group results: DM intake: 21.7 kg/day; milk yield: 32.3 kg/day; milk fat: 4.08%; milk protein: 3.74%; milk yield/DMI: 1.50; lying: 725 min/day).

The results showed that EQ up to 10× the maximum recommended level had no influence on the performance parameters when fed for 36 days.

### 3.4.6. Cats

In its previous opinion, the FEEDAP Panel could not conclude on the safety of the additive for cats owing their known limited capacity to conjugate aromatic substances. No additional studies of data have been provided in the current submission. Therefore, in the absence of tolerance studies or specific toxicity data, the FEEDAP Panel reiterates its former conclusion.

### 3.4.7. Conclusions on the safety for the target species

Based on the information reviewed above and those already evaluated in the previous opinion, 50 mg EQ/kg complete feed could be considered safe for chickens for fattening, laying hens, piglets, cattle for fattening and salmon. This conclusion could be extrapolated to all animal species and categories with the exception of cats, for which the Panel cannot conclude on a safe level. However, considering that the additive contains p-phenetidine, a possible mutagen, the Panel cannot conclude on the safety of EQ at any level for long-living and reproductive animals.

## 3.5. Safety for the consumer

In its former opinion, the Panel concluded that 'An estimate of consumer exposure to ethoxyquin-related residues in tissues and products from animals treated with ethoxyquin is not possible because there are considerable data gaps. An assessment of safety for the consumer is prevented by the lack of a safe level of exposure and the presence of p-phenetidine in the currently measured quantities in the additive' (EFSA FEEDAP Panel, 2015). In the present application, data on residues of EQ and its metabolites/EQ-TPs in tissues and products from different target species were made available. However, no data on p-phenetidine content in tissues and products was made available in the present submission.

### 3.5.1. Residues of EQ and EQ-TPs/metabolites in animal tissues and products

Residues of EQ and EQ-TPs/metabolites were identified and quantified in the tissues of piglets, cattle for fattening, laying hens and fish and in eggs and milk, sampled at the end of the tolerance studies described in Section 3.5. In all the studies, a negative control group was compared with groups fed 50 mg EQ/kg complete feed and its multifolds. Only the results of the control and the use level groups are reported below. The analysis of the residues for piglets, laying hens, calves, dairy cows followed the same protocol. All tissue samples were analysed in the same laboratory. Residues of EQ, EQDM, EQI and DHEQ were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), using a validated method. The method was extended to metabolites A (6-ethoxy-2,2,4-trimethyl-quinolin-8-one), B (1,2-dihydro-8-hydroxy-6-ethoxy-2,2,4-trimethylquinoline), C and C-oxy (structures not established). DEQ was proven to be unstable during the analytical process and converted to EQI; metabolite B was also unstable and converted to metabolite A. DEQ and metabolite B, as well as metabolite C (and the related metabolite C-oxy, an oxidised form of DEQ)

could not be quantified, although detected; only an LOD was estimated (10 μg/kg) for these metabolites/TPs.

Only residues of EQ, EQDM, EQI, DHEQ and metabolite A were used for the assessment of the safety for the consumer, considering the apparently inconsistent occurrence of the other metabolites/TPs (i.e. DEQ, metabolite B and C) due to their instability and the analytical uncertainties.

The residues analysis in salmon was done with HPLC with fluorescence detection and was limited to the determination of EQ and EQDM.

The results summarised in the following tables refer only to those metabolites/TPs that were detected above the respective LOQs in at least one tissue/product sample.

**Piglet tissues**

Tissues (liver, kidney, muscle and subcutaneous fat) were sampled from eight piglets per group after a 42-day feeding period.[41] Residues of EQ, EQDM, EQI, DHEQ, DEQ (in all samples) and metabolites A, B and C (in liver and muscle only) were determined. The results are summarised in Table 5. Results for DEQ, metabolite B and C were not considered (see Section 3.5.1) and not reported in the table.

**Table 5:** Residues of EQ and metabolites/TPs in weaning piglet tissues (μg/kg) following the administration of a control feed or the same feed supplemented with 50 mg EQ/kg for 42 days. Results are reported as mean $\pm$ SD

| Metabolite | EQ concentration (mg/kg feed) | Tissue | | | |
|---|---|---|---|---|---|
| | | Liver[a] | Kidney | Muscle | Subcutaneous fat |
| EQ | 0 | 0.3 ± 0.9 | < LOQ[b] | < LOQ | < LOQ |
| | 50 | 7.5 ± 4.5 | 4.9 ± 3.1 | < LOQ | 8.6 ± 5.9 |
| EQDM | 0 | 0.7 ± 1.4 | 4.0 ± 5.4 | 5.8 ± 7.3 | 374.0 ± 465.0 |
| | 50 | 8.2 ± 3.0 | 21.3 ± 6.5 | 31.6 ± 17.5 | 863.0 ± 392.4 |
| EQI | 0 | 0.5 ± 1.5 | < LOQ | < LOQ | < LOQ |
| | 50 | 1.5 ± 1.6 | 3.0 ± 1.5 | < LOQ | 25.6 ± 9.4 |
| Metabolite A | 0 | 1.5 ± 1.3 | NA | < LOQ | NA |
| | 50 | 29.4 ± 9.7 | | < LOQ | |

(a): After treatment with glucuronidase.
(b): LOQs = 1.5 to 2 μg EQ, EQDM and metabolite A/kg tissue, 0.5 μg EQI/kg tissue.

The Panel noted that EQDM concentration in fat of the control animals was absolutely very high (average > 370 μg/kg) as well as in relation to the treated group.

**Cattle for fattening**

Tissues (liver, kidney, muscle and subcutaneous fat) were sampled from four animals per group after a 42-day feeding period.[42] Residues of EQ, EQDM, EQI, DHEQ, DEQ (in all samples) and metabolites A, B and C (only in liver and muscle) were determined. The results are summarised in Table 6, reporting only the highest values (number of samples < 6). Results for DEQ, metabolites B and C were not considered (see Section 3.5.1) and not reported in the table.

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[41] FAD-2018-0029/Technical dossier/Annex 06.
[42] FAD-2018-0029/Technical dossier/Annex 07.

**Table 6:** Residues of EQ and metabolites/TPs in cattle tissue (μg/kg) following the administration of 50 mg EQ/kg complete feed for 42 days. Considering the low number of samples (four per group), the results reported include only the highest values

| Metabolite | EQ concentration (mg/kg feed) | Tissue/organ | | | |
| --- | --- | --- | --- | --- | --- |
| | | Liver[a] | Kidney | Muscle | Subcutaneous fat |
| EQ | 0 | 91 | 26.4 | < LOQ | 86.6 |
| | 50 | 34 | 19.9 | < LOQ | 95.9 |
| EQDM | 0 | < LOQ[b] | 6.2 | 2.4 | 66.3 |
| | 50 | < LOQ | 10.7 | 5.8 | 112.0 |
| EQI | 0 | 20 | 5.2 | < LOQ | 4.1 |
| | 50 | 20 | < LOQ | < LOQ | 14.1 |
| DHEQ | 0 | 4 | 2.4 | < LOQ | 5.0 |
| | 50 | < LOQ | < LOQ | < LOQ | 8.7 |
| Metabolite A | 0 | 61 | NA | < LOQ | NA |
| | 50 | 121 | | | |

(a): After treatment with glucuronidase.
(b): LOQs = 1.5–2 μg EQ, EQDM and metabolite A/kg tissue and 0.5–1 μg EQI and DHEQ/kg tissue.

The limited number of animals and dispersion of the results restricts the appraisal of differences between treatments. The Panel also noted that EQ and EQDM concentration in fat of the control animals were absolutely high (about 87 and 66 μg/kg, respectively) as well as in relation to the treated group.

**Laying hen's tissues and eggs**

Tissues (liver, kidney, muscle and skin/fat) were sampled from eight animals per group after a 56-day feeding period.[43] A total of 12 eggs/treatment were available for analysis (four eggs per day on three consecutive days, at the end of the experimental period). Residues of EQ, EQDM, EQI, DHEQ, DEQ and metabolites A, B and C were determined. The results are summarised in Table 7. Results for DEQ, metabolite B and C were not considered (see Section 3.5.1) and not reported in the table.

**Table 7:** Residues of EQ and metabolites/TPs in laying hen's tissues and in eggs (μg/kg tissue and liquid egg) from animals fed a complete feed supplemented with 50 mg EQ/kg for 56 days. Results are reported as mean ± SD

| Metabolite | EQ concentration (mg/kg feed) | Tissue/organ/product | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | Liver[a],[b] | Kidney[a] | Muscle[a] | Skin/fat[a] | Eggs[c] |
| EQ | 0 | 0.3 ± 0.8 | 0.6 ± 1.1 | 0.6 ± 0.6 | 4.6 ± 0.8 | < LOQ[d] |
| | 50 | 96.2 ± 50.6 | 189.5 ± 1.3 | 9.2 ± 5.3 | 297.9 ± 73.8 | 21.2 ± 3.1 |
| EQDM | 0 | < LOQ | < LOQ | 17.7 ± 3.0 | 12.5 ± 3.3 | < LOQ |
| | 50 | 3.0 ± 1.6 | 2.7 ± 1.3 | 0.3 ± 0.7 | 42.3 ± 19.2 | 6.6 ± 1.9 |
| EQI | 0 | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| | 50 | 5.6 ± 1.5 | 6.2 ± 2.7 | < LOQ | 25.6 ± 9.4 | < LOQ |
| DHEQ | 0 | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| | 50 | 0.8 ± 1.0 | 1.9 ± 1.3 | < LOQ | 1.8 ± 1.5 | < LOQ |

(a): 8 animals per treatment.
(b): After treatment with glucuronidase.
(c): 12 eggs/treatment.
(d): LOQs = 0.5 (muscle)−2 (other tissues) μg EQ/kg, 0.5–1 μg EQDM, 1.5 μg EQI and 0.5 μg DHEQ/kg all tissues; 2 μg EQ and metabolites/TPs/kg whole egg.

The Panel also noted that EQDM concentration in muscle and skin/fat of the control animals was absolutely high (about 18 and 13 μg/kg, respectively) as well as in relation to the treated group.

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[43] FAD-2018-0029/Technical dossier/Annex 05 and Annex 08.

