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Use of (Q)SAR genotoxicity predictions and fuzzy multicriteria decision-making for priority ranking of ethoxyquin transformation products

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ABSTRACT

Ethoxyquin (EQ; 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) has been used as an antioxidant in feed for pets and food-producing animals, including farmed fish such as Atlantic salmon. In Europe, the authorization for use of EQ as a feed additive was suspended, due to knowledge gaps concerning the presence and toxicity of EQ transformation products (TPs). Recent analytical studies focusing on the detection of EQ TPs in farmed Atlantic salmon feed and fillets reported the detection of a total of 27 EQ TPs, comprising both known and previously not described EQ TPs. We devised and applied an *in silico* workflow to rank these EQ TPs according to their genotoxic potential and their occurrence data in Atlantic salmon feed and fillet. Ames genotoxicity predictions were obtained applying a suite of five (quantitative) structure–activity relationship ((Q)SAR) tools, namely VEGA, TEST, LAZAR, Derek Nexus and Sarah Nexus. (Q)SAR Ames genotoxicity predictions were aggregated using fuzzy analytic hierarchy process (fAHP) multicriteria decision-making (MCDM). A priority ranking of EQ TPs was performed based on combining both fAHP ranked (Q)SAR predictions and analytical occurrence data. The applied workflow prioritized four newly identified EQ TPs for further investigation of genotoxicity. The fAHP-based prioritization strategy described here, can easily be applied to other toxicity endpoints and groups of chemicals for priority ranking of compounds of most concern for subsequent experimental and mechanistic toxicology analyses.

1. Introduction

Ethoxyquin (EQ; 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) is a quinoline-based synthetic antioxidant globally used as a technological additive to protect against lipid peroxidation and to stabilize fat-soluble vitamins (Błaszczyk et al., 2013). EQ has been used extensively in feed for pets and livestock and has commonly been added to fish meal to prevent rancidity and self-ignition under long-distance sea transport and storage (Bernhard et al., 2019). Because of its antioxidant nature, EQ can readily be chemically oxidized or bio-transformed into a series of transformation products (TPs) detectable both in fish feed and the edible tissue of farmed fish (Merel et al., 2019; Negreira et al., 2017).

In the European Union (EU), Council Directive 70/524/EEC (1970) and Regulation (EC) No 1831/2003 (2003) authorized the inclusion of EQ as a feed additive for all farmed species with a maximum content of 150 mg/kg feed. However, in 2017, due to concerns regarding the safety of EQ and its TPs (EFSA, 2015), the previously granted authorization was suspended, and a transition period was granted, which allowed feed produced from certain materials containing EQ to be placed on the market until end of March 2020 (Regulation (EU) 2017/962, 2017). By the end of 2020, and in any event after the adoption of a non-favorable opinion by the European Food Safety Authority (EFSA) on the safety or

Abbreviations: AD, applicability domains; CCS, collision cross section; DHMEQ, Dehydrodemethylethoxyquin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EQ, Ethoxyquin; EQDM, Ethoxyquin dimer; EQI, Ethoxyquin quinone imine; EPA, United States Environmental Protection Agency; f(AHP), fuzzy analytic hierarchy process; FDA, United States Food and Drug Administration; MCDM, multicriteria decision-making; OECD, Organisation for Economic Co-operation and Development; (Q)SAR, (Quantitative) structure–activity relationship; QTOF-MS, Quadrupole time-of-flight mass spectrometry; SMILES, Simplified molecular input line entry system; TP, Transformation Product; TWIMS, Traveling-wave ion mobility spectrometry; UHPLC, Ultra-high-performance liquid chromatography.

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efficacy of EQ, Regulation (EU) 2017/962 was due to be reviewed. Recently, the implementation of this regulation was amended; its review was postponed to the end of 2022 or any time after the adoption of a non-favorable opinion by EFSA (Regulation (EU) 2021/412, 2021).

In a previous risk assessment of EFSA, an EQ TP namely, ethoxyquin quinone imine (EQI; 2,6-dihydro-2,2,4-trimethyl-6-quinolone), indicated structural alerts for mutagenicity, carcinogenicity and DNA binding, and no conclusion on the absence of genotoxic effects of this TP was possible (EFSA, 2015). Partly based on these data, the previously granted EQ authorization was suspended (Regulation (EU) 2017/962, 2017) and further studies on the genotoxic properties of EQ TPs were advised (EFSA, 2015). Trials with Atlantic salmon fed EQ-enriched feed showed that in addition to EQI, in fillet, a total of 14 chromatographic peaks with fluorescence characteristics similar to those of EQ were detected (Bohne et al., 2007), indicating the potential presence of multiple EQ TPs in farmed Atlantic salmon. Indeed, more recent specific analytical studies using targeted and untargeted high-resolution mass spectrometry led to the detection and identification of 25 EQ TPs in commercially produced Atlantic salmon feeds (Negreira et al., 2017); the occurrence of many of these compounds was reported for the first time and has yet not been included in EQ risk assessments in Atlantic salmon farming. A follow-up study using the same high resolution analytical approach on commercial Atlantic salmon fillets and fillets of Atlantic salmon fed graded levels of EQ-enriched feed, respectively detected and identified over 24 different EQ TPs (Merel et al., 2019); of these approximately 10 compounds were again different to those earlier identified in salmon feeds.

Not all EQ TPs identified in Atlantic salmon fillet were present in similar relative amounts (Merel et al., 2019). Some EQ TPs such as the known EQ dimer (1,8′-di(6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline); EQDM) or the newly identified TP-403 or TP-234 were more abundant (~35–97% of abundance of all identified EQ TPs) compared to other EQ TPs, including dehydrodemethylethoxyquin (DHMEQ) and EQI (<1% of total abundance). Commonly, the most abundant chemical transformation products are potentially the most relevant ones concerning feed and food safety considerations, as these most likely may exceed threshold limits of effects. However, suspected genotoxic substances such as EQI, are in general not considered to have specific threshold limits and models are generally used to provide estimates of carcinogenic risk at very low dose levels (Hartwig et al., 2020). The detection and identification of novel and not yet assessed EQ TPs in salmon muscle, even at relatively low levels, therefore, calls for an assessment if these TPs too elicit structural alerts related to genotoxicity when subjected to computational toxicity analyses. The identified EQ TPs did not have a similar occurrence in the analyzed Atlantic salmon fillets. Some EQ TPs, such as the newly identified TP-403, were present in all surveyed Atlantic salmon fillets, while other TPs such as EQI was only present in a few fillets (Merel et al., 2019). In addition to identifying structural alerts for genotoxicity, the relative occurrence of EQ TPs is of importance in ranking the likelihood of the identified EQ TPs for possible genotoxicity when consuming Atlantic salmon.

