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# Comparative assessment of the sensitivity of fish early-life stage, daphnia and algae to the chronic ecotoxicity of xenobiotics – perspectives for alternatives to animal testing

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Running head: Comparison of FELS sensitivity to daphnia and algae

# Comparative assessment of the sensitivity of fish early-life stage, daphnia and algae to the chronic ecotoxicity of xenobiotics – perspectives for alternatives to animal testing

KEYWORDS: Fish early life stage test; Adverse outcome pathways; Mode of action; Alternatives to animal testing; Fish embryo test

Abstract

Predicted no effect concentrations (PNECs) are used in environmental hazard and risk assessment as well as classification and labeling of chemicals. They are derived from the lowest effect concentrations obtained in standard tests: typically, the fish early-life stage (FELS) toxicity test, the chronic daphnia reproduction test, and the algae growth inhibition test. Given the demand to replace and reduce animal tests we explored the impact of the FELS test on the PNEC by comparing the sensitivity of the FELS test to daphnia and algae acute or chronic toxicity tests. Therefore, a database of FELS data for 231 compounds was established. Corresponding daphnia and algae toxicity tests were identified using established databases (US EPA ECOTOX, OECD QSAR toolbox, eChemPortal, EnviroTox and OpenFoodTox database). About 12 percent of the investigated compounds showed a 10-fold higher sensitivity in FELS in

comparison to the lowest effect concentrations obtained with any of the other tests. Many of these compounds were known or discussed as endocrine disrupting or other non-narcotic chemicals indicating that the higher sensitivity in the FELS test is related to a specific mechanism of action. Targeting these mechanisms by alternative test systems or endpoints, for instance using fish embryos, may allow reduction or replacement of the FELS test or prioritize compounds for conduction of the FELS test.

## INTRODUCTION

For the environmental hazard and risk assessment of industrial chemicals, biocides and plant protection products, TER (toxicity exposure ratios) or PNECs (predicted no effect concentrations) are calculated. The TER and PNEC assessment is based on the comparison of effect concentrations and expected exposure concentrations in one or all of three trophic levels represented by a species of algae, daphnia and fish (Ahlers et al., 2006; Damalas and Eleftherohorinos, 2011). Finally, the TER or PNEC assessment is used in classification and labeling of hazards to the aquatic environment as requested by the global harmonization system for classification and labeling of chemicals (Nations, 2011). The trophic level or species, respectively that gives rise to the lowest effect concentration is driving the PNEC. Classification and labelling are conducted for acute and long-term aquatic hazards. For long-term aquatic hazard estimation in fish the determination of chronic toxicity is conducted using a specific test setup (OECD TG 210, Fish Early-Life Stage (FELS) toxicity test (OECD, 1992)). With respect to fish there has been ethical concern with regard to the use of vertebrate animal tests. Therefore, various approaches had been proposed to avoid or reduce the need to conduct FELS tests. For

instance, a tiered testing strategy and the use of the adverse outcome pathway (AOP) concept as a foundation to replace and reduce FELS tests was proposed, and potential AOPs that lead to FELS toxicity were discussed (Volz et al., 2011; Villeneuve et al., 2014). A systematic analysis regarding modes of action (MoAs) that may results in enhanced FELS toxicity revealed that baseline toxicity in the FELS test was similar to baseline toxicity in acute fish and fish embryo toxicity (Scholz et al., 2018). Enhanced toxicity (i.e. effect concentrations below baseline toxicity levels and a high acute-to-chronic ratio) was particularly caused by compounds with a specific or reactive mode of action such as neuromuscular toxicity, methemoglobin formation, extracellular matrix inhibition or endocrine disruption (Scholz et al., 2018). Some of these MoAs may be captured by alternative endpoints such as behavior or phenotypic assessment in the fish embryo test (FET, OECD TG 236 (OECD, 2013)) and could be used to predict the chronic fish toxicity appropriately. However, in the context of a comparative assessment with daphnia and algae test, the FELS test may not represent the most sensitive test and even for compounds with a specific MoA the FELS test may exhibit a weak impact on the TER and PNEC assessment and subsequent labeling and classification of products with regard to their environmental hazard. This minor impact of fish tests has recently been shown for acute toxicity PNEC determination (Rawlings et al., 2019). Daphnia and algae toxicities were driving to a large extent the calculation of a PNEC and fish embryo and acute fish toxicity exhibited similar sensitivity. Hence, it was concluded that replacement of acute toxicity tests by fish embryo test would result in a very similar classification and labeling.