**Cow milk**

Milk from five dairy cows per treatment was collected on the three last consecutive days of the study (from day 34 to 36).[44] Composite samples for each cow and day were prepared for analysis. Residues of EQ, EQDM, EQI, DHEQ, DEQ and metabolites A, B, and C were determined. At the end of the experimental period, all the five samples collected were analysed only in the group with 500 mg EQ/kg feed, three of them showing no EQ or any metabolite; instead, for the control, 50 mg/kg feed and the 150 mg/kg feed groups only two samples were analysed Considering the fact that in these last samples, residues were found, and the mismatch of the number of samples analysed, the applicant hypothesised an error in handling the samples. After 14 months, the stored samples, kept frozen, were reanalysed. In a first set of analysis, the composite samples collected on day 34 were reanalysed to compare the results with the one obtained in the original analysis. The results showed a change in the pattern of the metabolites, also showing an increase in the total residues. The applicant considered it as a worst case, and reanalysed individually all the available samples. Out of 15 samples expected for the group fed 50 mg EQ/kg feed (five cows fed 50 mg EQ/kg complete feed, three sampling days), certificates of analysis were made available only for 11 samples; only 13 certificates were made available for the control group.

Considering the error in sampling, the low number of samples per treatment, and the observed change in the pattern of metabolites between the two sets of analysis, the study cannot be further considered for the assessment of the consumer exposure.

**Fish flesh**

Flesh was sampled from 15 fish per group after a 90-day feeding period.[45] Residues of EQ and EQDM were determined by HPLC with fluorescence detection. EQ concentration was 40 ($\pm$ 4) μg/kg flesh and that of EQDM was 182 ($\pm$ 32) μg/kg flesh.

### 3.5.2. Health-based guidance value

The metabolic pathways of EQ in target animals (mammals, birds and fish) are qualitatively similar to those described in the laboratory animals. Therefore, the toxicological studies performed with EQ and EQDM cover the toxicological potential of EQ residues in tissues and products of the exposed target animals for the human consumer.

Based on a $BMDL_{10}$ of 1.1 mg EQDM/kg bw per day for microvescicular steatosis in liver of mice, the FEEDAP Panel derives a HBGV of 0.006 mg EQDM/kg bw per day, using an uncertainty factor (UF) of 200 (taking into account the additional UF of 2 for extrapolation from subchronic to chronic study duration in rodents) (EFSA Scientific Committee, 2012).

In its previous opinion (EFSA FEEDAP Panel, 2015), the Panel concluded that 'The toxicological profile of the ethoxyquin dimers is considered to reflect that of the precursor monomer'. The results of the mice study, in which a lowest $BMDL_{10}$ of 1.1 mg EQDM/kg bw per day for microvescicular steatosis in liver has been identified, confirm this conclusion (the lowest NOAEL observed in a dog study was 2 mg EQ/kg bw per day, based on hepatocellular necrosis).

As far as the critical toxicological endpoints and the highest safe levels established for EQ and EQDM are similar, the Panel applies the lowest health-based guidance value derived for EQDM for the sum of EQ and its TPs (EQDM, EQI, DHEQ and metabolite A).

### 3.5.3. Consumer exposure

Any estimate of consumer exposure following Regulation (EC) No 429/2008 has to consider total residue data for muscle, liver, kidney, skin (with natural proportion of fat), dairy milk and eggs. Reliable data on milk were not available, and therefore, the calculation of the consumer exposure did not include milk. Total residue values are usually derived from radio-labelled studies. Such studies are not available for the target species object of this application. The LC-MS/MS measurements of residues in the tissues sampled at the end of the tolerance studies performed instead offer a high sensitivity and allow identifying and quantifying EQ and its major metabolites/TPs (see Section 3.5.1).[46] Consequently, the sum of EQ and metabolites/TPs has been retained as a total residue surrogate of toxicological

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[44] FAD-2018-0029/Technical dossier/Annex 08.
[45] FAD-2017-0073/Technical dossier/Annex 21.
[46] For fish the residues were obtained with a HPLC.

relevance (Table 8). The sum of EQ and its metabolites/TPs included the value of the LOQ (for the respective matrices), when the result was reported as < LOQ.

**Table 8:** Maximised sum of EQ and metabolites/TPs concentrations ($\mu$g/kg) in target animal tissues retained for the calculation of consumer exposure

| Animal products/tissues | Sum EQ plus EQDM, EQI, DHEQ and metabolite A |
|---|---|
| **Piglet**[a] | |
| Liver | 84 |
| Kidney | 51 |
| Muscle | 71 |
| Fat | 1,713 |
| **Cattle**[b] | |
| Liver | 178 |
| Kidney | 33 |
| Muscle | 14 |
| Fat | 231 |
| **Laying hen**[a] | |
| Liver | 215 |
| Kidney | 214 |
| Muscle | 24 |
| Skin/fat | 575 |
| Whole egg | 46 |
| **Fish (salmon)**[a] | |
| Flesh | 294 |

(a): Average values plus two times standard deviation.
(b): The value reported is based in the highest values observed, due to the low number of samples analysed.

An estimate of consumer exposure was done following the methodology described in the Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017). The input data used to estimate exposure are reported in Table 9.

**Table 9:** Input data on EQ and its major metabolites/TPs content in food of animal origin used for the consumer exposure assessment

| Animal product | EQ (mg/kg wet tissue or product) |
|---|---|
| Birds fat tissue | 0.575 |
| Birds liver | 0.215 |
| Birds meat[a] | 0.079 |
| Birds offals and slaughtering products (other than liver)[b] | 0.214 |
| Fish (meat) | 0.294 |
| Mammals fat tissue | 1.713 |
| Mammals liver | 0.178 |
| Mammals meat[c] | 0.399 |
| Mammals offals and slaughtering products (other than liver)[b] | 0.051 |
| Whole eggs | 0.046 |

(a): Calculated by default as 90% muscles and 10% fat tissue.
(b): Kidney values taken by default.
(c): Calculated by default as 80% muscles and 20% fat tissue.

The results are summarised in Table 10. The detailed results are given in Appendix B.

**Table 10:** Chronic human dietary exposure to EQ and its major metabolites/TPs. Maximum highest reliable percentile expressed in mg/kg bw per day

| Population class | Number of surveys | Maximum HRP* | % HBGV[†] |
|---|---|---|---|
| Infants | 6 | 0.0034 | 57 |
| Toddlers | 10 | 0.0042 | 70 |
| Other children | 18 | 0.0046 | 78 |
| Adolescents | 17 | 0.0039 | 65 |
| Adults | 17 | 0.0027 | 46 |
| Elderly | 14 | 0.0018 | 30 |
| Very elderly | 12 | 0.0018 | 30 |

*Highest reliable percentile.
[†]HBGV: Health-based guidance value: 0.006 mg/kg body weight and day.

The highest exposure at the 95th percentile occurs in other children followed by toddlers and adolescents. For all consumer categories, exposure in the individual countries was constantly below 0.005 mg/kg bw per day, corresponding to approximately 80% of the HBGV.

The FEEDAP Panel noted that the analytical difficulties in identifying and quantifying EQ and its metabolites/TPs in feed and food, their chemical instability as well as the unknown feeding history of all animals used for the residue studies,[47] do not allow an exact quantification of EQ-related residues. These uncertainty elements contain factors which might equally result in over-/underestimation of residues.

The Panel concludes that the calculated exposure values can be taken as a valid estimate of the consumer exposure.

### 3.5.4. Conclusions on the safety for the consumer

The Panel concludes that the use of ethoxyquin at a maximum total concentration of 50 mg/kg in complete feed for all animal species, except dairy animals, would not result in residues of EQ or its metabolites/TP which would pose a risk for the consumer. In the absence of residue data in milk, the Panel cannot conclude on the safety for the consumer of EQ when used also in feed for milk-producing animals. The Panel reiterates its previous conclusion that, in the absence of data on the presence of p-phenetidine in tissues and products of animal origin, no conclusion on the safety of the consumer could be drawn.

It is noted that the conclusions on consumer safety are based on the assumption that the maximum total concentration of 50 mg EQ/kg feed is expressed as the sum of EQ, EQDM, EQI and DHEQ.

### 3.6. Safety for the user

In its previous opinion (EFSA FEEDAP Panel 2015), the FEEDAP Panel stated that 'In the absence of data, it is assumed that workers may be exposed by inhalation to a mist of the additive'. The additive contains p-phenetidine, classified as a possible mutagen. Therefore, exposure of the unprotected user to p-phenetidine via inhalation cannot be excluded. The Panel concludes that, to reduce the risk, the exposure of the users should be minimised.

### 3.7. Safety for the environment

The active ingredient is not a physiological/natural substance of established safety for the environment.

The FEEDAP Panel noted that, although the original dossier was submitted before the date of implementation (September 2019) of the updated FEEDAP guidance on the safety of feed additives for the environment, the assessment of the safety of EQ for the environment performed by the applicant for the present evaluation was prepared following the updated guidance. Therefore, the FEEDAP Panel assessment has followed the principles set in its updated guidance (EFSA FEEDAP Panel, 2019).

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[47] In some tissues of piglets, cattle and laying hens, unexplained relatively high level of EQ and EQ-related substances residues were found in the control groups. This may be a the result of a dietary exposure to EQ in a period before these animals were used in the study.

### 3.7.1. Phase I

**Physical–chemical properties of ethoxyquin**

The physical–chemical properties of EQ are summarised in Table 11.

**Table 11:** Physical–chemical properties of ethoxyquin (EFSA, 2010)

| Property | Value | Unit |
|---|---|---|
| Octanol/water partition coefficient (log Kow 25°C) | 3.18 (pH 5) 3.39 (pH 7) 3.19 (pH 9) | |
| Water solubility | 101 (pH 5), 60 (pH 7), and 70 (pH 9) at 20°C | mg/L |
| Vapour pressure | $3.46 \times 10^{-2}$ at 25°C | Pa |
| Dissociation constant | 4.56 at 22°C | – |

A phase I assessment was performed for ethoxyquin when used as a feed additive for both terrestrial and aquatic animals.

**Predicted environmental concentrations (PECs)**

*Additives for terrestrial animals*

The PECs for EQ in soil and groundwater were calculated based on inclusion of 50 mg EQ/kg feed, an estimated $K_{oc}$ of 1,190 L/kg (PCKOC Program v1.66 in EPIWIN v3.12) and 100% excretion. The highest $PEC_{soil}$ value calculated is 1.01 mg/kg soil dry weight (DW). The highest calculated $PEC_{groundwater}$ is 10.5 μg/L. Since both $PEC_{soil}$ and $PEC_{groundwater}$ exceed the respective trigger values, a phase II assessment is required for EQ when used on terrestrial animals.

*Additives for aquatic animals*

The PECs for EQ in sediment ($PEC_{sed}$) and in surface water from aquaculture ($PEC_{swaq}$) were calculated based on inclusion of 50 mg EQ/kg feed and 100% excretion.