In order to assess the likelihood of potential mutagenicity of newly identified contaminants, computer-based predictions such as (quantitative) structure activity relationship models ((Q)SAR) have been gaining importance, particularly for screening and prioritization purposes, as these *in silico* tools can provide faster, non-animal-based alternatives for the prediction of complex toxicological endpoints (Maunz et al., 2013; Pradeep et al., 2016; Rasinger et al., 2018). Several (Q)SAR models have been used and validated by United States (US) regulatory agencies, and a set of internationally agreed-upon validation principles for regulatory acceptance were laid out by the Organisation for Economic Co-operation and Development (OECD) (Pradeep et al., 2016). Also, in the EU, (Q)SAR have been gaining acceptance in the prediction of toxicity reference values and the classification of thresholds for human and environmental risk assessments (Benfenati et al.,2017; Benigni and Bossa, 2019; Dorne et al., 2021).

However, despite recent advances in computational toxicology, challenges do remain which hamper the use of (Q)SAR in chemical risk assessment. For example, one issue when using a multitude of (Q)SAR models in the prediction of toxicity endpoints, is the application of different mathematical algorithms and different training data sets which can result in conflicting toxicity predictions (Frenzel et al., 2017). To overcome this limitation, different strategies for the combination and aggregation of multiple (Q)SAR outputs for priority ranking of potentially mutagenic substances have been presented (Frenzel et al., 2017; Glück et al., 2018; Manganelli et al., 2018; Pradeep et al., 2016; Rusko et al., 2020; Van Bossuyt et al., 2017).

In the present work, to rank and prioritize EQ TP suspect mutagens for future toxicity testing, multiple (Q)SAR analyses predicting Ames genotoxicity (OECD, 2020) were run and aggregated using previously published combine-and-conquer strategies (Frenzel et al., 2017; Van Bossuyt et al., 2017). In addition, fuzzy analytical hierarchy process (fAHP) modeling was applied to jointly rank and combine (Q)SAR predictions and analytical occurrence data into a single EQ TP suspect mutagen priority list. Fuzzy AHP is a multiple criteria decision-making (MCDM) tool (Tesfamariam and Sadiq, 2006) commonly used to support subjective evaluations of performance criteria by decision makers (Mardani et al., 2015); fAHP aims to settle conflicts between practical demand and scientific decision making, effectively handles both qualitative and quantitative data, and has previously been employed successfully in different risk assessment scenarios (Mardani et al., 2015; Pereira et al., 2016; Zheng et al., 2012). The present study, to the best knowledge of the authors, for the first time applies fAHP to aggregate *in silico* toxicity predictions and analytical occurrence data for substance prioritization ranking in chemical risk assessment.

2. Material and methods

Ranking of EQ TPs was performed in three steps. First, *in silico* predictions of genotoxic properties of EQ TPs obtained from literature were made using five different (Q)SAR tools. Second, multiple (Q)SAR outputs were harmonized and aggregated into a priority rank using fAhP. Third, aggregated *in silico* predictions were combined with occurrence data in surveyed commercial feed and Atlantic salmon fillet and a EQ TP suspect mutagen priority list for future toxicity testing was generated.

2.1. Identification and occurrence of ethoxyquin transformation products

The EQ TPs included in the present work were first described in analytical chemistry-focused studies published earlier by our group (Merel et al., 2019; Negreira et al., 2017). Briefly, Negreira et al. (2017) reported EQ TPs generated by two common bench-top antioxidant activity tests, namely the hydrogen peroxide (H_2O_2) assay and the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. EQ TPs separated by ultra-high-performance liquid chromatography (UHPLC) were then detected and characterized by traveling-wave ion mobility spectrometry (TWIMS) coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS). An EQ TP database including retention times, accurate masses, characteristic fragment ions and collision cross section (CCS) values was established. In a follow-up study, 18 commercial Norwegian salmon feed samples were screened, and data were matched against the previously established EQ TP database. In addition, several EQ TPs, not detected in the antioxidant activity assays, were found in salmon feed following a newly developed untargeted screening strategy (Merel et al., 2019).

Using non-targeted screening on 12 commercial Norwegian Atlantic salmon and 45 experimental salmon muscle samples, EQ TPs previously detected in salmon feed and novel EQ TPs were identified (Merel et al., 2019). Experimental Atlantic salmon muscle samples originated from an EQ exposure trial in which fish were fed EQ-enriched diets (0.5, 118, 1173 mg/kg) for 90 days (Bernhard et al., 2019). The experimental diets with the two highest EQ levels exceeded concentrations usually found in

commercial feeds and were reflecting high-dose levels chosen to provoke the detection and identification of EQ TPs formed after biological transformation. For details on EQ TP chemical structures and unique identifiers introduced, see Merel et al.(2019) and Negreira et al. (2017).

2.2. Computer models for predicting mutagenicity and output processing

To perform (Q)SAR analyses for EQ and each of the 47 EQ TP described in Merel et al. (2019) and Negreira et al. (2017), simplified molecular input line entry system (SMILES) canonical codes were generated using the public PubChem data base (https://pubchem.ncbi.nlm.nih.gov/). As some newly detected EQ TPs can have different isomeric structures, several possible SMILES codes were used. This led to the creation of multiple SMILES entries for some EQ TPs, which for transparency and verifiability of the analysis presented here, were retained throughout and aggregated only at the final step of the ranking. An overview of EQ TP identifiers and associated SMILES codes and occurrence data is provided in Supplementary Table S1.