The $PEC_{sed}$ for marine aquaculture was 110 mg/kg wet weight (WW), far above the trigger value of 10 μg/kg WW. The values of $PEC_{swaq}$ were below the trigger value of 0.1 μg/L for salmon, trout and turbot, while it was 0.125 μg/L for seabass/seabream. A phase II assessment is required for EQ for both surface water and marine aquaculture.

### 3.7.2. Phase II

**Fate and behaviour**

*Fate in soil*

Adsorption/desorption in soil

The adsorption coefficient ($K_{oc}$) of EQ in soil was estimated in a study performed according to Good Laboratory Practice (GLP) using the retention time of the test item measured by HPLC, in accordance to OECD 121.[48] As EQ can be ionised at pH values relevant in soil, it was necessary to conduct tests at pH 6.5 and pH 2.5. For each pH tested, reference items were chromatographed before and after the duplicate injections of EQ, and the mean k' value determined. $K_{oc}$ values for EQ were then estimated by interpolation from the reference item regression line.

The estimated $K_{oc}$ value for EQ at pH 6.5 was 331 (Log $K_{oc}$ 2.52), showing therefore a medium mobility in soil. The estimated $K_{oc}$ value for EQ at pH 2.5 was 35 (Log $K_{oc}$ 1.55), showing a much higher mobility at very low pH. However, the FEEDAP Panel noted that this may not be a true representation of the mobility of EQ in low pH soil conditions as this study type does not replicate interactions associated with the cation exchange capacity of soil.

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[48] FAD-2018-0013/Spontaneous submission September 2020/Annex 1.

Degradation in soil

The route and rate of degradation of $[^{14}C]$-EQ were investigated in two US soils (Iowa (silt loam) and MCL-PF (clay loam)) and two European soils (Clipstone (loamy sand) and Speyer 2.4 (clay loam)) in a GLP study performed according OECD 307.[49] EQ was applied at a treatment rate equivalent to 2.13 mg/kg DW; the treated soils were incubated in the dark, at $20 \pm 2°C$ for up to 14 days, under aerobic conditions at a soil moisture between the pF 2 and pF 2.5. Duplicate samples were taken for analysis at 0, 0.5, 1, 2, 3, 7 and 14 days after treatment.

The overall recovery of radioactivity was good throughout the study. The mass balance for all individual soil samples ranged from 90.3% to 109.8% application ratio (AR). EQ was observed only in the Iowa soil at time zero (13.5% AR) with no significant levels detected thereafter, or in any of the other soils. EQ degraded very rapidly to the transformation product EQI; therefore, the rate of degradation in each soil was estimated not for EQ but for the next abundant significant metabolite, EQI. The degradation $DT_{50}$ and $DT_{90}$ values were calculated following the recommendations of the FOCUS working group 'Degradation Kinetics'[50] Best-fit kinetics (single first-order rate model, Gustafson and Holden model (FOMC) and double first-order in parallel (DFOP)) were used for EQI; for all the four soils the best fit was obtained through the DFOP kinetics. Results are reported in Table 12, together with the main characteristics of the tested soils.

**Table 12:** Degradation $DT_{50}$ and $DT_{90}$ values for EQI according to double first-order in parallel kinetics

| Soil | % OC | pH 0.01 M CaCl₂ (1:2 w/v) | $DT_{50}$* [days] | $DT_{90}$ [days] | $X^2$ | $R^2$ |
|---|---|---|---|---|---|---|
| Iowa (silt loam) | 1.9 | 6.7 | 2.2 | 6.3 | 10.4 | 0.9647 |
| MCL-PF (clay loam) | 2.4 | 7.5 | 2.0 | 5.7 | 11.6 | 0.9744 |
| Clipstone (loamy sand) | 1.5 | 5.2 | 1.7 | 4.4 | 6.2 | 0.9931 |
| Speyer 2.4 (clay loam) | 1.9 | 7.4 | 1.8 | 3.9 | 11.4 | 0.9585 |
| **Geomean** | – | – | **1.9** | **5.0** | –– | |

*The longest phase $DT_{50}$ was considered.

*Conclusion on fate and behaviour*

The geomean $DT_{50}$ of 1.9 days at 20°C (corresponding to a geomean $DT_{50}$ of 4 days at 12°C) for EQI does not highlight any concern of accumulation during years.

A $K_{oc}$ of 331 L/kg and a $DT_{50}$ of 4 days at 12°C (a $DT_{50}$ surrogated from EQI) will be used for the Phase II exposure assessment for EQ in the different environmental compartments.

**Exposure assessment**

*PECs calculation refined in Phase II*

The PECs for the different compartments were recalculated using the experimental values for $DT_{50}$ and $K_{oc}$. In Table 13, the highest PEC values obtained for terrestrial animals are reported.

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[49] FAD-2018-0013/Spontaneous submission September 2020/Annex 2.
[50] https://esdac.jrc.ec.europa.eu/projects/degradation-kinetics

**Table 13:** Predicted environmental concentration of EQ in soil, groundwater, surface water and sediment

| Input | Value |
|---|---|
| Dose | 50 |
| Molecular weight | 217.31 |
| Vapour pressure (Pa) | $3.5 \times 10^{-2}$ |
| Solubility (mg/L) at pH 7 | 60 |
| $K_{oc}$ (L/kg) | 331 |
| $DT_{50}$ in soil at 12°C (days) | 4 |
| **Output** | |
| $PEC_{soil}$ (μg/kg) | 1,007 |
| $PEC_{groundwater}$ (μg/L) | 37 |
| $PEC_{surface\ water}$ (μg/L) | 12 |
| $PEC_{sediment}$ (μg/kg) | 456 |

*$PEC_{soil}$ refined for metabolism*

The information provided does not allow a further refinement based on metabolism.

*$PEC_{soil}$ refined for multiple application*

Manure may be spread in more than one spreading event. Taking into account that all animal species have to be considered for EQ, just two applications are considered reasonable for the PEC refinement. Table 14 reports the highest PEC values obtained for terrestrial animals, considering two applications spaced 90 days and a soil $DT_{50}$ of 4 days.

**Table 14:** Refined predicted environmental concentration of EQ for multiple application in soil, groundwater, surface water and sediment

| Output | |
|---|---|
| $PEC_{soil}$ (μg/kg) | 504 |
| $PEC_{groundwater}$ (μg/L) | 19 |
| $PEC_{surface\ water}$ (μg/L) | 6 |
| $PEC_{sediment}$ (μg/kg) | 228 |

*$PEC_{groundwater}$ refinement*

Considering the geomean $DT_{50}$ of 1.9 days at 20°C and the $K_{oc}$ of 331 L/kg and applying the inequality described in the EFSA guidance on the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019) related to the requirements for the $K_{OM}$ (= $K_{oc}$/1.7) as a function of the leaching concentration as provided by the FOCUS groundwater modelling, EQ is considered not to pose any risk to groundwater.

**Ecotoxicity studies**

EQ is reactive in soil and it disappears quickly in water. This is also observed in the ecotoxicity studies submitted for the present assessment. In soil studies, no measurable concentrations can be expected immediately after dosing and the FEEDAP Panel agreed to base the evaluation of the studies on nominal concentrations. In water or water-sediment studies, the disappearance is not so immediate as in soil and when measurements were within an acceptable range (80–120% of the nominal concentrations), the evaluation was based on nominal concentrations. Otherwise, analytical measurements within 24 h after dosing were used in the assessment. This allowed checking for possible issues related to the dosing and it is consistent with the total residue approach.

*Toxicity of ethoxyquin on soil organisms*

Effects on soil microorganisms

The potential effects of EQ on soil microflora under aerobic conditions were investigated in a GLP study conducted according to OECD guidelines 216 (Nitrogen Transformation Test).[51] Due to low water solubility, the test item was thoroughly mixed with quartz sand. Subsequently, the obtained mixture was added and mixed with the soil by means of a hand stirrer. The experiment was carried out over a period of 28 days. EQ was tested in two concentrations (1.07 and 10.70 mg test item/kg soil DW), plus control (three replicates). A sample of each replicate of each treatment was taken at intervals of 3 h, 7, 14 and 28 days and the mineral nitrogen content of the soil was determined. Since the maximum variation was below 15%, the validity criterion was fulfilled. On day 28, at the highest tested concentration, EQ caused no adverse effects (deviation from control $< 25\%$, OECD 216) on soil nitrogen transformation. Therefore, the additive poses no risk for soil microbes.

Effects on earthworms

The sublethal effects of EQ on reproduction, mortality and growth of the earthworm *Eisenia andrei* was assessed in a GLP study conducted in accordance with OECD 222 (test duration 56 days).[52] EQ was tested at eight nominal concentrations (16, 29, 53, 95, 171, 309, 556, 1,000 mg/kg of soil DW) plus solvent control (four replicates for the test item treatments, eight replicates for the solvent control, 10 worms per replicate). The validity criteria for the solvent control group were met. The tested concentrations are expressed as a nominal concentration; EQ is readily degraded in soil and according to the OECD 222 for substances for which there is uncertainty in maintaining the nominal soil concentration, analytical measurements of the exposure concentrations at the beginning, during and at the end of the test, should be considered. The FEEDAP Panel noted that these concentrations were not recorded. The calculated $LC_{50}$ (mortality) value was 693 mg test item/kg soil DW. The NOEC for mortality and biomass was determined to be 556 and $\geq 556$ mg test item/kg soil DW, respectively. The NOEC for reproduction was determined to be 171 mg test item/kg soil DW.

Effects on terrestrial plants

A 21-day seedling emergence and seedling growth test (according to OECD Guideline 208) was performed to test the phytotoxicity of ethoxyquin to three plant species (the monocot *Avena sativa* (oat) and the dicots *Brassica napus* (oilseed rape) and *Pisum sativum* (pea)).[53] The tests were performed in a climate chamber with a photoperiod of 16 h light/8 h dark, using seven pots with three seeds per replicate for oilseed rape and pea as well as five pots with five seeds per replicate for oat. EQ was applied before sowing via incorporation into the soil at nominal test concentrations of 37, 111, 333 and 1,000 mg test item/kg soil DW. Since the test item is only poorly soluble in water, the test item was applied to the soil using acetone as a volatile solvent (according to OECD guideline 208). The measured concentrations of EQ in the analysed test solutions remained of 90% of nominal values. After 50% of the control plants had emerged, the seedling emergence, survival/mortality and visual phytotoxicity of the plants were observed weekly for 21 days. At the end of the study, seedling emergence, survival of emerged seedlings, biomass (shoot fresh weight) and visible detrimental effects were recorded. All validity criteria were met.

Statistically significant effects regarding seedling emergence and survival of emerged seedlings were calculated for oilseed rape and pea at a concentration of 1,000 mg test item/kg soil DW. No adverse effects on seedling emergence and survival of emerged seedlings for oat were found. Shoot fresh weight was the most sensitive endpoint, and for all species tested, statistically significant effects at concentrations of 111 mg test item/kg soil DW were found. Related $EC_{50}$ values derived from dose responses curves were 242.2 mg/kg for oat, 155.6 mg/kg for oilseed rape and 181.3 mg/kg for pea. The lowest calculated $EC_{50}$ of 155 mg test item/kg soil DW and $EC_{10}$ of 55.8 mg test item/kg soil DW can be used for terrestrial risk assessment. Although the ecotoxicity study in three terrestrial plants would allow to calculate a predicted no effect concentration (PNEC), the FEEDAP Panel notes that according to the updated guidance on the safety of feed additives for the environment (2019), this PNEC should have been derived from six terrestrial plants instead of three. The study does therefore not allow to conclude on the ecotoxicity of EQ for terrestrial plants.

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[51] FAD-2018-0013/Technical dossier/Annex 11.
[52] FAD-2018-0013/Technical dossier/Annex 10.
[53] FAD-2018-0013/Technical dossier/Annex 18.

*Toxicity of ethoxyquin on freshwater organisms*

Effects on algae

A 72-h toxicity test with the freshwater alga *Pseudokirchneriella subcapitata* according to OECD Guideline for Testing of Chemicals No. 201 was performed by Wildlife International (US) in 2006, under GLP as published by US EPA (2004).[54] In short, five test concentrations, nominal 0.63, 1.3, 2.5, 5 and 10 mg of EQ/L, based on a range finder were tested at 24°C and 7,500 lux light intensity, in three replicates (six in negative/solvent controls). Cell densities were sampled every 24 h using a coulter particle counter.

Nominal and measured concentrations matched relatively well, with deviations of max 12%, except for the highest treatment level where the measured concentration was 156% of the nominal. In all treatments, concentration of EQ was below the limit of quantification (0.4 mg/L) after 3 days. Growth rates in the six control replicates showed coefficients of variation of 11.7, 6.7 and 3.7% after 24, 48 and 72 h. Cell increase from hour 0 to hour 72 was approximately 182-fold (min 16-fold). The pH value increased by a maximum of 1.6 during the experiments, which is above the threshold value of 1 pH unit in the current guidelines. Based on the growth rate, also an NOEC of 2.3 mg/L was established, the $E_rC_{50}$ is > 16 mg/L.

Effects on crustaceans

Acute toxicity of ethoxyquin to *Daphnia magna* in a 48-h semi-static GLP test was performed according to OECD 202 (2004).[55] The study report is comprehensive, the experimental set-up, analytics and the study results have been presented in detail. Validity criteria were met and the test with the reference item confirmed the sensitivity of the test system.

Being fairly soluble within the selected exposure concentration range, EQ was dissolved directly into the test medium, without use of solvent or carrier. The five tested concentrations ranged from 0.5 to 8 mg/L (nominal). Due to the low stability of EQ in water, nominal concentrations were analytically checked immediately at the start of the test, after 3 h and after 24 h. In freshly prepared solutions for the full renewal, the same analytical scheduled was applied. The measured concentrations of test item in the test solutions were within ranges of 89–98% of nominal concentrations in the freshly prepared test solutions at the start of the test and at renewal after 24 h and within a range of 49–82% in the spent solutions at the renewal of the test solutions after 24 h and at the test end (48 h) based on nominal values. Considering the experimental set-up and test substance chemical properties, the results expressed on nominal concentration basis seem appropriate. Based on nominal concentrations, the 48h $EC_{50}$ value (immobilization) for EQ was 2.48 mg/L (95% confidence interval 2.08–2.95) and this value is used for risk assessment.

A chronic toxicity GLP study of ethoxyquin to *Daphnia magna* (21-day semi-static reproduction test) was performed according to OECD 221 (2012).[56] The study report is comprehensive, the experimental set-up, analytics and the study results have been presented in detail. Validity criteria were met and the test with the reference item confirmed the sensitivity of the test system.

EQ was dissolved directly into the test medium, without the use of solvent or carrier. The tested concentrations ranged from 2.96 to 992.7 μg/L of EQ based on nominal values. The offspring produced by each parent animal were removed and counted daily from the appearance of the first brood. Mortality amongst the parent animals was recorded daily and the test medium was renewed daily. The test duration was 21 days. Due to the low stability of EQ in water, nominal concentrations were analytically checked immediately at the start of the test, once a week at renewal processes and at the test end for the fresh and spent test solutions. The measured concentrations of the test item in the test solutions were within ranges of 84% of nominal concentrations in the freshly prepared test solutions at the start of the test and at renewal after week 1, week 2 and week 3 and within a range of 18–88% in the spent solutions at the renewal of the test solutions after 24 h based on nominal values. Considering the experimental set-up and test substance chemical properties, the results expressed on a nominal concentration basis seem appropriate. Based on nominal concentrations, the 21-day $EC_{10}$ value (number of offspring per introduced and survived parent) for EQ was 59.7 μg/L (95% confidence interval 46.9–76.0).

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[54] FAD-2018-0013/Spontaneous submission September 2020/Annex 3.
[55] FAD-2018-0013/Technical dossier/Annex 12.
[56] Technical dossier EFSA-Q-2021-00523/Annex 1.

Effects on fish

A 96-h non-GLP acute toxicity study with the rainbow trout (*Oncorhynchus mykiss*) according to ASTM E729-88a, which is equivalent to OECD TG 203, was performed (1996).[57] Five test concentrations in two replicates were tested, nominal 1.9, 3.8, 7.5, 15 and 30 mg/L of EQ, a solvent control and a negative (well water) control. Each test chamber contained 10 rainbow trout. Delivery of the test substance was initiated 43.5 h prior to the introduction of the fish to the test water in order to achieve equilibrium of the test substance in the test chambers. The fish were assigned to exposure chambers at test initiation without distinction. After 3.5, 24, 96 h, observations of mortality and other clinical signs were made. Cumulative percent mortality observed on the treatment groups was used to calculate $LC_{50}$ values at 24, 48, 72 and 96 h.

Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at the beginning of the test, at 48 and 96 h. Nominal and measured concentrations matched relatively well. Samples collected at the beginning of the test had measured values that ranged from 67% to 96% of nominal values. Measured values collected for samples taken at 48 h ranged from 84% to 114% of nominal values and at 96 h from 89% to 100% of nominal values, respectively. Temperatures were within the limits of the $12\pm1°C$ range, dissolved oxygen concentration exceeded 60% saturation throughout the test, measurements of pH ranged from 8.1 to 8.4. The 96-h $LC_{50}$ value for *O. mykiss* determined in test was 18 mg/L EQ with 95% confidence limits that ranged from 14 to 23 mg/L EQ, based on measured concentrations.

Effects on freshwater sediment-dwelling organisms

The effects of ethoxyquin on the development of the midge *Chironomus riparius* in a water-sediment system were determined in a GLP study carried out in accordance with the OECD guideline 218.[58] The dose response test (static, 28 days) was conducted by spiking the sediment layer with five concentrations of EQ (nominal concentrations of 62.5, 125, 250, 500 and 1,000 mg/kg sediment DW). All validity criteria were met throughout the study. As all test item concentrations on day 0 were in the range of 80–120%, all evaluation was based on the nominal values. Eighty first-instar larvae were exposed to each test concentrations and controls (four replicates with 20 larvae each). In the control, solvent control and the test groups 62.5 and 125 mg/kg sediment DW, the midges started to emerge 12 days after larvae insertion; the emergence finished on day 22 after larvae insertion. In the test group of 250 mg/kg, emergence of the midges started 13 days after larvae insertion and was finished at day 22 after larvae insertion. No emerge of the larvae was observed at the test groups of 500 and 1,000 mg/kg sediment DW. Based on the obtained results, it was concluded that EQ did not affect the emergence rate of *C. riparius* up to a concentration of 250 mg/kg sediment DW (NOEC emergence rate = 250 mg/kg sediment DW). However, the development rate was significantly affected at the EQ concentration of 250 mg/kg and higher ($EC_{10}$ development rate > 250 mg/kg sediment DW; NOEC development rate was 125 mg/kg sediment DW). The NOEC development rate of 125 mg/kg DW is used in the risk assessment.

The effects of EQ on the oligochaete *Lumbriculus variegatus* were determined in a water-sediment system.[59] The study was carried out in accordance with the OECD Guideline 225, and by spiking artificial sediment with the test item (nominal concentrations of 18.8, 37.5, 75, 150, 300 and 600 mg/kg sediment DW and four replicates per concentration). A control using untreated artificial sediment as well as a solvent control using artificial sediment treated with the solvent were set up (six replicates per control and solvent control). All validity criteria were fulfilled. After 28 days of exposure, EQ induced no mortality up to concentrations of 150 mg/kg sediment DW. However, at the test item concentrations of 300 and 600 mg/kg sediment DW 50 and 100% mortality occurred, respectively. Since a statistically significant difference between the control and the solvent control was identified in case of total worm number/reproduction, the solvent control was used for all further evaluation. The total number of worms did not statistically significantly differ from the solvent control up to concentration of 150 mg/kg sediment DW. However, at the test item concentrations of 300 and 600 mg/kg sediment DW the total number of worms was reduced significantly. Compared to the solvent control, the difference in total biomass per replicate was not statistically significant at the test item concentration up to 300 mg/kg sediment DW. Due to 100% mortality, no total biomass per replicate was determined at the test item concentration 600 mg/kg sediment DW. Overall, the NOEC for total number of worms and reproduction was determined to be 150 mg test item/kg sediment DW.

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[57] FAD-2018-0013/Spontaneous submission September 2020/Annex 4.
[58] FAD-2018-0013/Spontaneous submission September 2020/Annex 5.
[59] FAD-2018-0013/Spontaneous submission September 2020/Annex 6.

The NOEC for biomass amounts to 300 mg test item/kg sediment DW. The corresponding $EC_{10}$ for total number of worms/reproduction and total biomass per replicate was 148 mg test item/kg sediment DW that is used in the assessment.