Five different (Q)SAR tools were used for the prediction of mutagenic activity of EQ TPs, following combine-and-conquer approaches recently described in literature (Frenzel et al., 2017; Manganelli et al., 2018; Van Bossuyt et al., 2017). In the following, a short description of each tool used and the strategy for harmonization of output styles for the combined analyses is provided; a detailed description of each tool and strategy for harmonization of outputs can be found in the aforelisted references. (1) The Toxicity Estimation Software Tool (TEST; v4.2.1, provided by the United States (US) Environmental Protection Agency (EPA) predicts mutagenicity using three different (Q)SAR methodologies based on either (i) hierarchical clustering, (ii) a so-called FDA approach as applied by the US Food and Drug Administration (FDA), and (iii) a nearest neighbor approach. In addition, a consensus model is reported. The output given by TEST is a numeric value between 0 and 1; when no prediction is obtained, no output is produced. (2) The VEGA platform (v1.1.4) provides several different software tools for the prediction of physicochemical, ecotoxicological, and toxicological properties for compounds of interest. In addition, VEGA includes tools for read-across (ToxRead) and prioritization (JANUS), and allows for the integration of results using a weight-of-evidence approach (ToxWeight). Within VEGA, three tools are available for the estimation of mutagenicity namely, (i) CESAR, (ii) SarPy, and (iii) ISS whose predictions are combined and outputted as single final VEGA consensus score. (3) LAZAR (lazy, structure–activity relationship) (Maunz et al., 2013) comprises two models for mutagenicity yielding yes/no answers, which are presented alongside probability scores that indicate which class the prediction belongs to (Rusko et al., 2020). (4) Derek Nexus (Marchant et al., 2008) is a SAR tool whose predictions rely on expert-curated rules, which are derived from both open-source literature and confidential data sets. (5) Sarah Nexus (Hanser et al., 2014) is a (Q)SAR-based tool which splits query compounds into fragments whose activity is reviewed. Based on a network of hypotheses generated from meaningful fragments, an overall applicability domain-checked mutagenicity prediction is provided alongside a confidence score. Unlike the three open-source tools described above, Derek and Sarah are commercially available as part of the Lhasa Limited Knowledge Suite.

Following individual (Q)SAR analyses, different mutagenic scores were combined and ranked. Unless otherwise specified, all settings and parameters within each software, and the conversion of original model-specific classifications into common cumulated mutagenicity scores, were performed as previously described (Frenzel et al., 2017; Manganelli et al., 2018; Rusko et al., 2020; Van Bossuyt et al., 2017). Table 1 provides an overview of the three categories used in the present study (negative, positive and undefined) and a summary of how these were derived.

Table 1
Consensus categories for joint analysis of five different (Q)SAR tools.

	Positive	Negative	Undefined
TEST	Continuous prediction value > 0.7	Continuous prediction value < 0.3	Continuous prediction value between 0.3 and 0.7
VEGA	Mutagenic and consensus score > 0.3	Non-mutagenic and consensus score > 0.3	Mutagenic or non-mutagenic and consensus score ≤ 0.3
LAZAR	Mutagenic and odds ratio > 1.5	Non-mutagenic and odds ratio > 1.5	Mutagenic or non-mutagenic and odds ratio ≤ 1.5
Derek	Equivocal to certain	Inactive and no misclassified or unclassified features	Inactive and contains unclassified features
Sarah	Positive or equivocal	Negative	Outside domain

2.3. Ranking of priority substances

For hazard ranking and prioritization of EQ TPs, in addition to approaches described in literature (Frenzel et al., 2017; Manganelli et al., 2018; Van Bossuyt et al., 2017), fAHP modeling was applied as described by Zheng et al. (2012). Following fAHP ranking of (Q)SAR data, occurrence data also was ranked using fAHP modelling, assigning the highest priority to EQ TP detected in commercial salmon fillet, followed by experimental salmon fillets and commercial feed. Lastly, fAHP (Q)SAR priority ranks were combined with fAHP occurrence ranks to obtain a final ranking for recommendation for further investigation of the genotoxic risk of newly identified EQ TPs.

Priority ranking, data aggregation and analyses were performed in R (vers. 4.0.2) (R Core Team, 2020) running in RStudio (vers. 1.2.5019) (RStudio Team, 2019). Individual (Q)SAR outputs were recoded as described in Table 1 using tidyverse functions (vers. 1.3.0) (Wickham et al., 2019). The package FuzzyAHP (vers. 0.9.5) was used to perform fAHP ranking of EQ TPs. Plots were created using the packages ggplot2 (vers. 3.3.2), UpSetR (vers. 1.4.0), and ComplexHeatmap (2.4.3). All R code is available on request from the authors.

3. Results and discussion

In the present study, Ames genotoxicity of EQ, as well as legacy and novel EQ TPs detected in fish feed, experimentally produced and commercial Atlantic salmon were investigated *in silico*. Multiple (Q)SAR analyses predicting Ames genotoxicity were run and combined using previously published strategies. Using a novel approach, fAHP modeling, (Q)SAR predictions and analytical occurrence data were aggregated into a single EQ TP suspect mutagen priority list for future toxicity testing. The workflow presented here can easily be applied to other groups of chemicals and toxicity endpoints, respectively.

3.1. EQ and EQ TPs in fish feed and fish

Fig. 1 provides an overview of the total numbers of EQ TPs detected in the different matrices as reported by to recent studies (Merel et al., 2019; Negreira et al., 2017). As can be seen, in addition to EQ, a total of 47 EQ TPs were detected across all matrices analyzed.

The detection of EQ TPs using UHPLC-TWIMS-QTOF-MS does not always precisely characterize which part of EQ is altered. For instance, a hydroxylation may be confirmed although the exact location of the hydroxyl moiety remains uncertain. Therefore, employing a conservative approach, SMILES codes for all possible isomers for the 47 EQ TPs were generated. This increased the final number of possible substance structures to be analyzed by (Q)SAR from 48 to 108 considering EQ and all isomers of its 47 previously described EQ TPs (see Supplementary Table S1).