A sediment toxicity test on the nematode *Caenorhabditis elegans* was performed according to the Guideline ISO 10872 in a GLP study.[60] The effects of EQ were determined at 96 h after insertion of the test organisms and using concentrations of 25.6, 64, 160, 400 and 1,000 mg/kg sediment DW (four replicates per control and per test item concentration). Analytical evaluation was carried out via LC/MS-MS for the control and all test item concentrations. The recovery rates in the spiking solutions ranged from 53% to 108% of the nominal test item concentrations at day 1. The relatively low recovery rate of 53% was confirmed by reanalysis and may therefore be caused by a dilution mistake during spiking. As the corresponding test item concentration of 64.0 mg/kg sediment DW is far below the effective test item concentration of 1,000 mg/kg sediment DW, this finding is regarded to have no influence on the outcome of the study. All validity criteria were fulfilled. Since no statistically significant difference was observed between the control and the solvent control, both controls were pooled for further evaluation. No reduction of nematode reproduction was observed at the test item concentrations up to 400 mg/kg sediment DW. However, EQ concentration of 1,000 mg/kg sediment DW significantly reduced the reproduction of *C. elegans*. Moreover, ethoxyquin did not induce statistically significant growth reduction at all treatment rates compared to the pooled control. Overall, concerning the test item EQ the NOEC for fertility, reproduction and growth was determined to be 400 mg/kg sediment DW. The corresponding $EC_{10}$ was 727 mg/kg sediment DW for reproduction, whereas it was > 1,000 mg/kg sediment DW for fertility and growth. The NOEC of 400 mg/kg sediment DW is used in the assessment.

*Toxicity of ethoxyquin in marine organisms*

Effects on marine algae

The inhibitory effects of EQ on the growth of the marine microalgae *Skeletonema pseudocostatum* (strain NIVA BAC 1) were investigated according to ISO 10253:2016, Water quality – Marine algal growth inhibition test with *Skeletonema* sp. and *Phaeodactylum tricornutum*.[61] A series of test concentrations ranging from 0.1 to 10 mg/L of EQ was prepared from stock solutions in acetone. The final acetone concentration in all test solutions was 0.1 mL/L (0.01%). A solvent control with 0.01% of acetone (0.1 mL/L) was included in addition to a dilution water control (filtered natural sea water). The solutions were inoculated with approximately $5 \times 10^3$ cells/mL of an exponentially growing culture of *Skeletonema pseudocostatum*. Three replicates of each concentration were incubated in 25 mL glass flasks with 15 mL test volume, in an incubator with orbital shaking set to $20 \pm 2°C$ and under continuous light. Six replicate cultures in growth medium were used as controls in addition to the solvent control. Growth was monitored using a coulter counter at 24, 48 and 72 h. Measured concentrations showed considerable deviations from the nominal ones, with initial ratios of 68–208%. In all treatments, concentration of EQ was above the limit of quantification (0.05 mg/L) after 3 days. Growth rates in the six control replicates showed coefficients of variation of 4.1%, 4.1% and 5.5% after 24, 48 and 72 h. Cell increase from hour 0 to 72 h was approx. 112-fold (min 16-fold). The pH value in the controls increased by 0.85 during the experiments.

Based on the growth rates (geomean of measured concentrations at 0 and 72 h), an NOEC of 2.2 mg/L and an $E_rC_{50}$ of 3.3 mg/L were established. The FEEDAP Panel notes, however, that the use of the geomean of the measured concentrations at 0 and 72 h is considered invalid due to the large deviations between nominal and measured concentrations, so that rather the initially measured concentrations should have been used. In addition, the inhibition of algae growth showed no monotonous dose-response pattern, since the nominal treatment 1 mg/L (dosed with initially 1.4) showed larger inhibition as compared to the nominal concentration of 3.2, which was initially dosed with 6.6 mg/L. While problems with the dosing are potentially tolerable due to the difficulties in handling the test substances, the above reported issues lead to reject this test.

Effects on marine invertebrates (sediment-dwelling organisms and other marine invertebrates)

The effects of EQ on marine invertebrates have been evaluated following the requirement of the FEEDAP guidance on the assessment of the safety of feed additives for the environment (2019), which requires the submission of studies in three marine sediment species. Four new studies were submitted.

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[60] FAD-2018-0013/Spontaneous submission September 2020/Annex 07.
[61] FAD-2018-0013/Spontaneous submission September 2020/Annex 13.

A 10-day GLP acute toxicity study with the marine polychaete worm *Arenicola* sp., according to OSPAR Commission protocol on methods for the testing of chemicals used in the offshore oil industry (2006) and ICES Times No. 29 (2001), was performed with modifications.[62] The test animals were field collected specimens and field collection was not according to the protocol. The species was confirmed by the supplier. Five test concentrations (spiked overlying water) in three replicates ranging from 0.1 to 10 mg/L (nominal) of EQ were prepared from stock solution in acetone. Final acetone concentration in all test solutions was 0.1 mL/L (0.01%). A solvent control with 0.01% of acetone was included in addition to the dilution water control (natural seawater). Three replicate vessels, each containing five *Arenicola* worms were used for the controls and each treatment. No food was provided to the worms during the study (10 days) and survival was assessed at the end. The measured concentrations were in the range from 40.5% to 100% at day 0, control and solvent control samples were < LOQ. The data generated during the study showed a 10-day $LC_{50}$ 1.93 mg/L, based on initial measured concentrations; this value can be used for the risk characterisation. Since just concentration in water column is available, the corresponding PNEC for sediment organisms may be calculated through the equation of equilibrium partitioning.

A 24-h GLP embryo-larval developmental test with the Pacific oyster *Crassostrea gigas*, according to ASTM, 1994; Environment Agency, 2007; ICES 2013; ISO 17244, was submitted.[63] Conditioned Pacific oysters were obtained in spawning condition from a commercial farm. The bioassay considers the larval development phases from 16 to 32 cell stage to the stage D larvae, namely exposure to toxic substances and components which could have sublethal teratogenic effects. Five test concentrations ranging from 0.1 to 10 mg/L of EQ were prepared from stock solutions in acetone, the final acetone concentration was 0.1 mL/L. A solvent control with 0.1 mL/L of acetone was included in addition to the dilution water (filtered natural sea water) control and the positive control series with zinc (Zn). After 24 h of exposure, the organisms were fixated and stored under cold condition until they were scored. The chemical analysis indicated that EQ concentrations were in the range from 49.4% to 91.2% at day 0, control and solvent control samples were < LOQ. A significant effect on development was observed at the highest tested concentration of EQ, 4.9 mg/L (initially measured), resulting in a 24-h NOEC of 2.9 mg/L. The predicted $EC_{50}$ value based on the initial measured concentrations was 4.3 mg/L. The FEEDAP Panel noted that the embryo-larval development of Pacific oyster *Crassostrea gigas* was considered methodologically acceptable but of marginal relevance for the assessment because this organism is living in the water column rather than in sediment (the test is sediment-free).

The applicant submitted two additional studies: an acute and a chronic toxicity study in marine copepod *Tisbe battagliali*, again an organism that is living in the water column instead of in sediment.[64,65] The *T. battagliali* studies showed unexpected results (in the acute study for instance the measured concentrations were higher than nominal concentrations and $EC_{50}$ of measured concentrations would be higher than that of the nominal concentrations; in the chronic study, there was high parent mortality in test treatments during the exposure, reverse concentration – response curve for the mortality and non-monotonous concentration – response curve for reproduction) and were not further considered in the assessment.

In conclusion, out of the four studies submitted, only the one performed in *Arenicola* sp. can be used for the assessment of the effects of EQ on marine invertebrates; a 10-day $LC_{50}$ of 1.93 mg/L was used for the risk characterisation. The FEEDAP Panel notes that studies in at least two additional marine sediment species are missing to fulfil the requirements of the FEEDAP guidance (EFSA FEEDAP Panel, 2019).

### Risk characterisation (PEC/PNEC ratio)

*Terrestrial animals*

For the terrestrial compartment, the PEC/PNEC ratio for earthworms is below (Table 15) and based on the results of a nitrogen transformation test, EQ poses no risk for soil microbes. The calculation of the PEC/PNEC ratio for plants using the results of the test submitted in the dossier with three plants gives a value below 1. The FEEDAP Panel notes that according to the updated guidance on the safety of feed additives for the environment (2019), the PNEC for plants should have been derived from six

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[62] FAD-2018-0013/Spontaneous submission September 2020/Annex 9.
[63] FAD-2018-0013/Spontaneous submission September 2020/Annex 11.
[64] FAD-2018-0013/Spontaneous submission September 2020/Annex 12.
[65] FAD-2018-0013/Spontaneous submission September 2020/Annex 10.

terrestrial plants instead of three. In the absence of an adequate study in plants, the FEEDAP Panel cannot conclude on the safety of EQ for the terrestrial compartment when fed to terrestrial animals.

The risk characterisation for aquatic compartment is reported in Table 16.

**Table 15:** Risk characterisation (PEC/PNEC ratio) for terrestrial compartment

| Taxa | PEC$_{soil}$ ($\mu$g/kg) | NOEC (mg/kg) | AF | PNEC ($\mu$g/kg) | PEC/PNEC |
|---|---|---|---|---|---|
| **Earthworm** | 504 | 171 | 10 | 17,100 | 0.03 |

From the table above a risk cannot be excluded for aquatic organisms when EQ is used in

**Table 16:** Risk characterisation (PEC/PNEC ratio) for freshwater compartment

| Taxa | PEC$_{sw}$ ($\mu$g/L) | E$_{(r)}$(L)C$_{50}$/EC$_{10}$/NOEC (mg/L) | AF | PNEC ($\mu$g/L) | PEC/PNEC |
|---|---|---|---|---|---|
| **Algae** *Pseudokirchneriella subcapitata* | 6 | 16* 2.3** | 50 | 1.2 | 5 |
| **Aquatic invertebrates** *Daphnia magna* | | 2.48* 0.06*** | | | |
| **Fish** *Oncorhynchus mykiss* | | 18* | | | |

*E$_{(r)}$(L)C$_{50}$.
**NOEC.
***EC$_{10}$.

terrestrial animals. The applicant also provided a water sediment study,[66] performed according to OECD Guidance 308, showing a rapid dissipation of EQ both from surface water and from sediment. However, the results of this study were not used by the applicant to refine the aquatic exposure. The FEEDAP Panel notes that a higher tier FOCUS SW models could provide far lower values for the predicted concentration in surface water and in sediments. The risk for aquatic organisms might therefore be addressed with a refined calculation.

The risk characterisation for freshwater sediment is reported in Table 17.

Unacceptable risk is not expected for sediment dwelling organisms when EQ is used in terrestrial

**Table 17:** Risk characterisation (PEC/PNEC) for freshwater sediment

| Taxa | PEC$_{sed}$ ($\mu$g/kg) | NOEC/EC$_{10}$ (mg/kg) | AF | PNEC ($\mu$g/kg) | PEC/PNEC |
|---|---|---|---|---|---|
| *Ch. Riparus* | 228 | 125 | 10 | 12,500 | 0.02 |
| *L. variegatus* | | 148* | | | |
| *C. elegans* | | 400 | | | |

*EC$_{10}$.

animals.

*Fish farmed land-based system*

The PEC/PNEC ratio for aquaculture is reported in Table 18.

**Table 18:** Risk characterisation (PEC/PNEC) for aquaculture

| Taxa | PEC$_{sw}$ ($\mu$g/L) | E$_{(r)}$(L)C$_{50}$/EC$_{10}$/NOEC (mg/L) | AF | PNEC ($\mu$g/L) | PEC/PNEC |
|---|---|---|---|---|---|
| **Fish farmed in land-based systems** | | | | | |
| | PEC$_{swaq}$ ($\mu$g/L) | E(L)C$_{50}$ (mg/L) | AF | PNEC ($\mu$g/L) | PEC/PNEC |
| | 0.125 | – | – | 1.2 | 0.10 |

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[66] FAD-2018-0013/Technical dossier/Annex 19.

No unacceptable risk for aquatic organisms is expected for use of EQ in fish farmed in land-based system.

*Aquaculture in seacages*

The PEC/PNE ratio for marine sediment from marine aquaculture in seacages is reported in Table 19.

**Table 19:** Risk characterisation (PEC/PNEC) for marine sediment

| Taxa | $PEC_{sed}$ (µg/kg) | $LC_{50}$ (mg/L) | AF | $PNEC_{sed}$ (µg/kg) | PEC/PNEC |
|---|---|---|---|---|---|
| *Arenicola* sp. | 110,000 | 1.93 | 1,000 | 70.8 | 1,550 |

As regards marine sediment, the FEEADP Panel has assessed $PNEC_{sed}$ using the equilibrium partitioning approach, the corresponding value was 70.8 µg/kg. The study provided do not allow to exclude a risk for marine sediment-dwelling organisms when EQ is used in sea cages. Although the calculated $PEC_{sed}$ of phase I is considered extremely conservative, there are no advanced models accepted at EU level which can be suggested for the refinement of the exposure (PEC) for marine sediment. Further investigations should be conducted, using other modelling tools, more studies or relevant arguments provided that these models, studies and/or arguments are scientifically underpinned. As regards the effect, the potential of additional ecotoxicity studies (acute/chronic) to contribute to increase the PNEC (either via increasing the concentration of the toxicity endpoint considered; and/or by reducing the assessment factor to be used) could also be explored.

*Bioaccumulation and assessment of secondary poisoning*

To assess risk for secondary poisoning for EQ, the method proposed in the relevant Guidance from the European Medicines Agency (EMA) has been considered (EMA, 2016). Based on log Kow of 3.39, EQ has a potential for bioaccumulation, but no bioconcentration factor (BCF) values for earthworm and fish has been provided for this substance. Risk for secondary poisoning has been assessed for EQ, consistent with a total residue approach used by the FEEDAP Panel to evaluate the ecotoxicity studies.

The lowest NOAEL for the parent compound EQ from the toxicological data set has been identified in a 90-day subchronic oral toxicity study in Beagle dogs, adopted by the FEEDAP Panel in 2015 (EFSA FEEDAP Panel, 2015). It was determined as 2 mg/kg bw per day based on hepatocellular necrosis in the liver and kidneys. NOEC for ethoxyquin was 80 mg/kg feed and was calculated from NOAEL considering conversion factor of 40 for dogs. Using the assessment factor of 90, the corresponding $PNEC_{oral}$ for EQ was equivalent to 0.89 mg/kg feed. This value is higher than the estimated concentration in the worms of 0.054 and in the same concentration range than the estimated concentration in fish of 0.91 mg/kg. Predicted concentrations in worms and fish are derived using $PEC_{soil}$ and $PEC_{surface\ water}$ presented in Table 14 and $PEC_{groundwater}$ based on refinement calculation. The PEC/PNEC ratios for terrestrial and aquatic food chain for EQ are given in Table 20. A risk for secondary poisoning for EQ for worm-eating birds and mammals is not likely to occur. For fish-eating birds and mammals, the risk quotient ratio is 1 and a risk cannot be excluded.

**Table 20:** Assessment of secondary poisoning for ethoxyquin

| | $PEC_{fish}$[a] (mg/kg) | $PEC_{worm}$[b] (mg/kg) | $NEC_{oral}$ (mg/kg) | $PEC_{fish}/$ $PNEC_{oral}$ | $PEC_{worm}/$ $PNEC_{oral}$ |
|---|---|---|---|---|---|
| Ethoxyquin | 0.91 | 0.054 | 0.89 | 1.0 | 0.06 |

(a): $PEC_{fish\ (oral,predator)}$ for the assessment of secondary poisoning via the aquatic food chain.
(b): $PEC_{worm\ (oral,predator)}$ for the assessment of secondary poisoning via the terrestrial food chain.

To further refine the assessment of secondary poisoning via the aquatic food chain, a higher tier calculation according to FOCUS SW models is expected to provide far lower values for the predicted concentration in surface water. Another possibility to refine this assessment is to replace the QSAR estimate of BCF for fish by an experimental value determined in a study conducted in accordance with OECD TG 305. The risk for secondary poisoning via the aquatic food chain might therefore be addressed with a refined calculation.

### 3.7.3. Conclusion on environmental risk assessment

Ethoxyquin, when used on terrestrial animal, is considered not to raise safety concerns for groundwater, soil microorganisms and for earthworms. Although there are no indications of safety concerns from a test conducted in three terrestrial plants, ecotoxicological data on three additional terrestrial plants would be needed to conclude on the safety of EQ for the terrestrial compartment. A risk for the aquatic compartment cannot be excluded; nevertheless, a higher tier calculation according to FOCUS SW models is expected to provide far lower values for the predicted concentration in surface water and in sediment. The risk for aquatic organisms might therefore be addressed with refined calculation. Unacceptable risk is not expected for freshwater sediment-dwelling organisms. Ethoxyquin is considered not to pose the risk of secondary poisoning via the terrestrial food chain, whereas a risk for secondary poisoning via the aquatic food chain cannot be excluded.

Ethoxyquin, when used in fish farmed in land-based system, is considered not to raise safety concerns for aquatic organisms.

A risk cannot be excluded for ethoxyquin used in sea-cages for marine sediment-dwelling organisms, although the calculated exposure in the sediment is considered to be extremely conservative. Further investigations could be conducted on exposure in sediment and/or additional ecotoxicological studies may be provided to contribute to increasing the PNEC.

### 3.8. Efficacy

In its previous opinion, the FEEDAP Panel evaluated the results of three published studies which demonstrated the efficacy of ethoxyquin as an antioxidant, but at inclusion levels higher than the intended one. Two stability studies were also evaluated, in which efficacy of ethoxyquin as an antioxidant was demonstrated, however, only in one of these studies, the inclusion level in complete feed (37.5 mg/kg) supported the efficacy at the proposed conditions of use. Therefore, the FEEDAP Panel could not conclude on the efficacy of EQ at the recommended inclusion level of 50 mg/kg complete feed.

Four trials were submitted to support the efficacy of ethoxyquin at the inclusion level of 50 mg/kg complete feed.

In the first study, a typical pelleted complete feed for chickens for fattening (mainly composed of wheat, barley, soybean (meal and extruded) and soybean oil) was prepared with no added antioxidants.[67] The feed, after being grinded, was either unsupplemented or supplemented with (i) 50 mg EQ/kg complete feed (EQ), (ii) 25 mg xanthophylls/kg feed (from an additive providing also 25 mg ethoxyquin/kg feed) (XP) or (iii) 25 mg xanthophylls/kg feed (providing 25 mg ethoxyquin/kg feed) and 25 mg ethoxyquin/kg feed (XPEQ). The intended concentrations of EQ and xanthophylls in feed were confirmed by analysis. Three samples of each feed were stored in three-layer paper bags for 3 months at ambient temperature ($25-30°C$ and $55-65\%$ RH). The samples were analysed after manufacturing and after 1, 2 and 3 months of storage for EQ, total xanthophylls and lutein content, and for peroxide value. The results showed a reduction of EQ content over time in the three supplemented feeds (approximately 40%, 70% and 60% loss after 3 months in the EQ50, XP and XPEQ feeds, respectively). Xanthophylls and lutein content showed also a decrease over time; the XPEQ feed showed a marked lower reduction compared to the XP feeds after 3 months of storage (xanthophylls reduction approx. 41% vs. 29% in the XP and XPED, respectively; lutein reduction approx. 26% vs. 14% in the XP and XPED, respectively). Peroxide values measured after manufacturing were in the same range in the four feeds (1.87, 1.61, 2.14 and 1.7 meq $O_2$/kg fat in the basal feed and in the EQ50, XP and XPEQ, respectively). After 3 months of storage, the peroxide value of the basal feed was 17.2 meq $O_2$/kg fat, while the EQ50, XP and XPEQ feeds showed peroxide values of 5.6, 4.6 and 3.8, respectively.

In the second study, a complete feed for sows (mainly composed of barley, wheat bran, wheat meal, corn, soybean meal) was prepared with no added antioxidants.[68] The basal feed was either unsupplemented or supplemented with 25 mg EQ/kg feed (EQ25) or 50 mg EQ/kg feed (EQ50). The intended concentrations of ethoxyquin were confirmed by analysis. Three samples of each feed were stored in three-layer paper bags for 3 months at ambient temperature ($25-30°C$ and $55-65\%$ RH). The samples were analysed after manufacturing and after 1, 2 and 3 months of storage for EQ concentration and for peroxide value. The results showed a reduction of ethoxyquin content over time in the two supplemented feeds; in the EQ25 feed, EQ completely disappeared after 3 months of

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[67] FAD-2018-0029/Technical dossier/Annex 14.
[68] FAD-2018-0029/Technical dossier/Annex 15.

storage, while in the EQ50, the reduction of EQ reached approx. 80%. Peroxide values measured after manufacturing were in the same range in the three feeds (1.12, 0.89 and 1.36 meq $O_2$/kg fat in the basal feed and in the EQ25 and EQ50, respectively). After 3 months of storage, the peroxide value of the basal feed was 47.3 meq $O_2$/kg fat, while the EQ25 and the EQ50 feeds showed peroxide values of approx. 23.8 and 13.6, respectively.