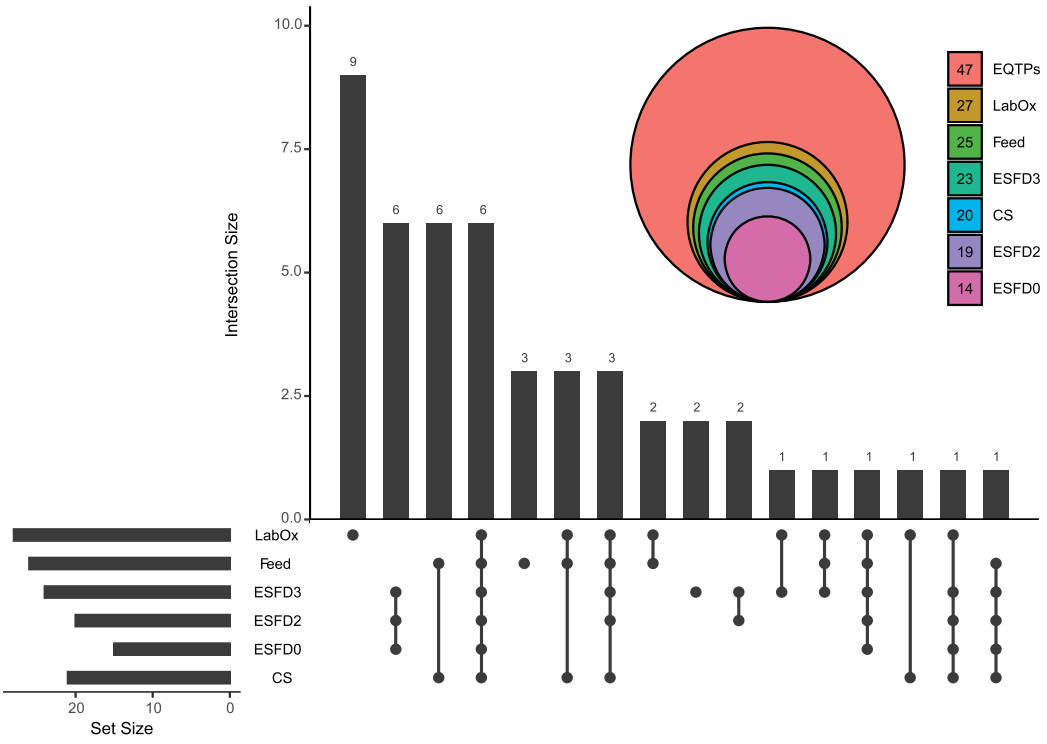

Fig. 1. Overview of EQ TPs considered in the present study. EQ TPs refers to the (i) total number EQ TPs listed in Negreira et al. (2017) and Merel et al. (2019). Negreira et al. (2017) reported on the occurrence of EQ TPs detected in (i) oxidation experiments (LabOx) and (ii) fish feed (Feed); Merel et al. (2019) on the occurrence of EQ TPs in (iii) commercial (CS) and (iv) experimentally farmed (ES) Atlantic salmon fed control diets (ESFD0: 0.47 mg/kg) or diets spiked with EQ (ESFD2: 119 ± 7 mg/kg and ESFD2: 1173 ± 113 mg/kg). In addition to EQ TPs, EQ was detected in all matrices and was included in all (Q)SAR analyses.

3.2. Individual models and direct combination of models

All 108 EQ TP SMILES were subjected to *in silico* mutagenicity prediction analysis using a suite of five different (Q)SAR tools, namely VEGA, TEST, LAZAR, Derek Nexus and Sarah Nexus. Original model-specific classifications for genotoxicity were re-coded as previously described (Frenzel et al., 2017; Manganelli et al., 2018; Rusko et al., 2020; Van Bossuyt et al., 2017) to allow for a concomitant analysis of the predictions made by each tool. Original outputs and re-coded values for each tool are presented in Supplementary Tables S2 (Derek), S3 (Sarah), S4 (LAZAR), S5 (TEST) and S6 (VEGA).

Fig. 2A provides an overview of the re-coded harmonized prediction outputs obtained for genotoxicity by each of the five different (Q)SAR tools; Fig. 2 (B, C, D) shows Venn diagrams depicting the degree of overlap between positive, negative and undefined genotoxicity predictions.

As can be seen in Fig. 2A, the positive prediction rate for the commercial tools Derek and Sarah were 24.1% (26/108) and 14.8% (16/108), respectively. The open-source tools, LAZAR, TEST and VEGA, displayed positive prediction rates of 27.8% (30/108), 4.6% (5/108) and 8.3% (9/108), respectively. Overall, LAZAR yielded the highest number of positive structural alerts for mutagenicity, followed by Derek, Sarah, VEGA and TEST. The negative prediction rate, which based on the chosen method of re-coding of (Q)SAR outputs should be interpreted as "no positive response reported" (Van Bossuyt et al., 2017) rather than regarded as a proof of absence of genotoxic effects, was highest for TEST (67.6%; 73/108), followed by Derek with (63.9%; 69/108), VEGA (45.4%; 49/108), LAZAR (27.8%; 30/108) and Sarah (21.3%; 23/108).

In addition to the output categories positive and negative, also a third output category, undefined, was included in the present study. This conservative approach first described by (Van Bossuyt et al., 2017) was adopted to ensure that any incorrect mis-labeling of substances as non-mutagenic would be avoided to the greatest extent possible. As can be seen in Table 1, the category undefined was attributed to ambiguous (Q)SAR outputs, i.e. for mutagenicity predictions, which were of moderate reliability as model assumptions were not met or were outside predefined Ames mutagenicity applicability domains (AD). Based on the categories defined in Table 1, 63.9% (69/108), 46.3% (50/108), 44.4% (48/108), 27.8% (30/108) and 12.0% (13/108) of Sarah, VEGA, LAZAR, TEST and Derek predictions, respectively, were classified as undefined.

Venn diagrams in Fig. 2 (B,C,D) display the degree of overlap between the harmonized *in silico* mutagenicity predictions made by the five different computational analyses. No overlap of output of all five (Q)SAR tools was observed for positive and undefined genotoxicity predictions (Fig. 2B and C); and only seven out of 108 EQ TP SMILES tested were placed into the negative prediction category by all five tools (Fig. 2D). The low degree of overlap between genotoxicity predictions could in parts be explained by the choice of conditions set for each of the three harmonized categories used in the present study. For example, when using less stringent cut-offs for mutagenicity predictions and pooling outcomes from both positive and undefined categories, a total of 14 SMILES codes representing five EQ TPs (DHMEQ, TP-218A, TP-218B, TP-218C and TP-232A) would be categorized as suspect mutagens by all five tools.