Two additional studies were submitted in which the protective effects of EQ against oxidation were measured using an oxygen bomb method. The experiment determined the consumption of oxygen at elevated temperature (90–120°C) and high pressure (5 bar) by a specific amount of feed. The time (measured in h) needed to consume oxygen (measured as relative change of pressure) was recorded (induction period). The ratio 'induction period of the feed with antioxidant/ induction period of the basal feed sample' is called protection factor.

The same complete feeds for chickens for fattening and for sows described above were used in the two additional studies, with the only difference that in the study with feed for chickens for fattening an additional group (25 mg ethoxyquin/kg feed, EQ25) was included.

The results of the experiment (duplicate determination) with the feed for chickens for fattening showed an induction period for the basal feed of 7 h.[69] The two feeds supplemented with 25 mg ethoxyquin/kg feed (from ethoxyquin (EQ25) and from xanthophylls (XP) showed induction periods of 7.6 h and 7.8 h, respectively). These values correspond to protection factors of 1.12 and 1.10, respectively. The two feeds supplemented with 50 mg ethoxyquin/kg feed (from ethoxyquin (EQ50) and from xanthophylls and ethoxyquin (XPEQ) showed induction periods of 9.9 h and 9.2 h, respectively). These values correspond to protection factors of 1.46 and 1.29, respectively.

The results of the experiment (duplicate determination) with the feed for sows showed an induction period for the basal feed of 0.9 h, while the induction periods for the feed supplemented with 25 or 50 mg ethoxyquin/kg feed were 1.35 and 1.6 h, respectively.[70] The corresponding protection factors were 1.50 and 1.78 for the EQ25 and EQ50 feeds, respectively.

The FEEDAP Panel notes that the studies assessed in its previous opinion (EFSA FEEDAP Panel, 2015) on the stability and efficacy of EQ when added to a feed material (fishmeal) or a vitamin premixture could be considered as supporting evidence of efficacy, in addition to the studies in complete feedingstuffs: (i) the inclusion levels of 400 mg EQ/kg fishmeal preventing heat generation would correspond to approximately 50 mg EQ/complete feed, considering an inclusion level of fishmeal in complete feedingstuffs of about 12.5% and the absence of EQ in the other feed materials used; (ii) the inclusion levels of 2500 mg EQ/kg vitamin/mineral premixture reducing vitamin A loss during storage, would correspond to approximately 25 mg EQ/complete feed, considering an inclusion level of the premixture in complete feedingstuffs of about 1% and the absence of EQ in the other feed materials used.

### 3.8.1. Conclusion on the efficacy of ethoxyquin

Based on the results of new studies on two complete feed, one study in complete feed and the supporting evidence of efficacy in a premixture and a feed material already assessed in its previous opinion, the FEEDAP Panel considered ethoxyquin an efficacious antioxidant in feed in the range of 25–50 mg/kg complete feed.

## 4. Conclusions

Ethoxyquin is synthetised from p-phenetidine, a recognised possible mutagen, which remains in the additive as an impurity. The applicant is proposing a new specification for p-phenetidine of < 2.5 mg/kg additive, which was shown to be effectively met in pilot batches of the additive.

As a conclusion from tolerance studies, 50 mg EQ/kg complete feed could be considered safe for chickens for fattening, laying hens, piglets, cattle for fattening and salmon. This conclusion could be extrapolated to all animal species and categories with the exception of cats, for which the Panel cannot conclude on a safe level. However, considering that the additive contains p-phenetidine, a possible mutagen, the Panel cannot conclude on the safety of EQ at any level for long-living and reproductive animals.

The Panel concludes that the use of EQ at a maximum total concentration of 50 mg/kg in complete feed for all animal species, except dairy animals, would not result in residues of EQ or its metabolites/ TP which would pose a risk for the consumer. In the absence of residue data in milk, the Panel cannot

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[69] FAD-2018-0029/Technical dossier/Annex 16.
[70] FAD-2018-0029/Technical dossier/Annex 17.

conclude on the safety of EQ when used in feed for milk-producing animals. Owing to the presence in the additive of p-phenetidine, and in the absence of data on the residues of p-phenetidine in tissues and products of animal origin, no conclusion on the safety of the consumer could be drawn. The conclusions on consumer safety are based on the assumption that the maximum total concentration of 50 mg EQ/kg feed is expressed as the sum of EQ, EQDM, EQI and DHEQ.

Exposure of the unprotected user to p-phenetidine via inhalation cannot be excluded. The Panel concludes that, to reduce the risk, the exposure of the users should be minimised.

Regarding the safety for the environment, no safety concerns for groundwater are expected. Ecotoxicity data on three additional terrestrial plants would be needed to conclude on the safety of EQ for the terrestrial compartment. A risk for the aquatic compartment cannot be excluded when the additive is used in terrestrial animals. Unacceptable risk is not expected for freshwater sediment-dwelling organisms. Ethoxyquin is considered not to pose a risk of secondary poisoning via the terrestrial food chain, whereas a risk for secondary poisoning via the aquatic food chain cannot be excluded. Ethoxyquin, when used in fish farmed in land-based system, is considered not to raise safety concerns for aquatic organisms. A risk cannot be excluded for ethoxyquin used in sea-cages for marine sediment-dwelling organisms.

Ethoxyquin is considered an efficacious antioxidant in the range of 25–50 mg/kg complete feed.

## 5. Recommendations

The Panel recommends that the maximum total concentration of EQ in complete feedingstuff is expressed as the sum of EQ, EQDM, EQI and DHEQ.

## 6. Documentation provided to EFSA/Chronology

| Date | Event |
|---|---|
| 29/03/2016 | Reception mandate from the European Commission for EFSA-Q-2016-00267 |
| 30/03/2016 | Reception Fefana asbl additional data from Antoxiac EEIG for EFSA-Q-2016-00267 |
| 7/042016 | Acceptance mandate from the European Commission by EFSA for EFSA-Q-2016-00267 |
| 29/072016 | Request of supplementary information for EFSA-Q-2016-0026767 to the applicant in line with Article 7(3) of Commission Regulation (EC) No 1304/2003 – Scientific assessment suspended. *Issues: genotoxicity studies* |
| 18/12/2017 | Reception Fefana asbl additional data from Antoxiac EEIG for EFSA-Q-2018-00013 |
| 11/01/2018 | Reception mandate from the European Commission for EFSA-Q-2018-00013 |
| 12/01/2018 | Acceptance mandate from the European Commission by EFSA for EFSA-Q-2018-00013 |
| 23/04/2018 | Reception Fefana asbl additional data from Antoxiac EEIG for FSA-Q-2018-00372 |
| 2/05/2018 | Reception mandate from the European Commission for EFSA-Q-2018-00372 |
| 28/05/2018 | Request of supplementary information for EFSA-Q-2018-00013 to the applicant in line with Article 7(3) of Commission Regulation (EC) No 1304/2003 – Scientific assessment suspended. *Issues: Safety for the target species* |
| 28/05/2018 | Acceptance mandate from the European Commission by EFSA for EFSA-Q-2018-00372 |
| 6/08/2018 | Reception of supplementary information for EFSA-Q-2016-0026767 from the applicant - Scientific assessment re-started |
| 7/08/2018 | Reception of supplementary information for EFSA-Q-2018-00013 from the applicant - Scientific assessment re-started |
| 19/11/2018 | Request of supplementary information for EFSA-Q-2018-00372 to the applicant in line with Article 7(3) of Commission Regulation (EC) No 1304/2003 – Scientific assessment suspended. *Issues: safety for the consumers* |
| 28/02/2019 | Reception of supplementary information for EFSA-Q-2018-00372 from the applicant - Scientific assessment re-started |
| 24/09/2020 | Spontaneous submission for EFSA-Q-2018-00372 |
| 16/08/2021 | Reception mandate from the European Commission for EFSA-Q-2021-00523 |
| 27/08/2021 | EFSA received from FEFANA ASBL the supplementary information and data for EFSA-Q-2021-00523 |
| 1/10/2021 | Acceptance mandate from the European Commission by EFSA for EFSA-Q-2021-00523 |
| 27/01/2022 | Opinion adopted by the FEEDAP Panel. End of the Scientific assessment |

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## Abbreviations

| | |
|---|---|
| $\gamma$GT | gamma-glutamyl transferase |
| ADME | absorption, distribution, metabolism and excretion |
| ALT | alanine aminotransferase |
| APH | alkaline phosphatase |
| AST | aspartate aminotransferase |
| BHA | butylated hydroxyanisole |
| BHT | butylated hydroxy toluene |
| CK | creatinine kinase |
| CP | crude protein |
| DEQ | de-ethylated ethoxyquin |
| DHEQ | dihydro ethoxyquin |
| $DT_{50}$ | time to degradation of 50% of original concentration of the compound in the testedsoils |
| $DT_{90}$ | time to degradation of 90% of original concentration of the compound in the testedsoils |
| DNA | deoxyribonucleic acid |
| DW | dry weight |
| EINECS | European Inventory of Existing Commercial chemical structures |
| EMA | European Medicines Agency |
| EQDM | ethoxyquin dimer |
| EQI | ethoxyquin quinone imine |
| EURL | European Union Reference Laboratory for Feed Additives |
| FAO | Food and Agriculture Organisation |
| FEEDAP | Panel on Additives and Products or Substances used in Animal Feed |
| GGT | gamma-glutamine transpeptidase |
| GLP | good laboratory practice |
| GSH | glutathione |
| HB | haemoglobin |
| HPLC | high-performance liquid chromatography |
| HT | Haematocrit |
| LDH | lactate dehydrogenase |
| LOD | limit of detection |
| LOQ | limit of quantification |
| MCH | mean corpuscular haemoglobi |
| MCHC | mean corpuscular haemoglobin concentration |
| MCV | mean corpuscular volume |
| ME | metabolisable energy |
| MJ | Mega Joule |
| MRLs | maximum residue levels |
| MTD | maximum tolerated dose |
| NOAEL | no observed adverse effect level |
| OECD | Organisation for Economic Co-operation and Development |
| PEC | predicted environmental concentration |
| pF | unit for soil moisture, logarithm of the absolute value of soil matric potential |
| PNEC | predicted no effect concentration |

| QSAR | quantitative structure-activity relationship |
| RH | relative humidity |
| SCAN | Scientific Committee on Animal Nutrition |
| SCF | Scientific Committee for Food |
| UN | United Nations |
| WHO | World Health Organisation |
| XP | xanthophylls |
| XPEQ | xanthophylls and ethoxyquin |