Irrespective of the categorization approaches chosen, it has been observed previously that conflicting predictions of toxicity can arise when using a screening battery of (Q)SAR tools based on different mathematical models and different training data sets, respectively (Frenzel et al., 2017; Rusko et al., 2020; Van Bossuyt et al., 2017). To overcome this issue, several combine-and-conquer strategies have been proposed in the literature (Frenzel et al., 2017; Manganelli et al., 2018; Van Bossuyt et al., 2017). These approaches are based on the assumption that substances, which are classified as suspect mutagens in multiple complementary (Q)SAR tools, are associated with increased prediction

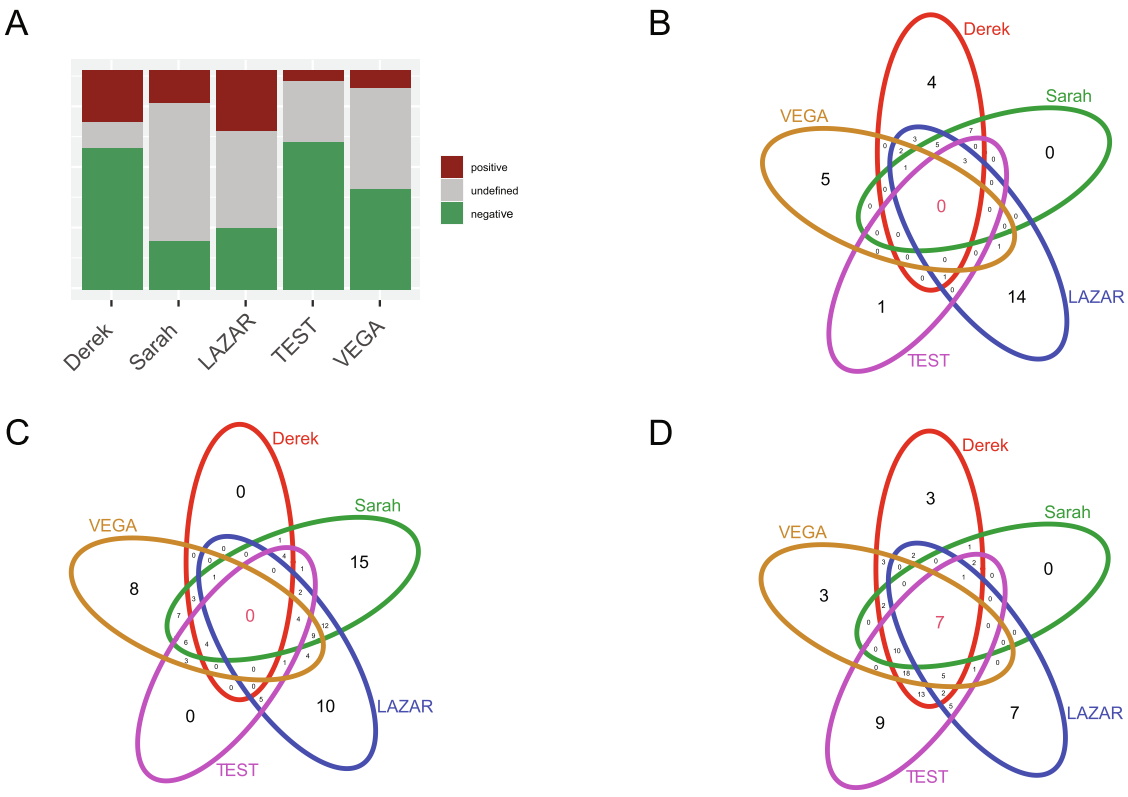

Fig. 2. Overview of outcome and overlap of (Q)SAR predictions. (A) Total number of EQ TPs predicted to elicit negative (green), positive (red) or undefined (gray) responses in Ames mutagenicity assays in a series of (Q)SAR analyses. None of the positive (B) or undefined (C) Ames mutagenicity predictions were consistent across the different tools used; only negative predictions (D) displayed some agreement across all five tools used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confidence for experimental mutagenicity and thus, are of higher concern and are to be prioritized in the selection for subsequent experimental validation *in vitro* or *in vivo* (Frenzel et al., 2017; Rusko et al., 2020; Van Bossuyt et al., 2017).

The combined ranked mutagenicity prediction outcome based on the output of five different (Q)SAR tools is provided in Supplementary Table S7; an overview of the ranking is displayed in Fig. 3. For priority ranking, the categorical prediction categories used in the present study were transformed into integer numeric values comprising −1 (negative), 0 (undefined) and 1 (positive), average rank sums were calculated and subsequently re-scaled to reflect a previously proposed mean mutagenic score ranging from 0 to 1 (Frenzel et al., 2017). The higher the numeric value of the mean summary mutagenic score, the higher the probability of mutagenicity for the EQ TP SMILES in question.

When applying mutagenicity summary score cut-offs previously used in literature (Frenzel et al., 2017), 26 and 30 EQ TP SMILES would be considered probable mutagens (mutagenicity score > 0.66) and probable non-mutagens (mutagenicity score < 0.33), respectively; 52 EQ TP

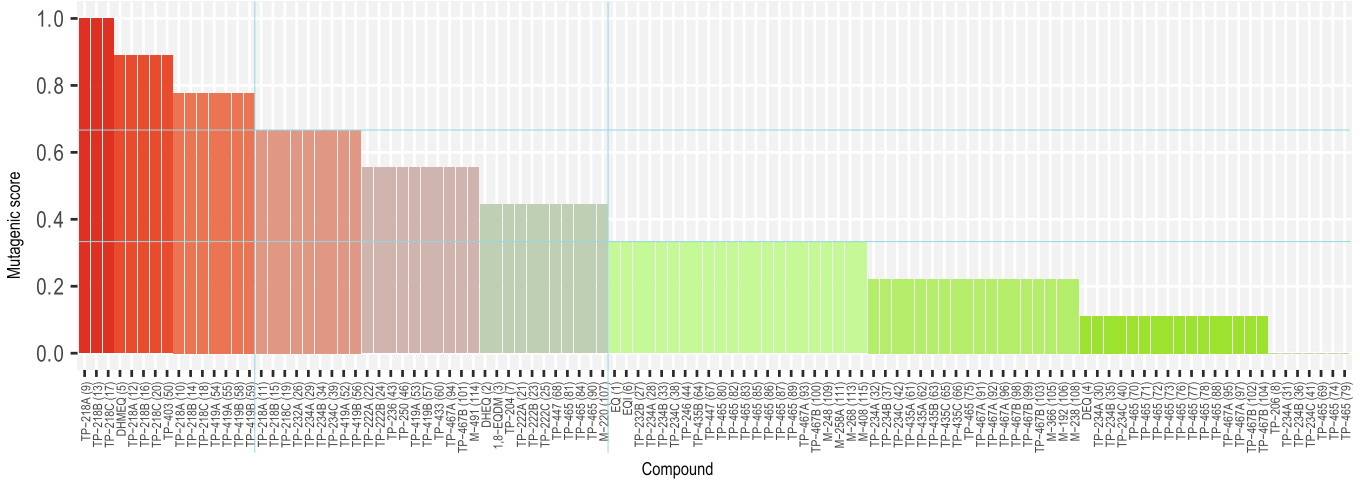

Fig. 3. Schematic presentation of the priority ranking of EQ TP SMILES based on *in silico* Ames genotoxicity predictions using five different (Q)SAR tools. Prioritization ranking was based on mean mutagenic scores recently described in literature (Frenzel et al., 2017). Prediction scores were divided into three different groups: probable mutagens (mutagen summary score > 0.66, probably non-mutagens (mutagen summary score < 0.33), and equivocal predictions (mutagen summary score ≥ 0.33 and ≤ 0.66).

SMILES would yield equivocal predictions (score $\geq$ 0.33 and $\leq$ 0.66).

Substance priority ranking based on mean mutagenic scores (Frenzel et al., 2017) or the proportion of positive findings (Van Bossuyt et al., 2017), are convenient and easy to perform metrics to derive shortlists for subsequent safety evaluation of not yet characterized substances. However, these basic summary scores lack granularity, may be prone to outliers and do not allow for a clear representation of contradictory predictions (Rusko et al., 2020). What is more, currently used scoring systems for priority ranking cannot easily be extended to include occurrence data into the ranking. The latter is key since, as was for example shown for EQ (Merel et al., 2019; Negreira et al., 2017), not all currently known EQ TPs were detected in all matrices tested (see Fig. 1). In other words, when ranking contaminants according to their mutagenicity in human health risk assessment, ideally, compounds detected in commercial food samples should be ranked higher than those exclusively detected in animal feed or samples obtained from laboratory experiments. An integrated fuzzy-logic based analyses workflow (as described in detail below), allows for such a weighted approach and

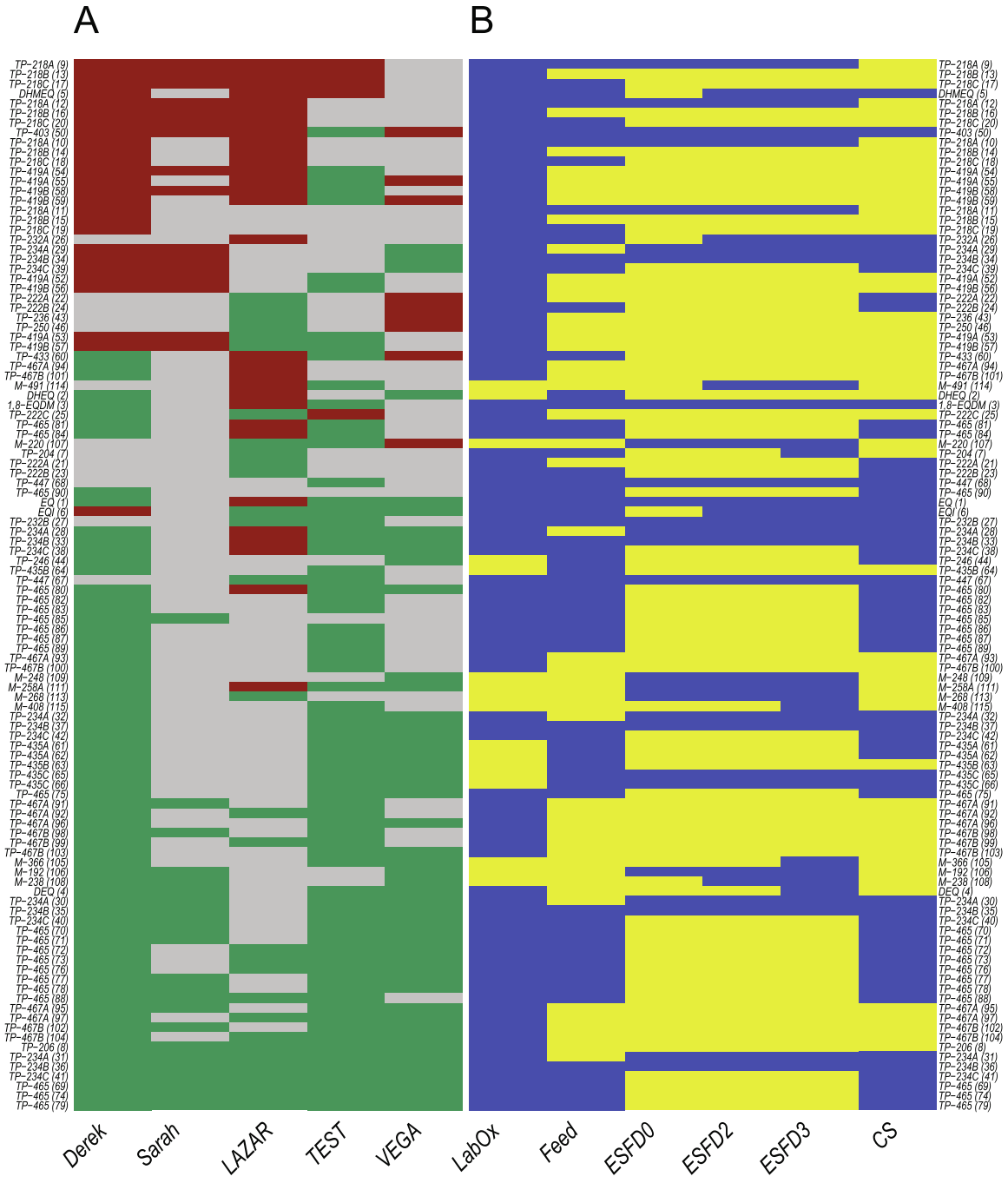

Fig. 4. The heatmap to the left lists recently described EQ TPs ordered according to the mutagenicity priority index. Compounds with agreeing positive mutagenicity predictions were ranked highest, compounds with agreeing negative mutagenicity predictions were ranked lowest, and compounds with conflicting predictions were ranked in between. Red, gray and green boxes indicate positive, undefined and negative (Q)SAR predictions, respectively. The heatmap to the right shows if specific EQ TPs were detected in oxidation experiments (LabOx), commercial fish feed (Feed), muscle of experimental Atlantic salmon (ES) fed control diets (ESFD0) or diets spiked with additional EQ (ESFD2 and ESFD3), as well as muscle samples of Norwegian produced commercial Atlantic salmon (CS). Blue and yellow boxes indicate presence or absence of the ranked compound to the left as determined by TWIMS-QTOF-MS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

efficiently combines different (Q)SAR models and EQ TP occurrence data. In other words, the workflow presented here, provides, assembles, weighs and integrates data in a manner that reflects current harmonized weight of evidence approaches whose use are considered key when working with new approach methodologies (NAMs) in chemical risk assessment (Dorne et al., 2021).