## Appendix A – Ethoxyquin: tentative transformation pathways
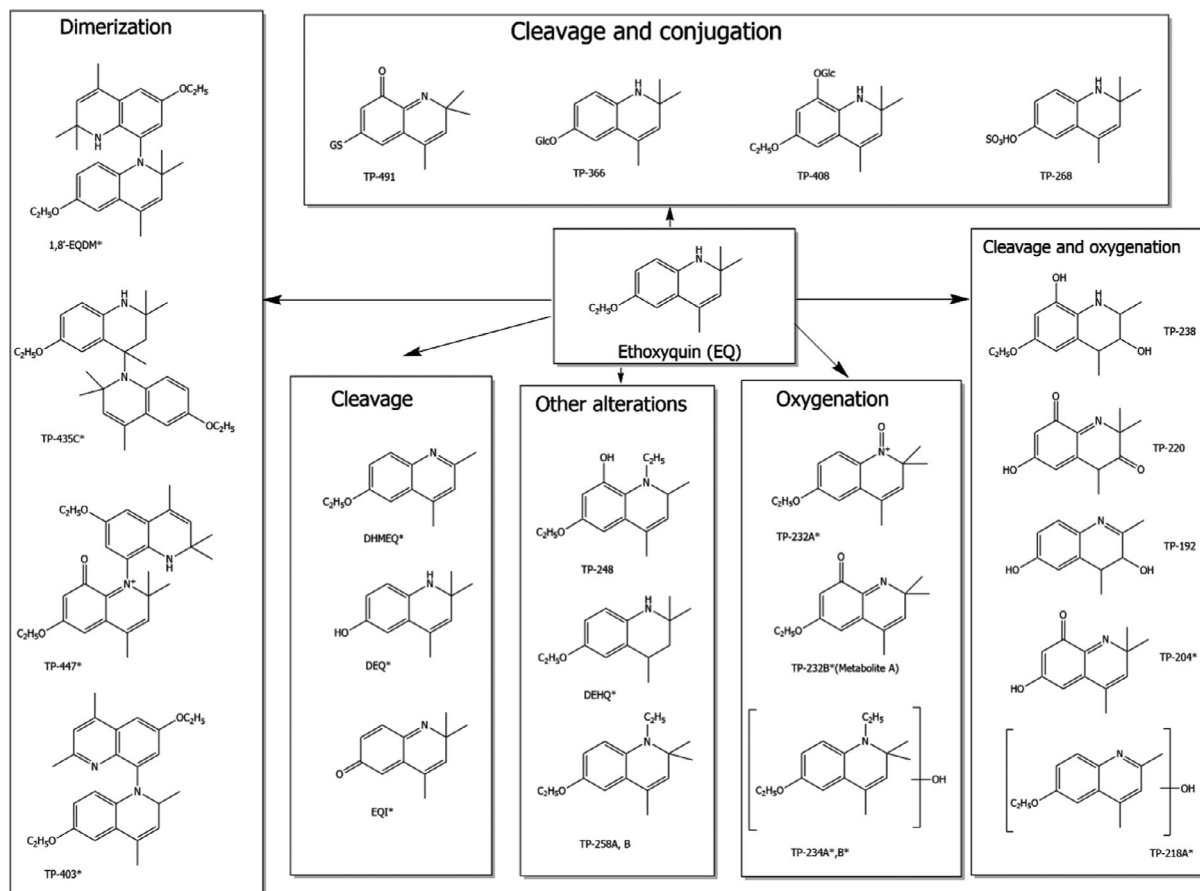


**Figure A.1:** Transformation products (TQ) of ethoxyquin (EQ) grouped according to different transformation pathways, as proposed by Merel et al. (2019). The metabolites were identified in Atlantic salmon (Salmo salar L.) after controlled dietary exposure to EQ via fish feed for 90 days. Metabolites identified with an asterisk (TP*) were previously identified by Negreira et al. (2017) during bench scale oxidation of EQ or in fish feed (Scheme adapted from Merel et al., 2019)

## Appendix B – Detailed results of chronic exposure calculation

**Table B.1:** Chronic dietary exposure of consumers to residues of EQ and its major metabolites/TPs and per population class, country and survey (mg/kg body weight per day) based on residue data

| Population class | Survey's country | Number of subjects | Highest reliable percentile value | Highest reliable percentile description |
|---|---|---|---|---|
| Infants | Bulgaria | 523 | 0.00230 | 95th |
| Infants | Germany | 142 | 0.00172 | 95th |
| Infants | Denmark | 799 | 0.00340 | 95th |
| Infants | Finland | 427 | 0.00185 | 95th |
| Infants | Italy | 9 | 0.00001 | 50th |
| Infants | United Kingdom | 1,251 | 0.00218 | 95th |
| Toddlers | Belgium | 36 | 0.00315 | 90th |
| Toddlers | Bulgaria | 428 | 0.00378 | 95th |
| Toddlers | Germany | 348 | 0.00342 | 95th |
| Toddlers | Denmark | 917 | 0.00419 | 95th |
| Toddlers | Spain | 17 | 0.00288 | 75th |
| Toddlers | Finland | 500 | 0.00338 | 95th |
| Toddlers | Italy | 36 | 0.00359 | 90th |
| Toddlers | Netherlands | 322 | 0.00337 | 95th |
| Toddlers | United Kingdom | 1,314 | 0.00314 | 95th |
| Toddlers | United Kingdom | 185 | 0.00379 | 95th |
| Other children | Austria | 128 | 0.00305 | 95th |
| Other children | Belgium | 625 | 0.00347 | 95th |
| Other children | Bulgaria | 433 | 0.00408 | 95th |
| Other children | Germany | 293 | 0.00269 | 95th |
| Other children | Germany | 835 | 0.00293 | 95th |
| Other children | Denmark | 298 | 0.00317 | 95th |
| Other children | Spain | 399 | 0.00394 | 95th |
| Other children | Spain | 156 | 0.00455 | 95th |
| Other children | Finland | 750 | 0.00366 | 95th |
| Other children | France | 482 | 0.00325 | 95th |
| Other children | Greece | 838 | 0.00318 | 95th |
| Other children | Italy | 193 | 0.00382 | 95th |
| Other children | Latvia | 187 | 0.00346 | 95th |
| Other children | Netherlands | 957 | 0.00286 | 95th |
| Other children | Netherlands | 447 | 0.00293 | 95th |
| Other children | Sweden | 1,473 | 0.00358 | 95th |
| Other children | Czechia | 389 | 0.00397 | 95th |
| Other children | United Kingdom | 651 | 0.00287 | 95th |
| Adolescents | Austria | 237 | 0.00222 | 95th |
| Adolescents | Belgium | 576 | 0.00175 | 95th |
| Adolescents | Cyprus | 303 | 0.00149 | 95th |
| Adolescents | Germany | 393 | 0.00229 | 95th |
| Adolescents | Germany | 1,011 | 0.00205 | 95th |
| Adolescents | Denmark | 377 | 0.00213 | 95th |
| Adolescents | Spain | 651 | 0.00281 | 95th |
| Adolescents | Spain | 209 | 0.00336 | 95th |
| Adolescents | Spain | 86 | 0.00272 | 95th |
| Adolescents | Finland | 306 | 0.00179 | 95th |
| Adolescents | France | 973 | 0.00193 | 95th |

| Population class | Survey's country | Number of subjects | Highest reliable percentile value | Highest reliable percentile description |
|---|---|---|---|---|
| Adolescents | Italy | 247 | 0.00210 | 95th |
| Adolescents | Latvia | 453 | 0.00270 | 95th |
| Adolescents | Netherlands | 1,142 | 0.00201 | 95th |
| Adolescents | Sweden | 1,018 | 0.00229 | 95th |
| Adolescents | Czechia | 298 | 0.00388 | 95th |
| Adolescents | United Kingdom | 666 | 0.00167 | 95th |
| Adults | Austria | 308 | 0.00199 | 95th |
| Adults | Belgium | 1,292 | 0.00172 | 95th |
| Adults | Germany | 10,419 | 0.00179 | 95th |
| Adults | Denmark | 1,739 | 0.00149 | 95th |
| Adults | Spain | 981 | 0.00236 | 95th |
| Adults | Spain | 410 | 0.00214 | 95th |
| Adults | Finland | 1,295 | 0.00178 | 95th |
| Adults | France | 2,276 | 0.00147 | 95th |
| Adults | Hungary | 1,074 | 0.00240 | 95th |
| Adults | Ireland | 1,274 | 0.00170 | 95th |
| Adults | Italy | 2,313 | 0.00153 | 95th |
| Adults | Latvia | 1,271 | 0.00229 | 95th |
| Adults | Netherlands | 2,055 | 0.00158 | 95th |
| Adults | Romania | 1,254 | 0.00205 | 95th |
| Adults | Sweden | 1,430 | 0.00200 | 95th |
| Adults | Czechia | 1,666 | 0.00274 | 95th |
| Adults | United Kingdom | 1,265 | 0.00136 | 95th |
| Elderly | Austria | 67 | 0.00142 | 95th |
| Elderly | Belgium | 511 | 0.00157 | 95th |
| Elderly | Germany | 2,006 | 0.00156 | 95th |
| Elderly | Denmark | 274 | 0.00127 | 95th |
| Elderly | Finland | 413 | 0.00154 | 95th |
| Elderly | France | 264 | 0.00126 | 95th |
| Elderly | Hungary | 206 | 0.00177 | 95th |
| Elderly | Ireland | 149 | 0.00165 | 95th |
| Elderly | Italy | 289 | 0.00139 | 95th |
| Elderly | Netherlands | 173 | 0.00146 | 95th |
| Elderly | Netherlands | 289 | 0.00138 | 95th |
| Elderly | Romania | 83 | 0.00155 | 95th |
| Elderly | Sweden | 295 | 0.00174 | 95th |
| Elderly | United Kingdom | 166 | 0.00108 | 95th |
| Very elderly | Austria | 25 | 0.00092 | 75th |
| Very elderly | Belgium | 704 | 0.00146 | 95th |
| Very elderly | Germany | 490 | 0.00150 | 95th |
| Very elderly | Denmark | 12 | 0.00089 | 75th |
| Very elderly | France | 84 | 0.00116 | 95th |
| Very elderly | Hungary | 80 | 0.00179 | 95th |
| Very elderly | Ireland | 77 | 0.00163 | 95th |
| Very elderly | Italy | 228 | 0.00116 | 95th |
| Very elderly | Netherlands | 450 | 0.00133 | 95th |
| Very elderly | Romania | 45 | 0.00161 | 90th |
| Very elderly | Sweden | 72 | 0.00161 | 95th |
| Very elderly | United Kingdom | 139 | 0.00116 | 95th |