3.3. Fuzzy analytical hierarchy process

In the present study, in addition to ranking substances by proportion ranking and mean summary scores, we adopted fAHP, a MCDM tool recently proposed in the assessment of workplace safety in extreme environments (Zheng et al., 2012), for use with (Q)SAR genotoxicity predictions. The output of the fAHP ranking is presented in Supplementary Table S7.

AHP, which was established by Saaty (1977), is an extensively used method that has been employed successfully in environmental decision making, environmental risk assessment, and health and safety analyses (Mardani et al., 2015). Classic AHP is designed to reflect human decision making, is based on crisp numbers and can efficiently handle both qualitative and quantitative data (Zheng et al., 2012). However, uncertainties and vagueness associated with the mapping of conflicting outcomes such as, contradictory mutagenicity predictions from a battery of (Q)SAR tools, cannot easily be considered. In such cases, fuzzy MCDM tools like fAHP can be employed, as classes and groupings of conflicting data can be analyzed and ranked although boundaries are not concisely defined (Mardani et al., 2015). Unlike binary systems, fuzzy numbers can appropriately express linguistic variables (e.g. safe, harmful, acceptable, unacceptable etc.) and, when compared to other existing safety evaluation methods, fuzzy MCDM tools are more efficient when dealing with imprecise expert judgements (Zheng et al., 2012).

Using fAHP modeling on the harmonized *in silico* mutagenicity predictions (positive, undefined, and negative) obtained by the five different (Q)SAR tools in the present study allowed for the creation of a priority ranking similar to the one proposed by Frenzel et al. (2017) or Van Bossuyt et al. (2017). As can be seen in Fig. 4A, the order and priority rank of substances according to fAHP analysis is the same as the one obtained previously using mean mutagenic scores (Fig. 3). However, unlike previously used combine-and-conquer approaches, fAHP ranking preserves the original classification of each individual (Q)SAR output. In other words, conflicting mutagenicity predictions can easily be spotted and investigated further. For example, EQI, which has previously been flagged to be further investigated due to structural alerts for mutagenicity (EFSA, 2015), was ranked lower than many newly identified EQ TPs in the present study, as only one out of the five different (Q)SAR tools used yielded a positive mutagenicity prediction (Supplementary Table S10).

Using fAHP also allows for the assignment of relative importance and weighting of (Q)SAR outputs. For allowing comparison with other prioritization strategies, in the present study, equal weights were assigned to all pairwise (Q)SAR comparisons. However, the fAHP comparison matrix is flexible and can easily be adjusted by experienced (Q)SAR users to increase (or decrease) the relative importance of a particular analysis tool. In other words, (Q)SAR outputs can be treated as expert opinions, and the opinion of each multiple decision maker can be modeled using reciprocal matrices (Rahimianzarif and Moradi, 2018).

For example, *in silico* predictions of VEGA comprise three different Ames mutagenicity models which are combined into one final VEGA consensus score (Van Bossuyt et al., 2017); this is done as it was found that the combined VEGA score displayed increased prediction performance when compared to individual genotoxicity prediction models (Cassano et al., 2014). LAZAR predictions on the other hand, rely on single predictions based on the same algorithm applied to different training data sets (Frenzel et al., 2017). Applying fAHP would allow, for example, to increase the relative weight of consensus outputs obtained from VEGA and decrease the relative weight of LAZAR outputs. Alternatively, the relative weights of SAR-based rules as for example implemented in Derek could be increased and the one of (Q)SAR based tools decreased if these would be expected to yield more reliable results for a particular set of substances in question. In other words, fAHP could efficiently be applied to aid expert-based integration of *in silico* results, which recently was highlighted as one possible alternative for the integration of predictions of individual (Q)SAR models (Benfenati et al., 2019).

3.4. Priority ranking of EQ TP in farmed Atlantic salmon

In addition to the possibility of the adjustment of the relative importance of complementary (Q)SAR models, fAHP also can be used for the priority ranking of occurrence data and, if available, detection levels of chemicals in different matrices. For example, Fig. 4B depicts occurrence data for the 108 EQ TP SMILES as described in Merel et al. (2019) and Negreira et al. (2017). As can be seen, the highest ranked EQ TP SMILES according to (Q)SAR analyses were not necessarily the ones detected in commercial salmon, the matrix of most concern for human safety. Furthermore, EQ TPs have a different occurrence pattern among commercial salmon feeds compared to commercial salmon fillets on the market. Some EQ TPs (e.g., TP-403 or TP-234B) were found in nearly all surveyed commercial salmon fillets, while other (e.g. DHMEQ) were only rarely observed in salmon.

Based on the combined fAHP analyses of both (Q)SAR Ames genotoxicity predictions and UHPLC-TWIMS-QTOF-MS occurrence data from literature, a final ranking of prioritization for mutagenicity testing of the newly identified EQ TP has been made. The fAHP comparison matrix for occurrence data is shown in Supplementary Table S8; the fAHP analysis output is presented in Supplementary Table S9. Table 2 provides a summary of EQ TPs ranking highest according to the combined fAHP analyses of both (Q)SAR genotoxicity predictions and occurrence data; a ranked list of all EQ TPs is provided in Supplementary Table S10. Suggested structural SMILES codes for the highest ranked EQ TPs in Atlantic salmon are shown in Table 2; associated suggested chemical structures are depicted in Fig. S1 in the supplementary material.

EQ TPs shown in Table 2 namely, TP-403, TP 234B, DHMEQ and TP-234A had (i) at least one associated SMILES annotation predicted to have a high chemical structural likelihood for causing an Ames mutagenicity by at least two of the five (Q)SAR models tested, and (ii) were detected in commercial salmon fillet. Thus, these TPs were considered to be of most concern and should be prioritized for further toxicity testing using for example, a battery of in vitro genotoxicity tests described in a recent EFSA guidance on the assessment of the safety of feed additives for the consumer (EFSA, 2017). In short, this includes the application of the bacterial reverse mutation test (Test guideline 471; OECD, 2020), and the vitro mammalian cell micronucleus test (Test guideline 487; OECD, 2016). If deemed necessary, in addition to the initial in vitro tests, appropriate in vivo studies also could be conducted to assess whether the genotoxic potential observed in vitro is expressed in vivo. In such cases, in the above listed recommendation, EFSA recommends the use of the mammalian erythrocyte micronucleus test (Test guideline 474; OECD, 2016), transgenic rodent somatic and germ cell gene mutation assay (Test guideline 488; OECD, 2020) or an in vivo Comet assay (Test guideline 489; OECD, 2016).

When an earlier safety assessment of EQ for food producing animals was performed by EFSA, only four main EQ TPs, namely, 2,4-dimethyl-6-ethoxyquinoline, ethoxyquin N-oxide, ethoxyquin dimer and EQI, were described in fish meal and salmon, respectively (EFSA, 2015). A recent study exposing humans to the pure parent compound EQ, identified EQI as the main urinary metabolite (Stoeckelhuber et al., 2020). According to *in silico* analyses using the OECD (Q)SAR toolbox, EQI showed structural alerts for mutagenicity, carcinogenicity and DNA-binding (EFSA, 2015). Further in vitro mutagenicity studies with EQI were inconclusive, but it could not be excluded that EQI is clastogenic (EFSA, 2015).

Table 2
Ranked overview of multiple (Q)SAR analyses for mutagenicity alerts of ethoxyquin transformation products (EQ TPs) identified in commercial fish feed (Negreira et al., 2017), and commercial and experimental Atlantic salmon fillets (Merel et al., 2019). ID refers the EQ TP identifier; SMILES* indicates the number of possible SMILES annotations for isomers of the respective EQ TP; SMILES Code displays the structural information of the EQ TP isomer causing an Ames mutagenicity alert; Rank denotes the combined (Q)SAR-occurrence data priority rank, where p, n and u denote positive, undefined and negative (Q)SAR predictions for Ames mutagenicity, respectively. For commercial feed and Atlantic salmon fillets (number) gives the amount on positively identified samples, for experimental Atlantic salmon ≥ mg/kg gives the concentration of EQ in the experimental diets which causes the positive identification of the EQ TPs. High relative likelihood is defined as EQ TPs that are identified as positive for at least 2 out of the 5 (Q)SAR tests.

ID	SMILES*	SMILES Code	Rank	(Q)SAR	Commercial feed	Commercial salmon	Experimental salmon
TP-403	1	O(c1cc2c(N(c3c4c(c(cc(n4)C)C)cc(OCC)c3)C(C=C2C)C)cc1)CC	1	p/p/p/p/n	Yes	Yes (12/12)	Yes (≥0.5 mg/kg)
TP-234B	5	O(c1cc2c(N(C(C=C2C)(C)C)O)cc1)CC	2	p/p/n/u/u	Yes	Yes (11/12)	Yes (≥0.5 mg/kg)
DHMEQ	1	O(c1cc2c(nc(cc2C)C)cc1)CC	3	u/p/u/p/p	Yes	Yes (3/12)	Yes (≥119 mg/kg)
TP-234A	5	O(c1cc2c(N(C(C=C2C)(C)C)O)cc1)CC	4	p/p/n/u/u	Yes	Yes (11/12)	Yes (≥0.5 mg/kg)

In the present workflow, EQI was identified as a potential genotoxic substance. However, EQI had a lower occurrence in surveyed commercial Atlantic salmon and fewer positive (Q)SAR identifications than some of the newly identified EQ TPs (Supplementary Table S10). The present study indicates that SMILES codes created for at least three more EQ TPs detected in farmed Atlantic salmon, two previously not described (TP-403, TP-234A and TP-234B) and DHMEQ, show a higher likelihood for potential genotoxicity for consumers of Atlantic salmon than EQI. Thus, as for EQI, to verify the *in silico* predictions, also for these newly ranked EQ TPs further experimental studies such as the ones listed in the EFSA guidance on the assessment of the safety of feed additives for the consumer (EFSA, 2017) are warranted. Before such validation studies can be performed, chemical standards for the highest-ranked EQ TPs need to be synthesized; this and the running of the in vitro validation experiments was beyond the scope of the present study which focused on the provision of a novel integrated (multi) (Q)SAR genotoxicity prediction and hazard ranking workflow. The ranked list of newly identified EQ TPs presented here provides a helpful guide for which substance to synthesize and test first if experimental follow-up research is to be performed in future work.

4. Conclusions

Chemical risk assessment depends on the availability of both toxicity and occurrence data of substances in food and the environment. Using high throughput non-targeted mass spectrometry-based screening methods, an increasing number of novel chemicals and their breakdown and metabolism products are being detected in different matrices. For the prioritization of which substances to subject to further toxicological screening, transparent and comprehensible strategies must be in place to, in the absence of experimental toxicity data, rank and identify compounds of most concern. The fAHP multi (Q)SAR priority ranking strategy presented here, allowed for the creation of a priority rank of non-evaluated EQ TPs found in Atlantic salmon feed and fillet. Contrary to traditional multi (Q)SAR priority rankings, which categorize suspect mutagen compounds based on their aggregated predicted toxicity scores only, the approach presented here also allows for the consideration and incorporation of analytical occurrence data into the decision making. Based on the integrated weighted hazard ranking of the 47 EQ TPs performed in the present study, we suggest to prioritize four EQ TPs (TP-403, TP234 A, TP234 B, and DHMEQ, see Table 2 for SMILES codes for suggested chemical structures) for future genotoxicity assessments using a battery of in different vitro tests as recently described in a EFSA guidance on the assessment of the safety of feed additives for the consumer (EFSA, 2017). In the present study, genotoxicity prediction of recently published EQ TP data was used as an example, but a wider range of application domains of the fAHP-based MCDM approach presented here can be anticipated. With more data available on human exposure, it is envisaged that the workflow presented here also could be expanded to include human relevance as additional factor in the integration and weighing of evidence in fAHP-based priority rankings of *in silico* predictions and chemical occurrence data. The approach described here is adding an additional method to the growing toolbox of NAMs that currently is driving the ongoing paradigm shift in toxicity testing (Krewski et al., 2020) and ultimately, is contributing to the reduction of animal testing in chemical risk assessment (Dorne et al., 2021).

CRediT authorship contribution statement

J.D. Rasinger: Project administration, Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Funding acquisition, Writing – original draft, Writing – review & editing. **F. Frenzel:** Methodology, Data curation, Formal analysis, Visualization, Writing – review & editing. **A. Braeuning:** Project administration, Supervision, Funding acquisition, Writing – review & editing. **A. Bernhard:** Formal analysis, Validation, Writing – original draft, Writing – review & editing. **R. Ørnsrud:** Project administration, Funding acquisition, Writing – review & editing. **S. Merel:** Data curation, Formal analysis, Writing – review & editing. **M.H.G. Berntssen:** Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106875.

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