It is also important to understand the relation of the FELS test effect concentrations to other aquatic toxicity endpoints, for two reasons: (i) In case that the FELS test would not represent the test with the highest sensitivity, the impact on TER or PNEC assessment would be low, since the

compounds with weak sensitivity in the FELS test may provoke the strongest effects in daphnids or algae (similar as observed for acute toxicity in (Rawlings et al., 2019)). (ii) The development of alternative test systems such as the FET could be optimized and prioritized for MoAs that exhibit a significant higher toxicity in the FELS test if compared to other aquatic toxicity endpoints (i.e. algae and daphnia toxicity).

Such an analysis may lead to the identification of compound characteristics that are most influential and provide the basis to the potential development of new endpoints in alternative test systems and assessment strategies with sufficient protection to environmental hazards but without the requirement to conduct (vertebrate) animal tests.

Our study had three main objectives: (i) Extension of an existing FELS test database (Scholz et al., 2018) and providing corresponding acute and chronic daphnia and chronic algae toxicity data by searching ECHA registration dossiers, and the EFSA OpenFoodTox databases, and using various other search tools to identify potential data on FELS, daphnia and algae toxicity (eChem Portal, EnviroTox database and AMBIT). (ii) Comparison of the effect concentrations of the different taxonomic levels (fish, daphnia, algae), aiming at the identification of cases in which the FELS test would have the highest influence on the TER or PNEC assessment. We used a threshold of 10 to identify compounds with highest sensitivity in the FELS test as suggested by the European Chemical Agency. According to the European REACH guidance document R7B, no further requirements for fish toxicity testing is indicated if there is compelling evidence to suggest that the fish value is likely to be at least a factor of about 10 less sensitive than invertebrates or algae (ECHA, 2017). It was anticipated that using this threshold would allow identifying potential major MoAs leading to high sensitivity in the FELS test if compared to other aquatic toxicity tests. (iii) Providing a strategy to improve prediction of FELS toxicity by

alternative testing strategy or identify for which compounds a FELS test may be required for PNEC assessment.

## MATERIAL AND METHODS

*Compilation of toxicity data*

Toxicity data were collected from five public databases, the US-EPA ECCOTOX database (https://cfpub.epa.gov/ecotox/), the database included in the OECD toolbox (http://www.qsartoolbox.org/), the database in eChem Portal (https://www.echemportal.org), the EnviroTox database (Health and Environmental Sciences Institute (HESI), 2018) and the OpenFoodTox database available via the AMBIT search tool (https://ambitlri.ideaconsult.net/tool2). The search in the OECD QSAR Toolbox was limited to the chemical inventory lists of available databases. It was restricted to databases that include data on aquatic toxicology, such as Aquatic ECETOC, Aquatic Japan MoE, Aquatic OASIS, Bioaccumulation Canada, Bioaccumulation fish, Carcinogenicity&mutagenicity ISSCAN, Genotoxicity OASIS, Toxicity Japan MHLW, and ToxRefDB US-EPA. In order to search for available toxicity data each of these databases had to be analysed separately. Therefore, the entire list of compounds of each database was used to search for aquatic ecotoxicity data in the whole set of the databases. The US EPA ECOTOX database was not included in the search of the OECD QSAR toolbox but was analysed separately. Received data were subsequently filtered for availability of the appropriate endpoints. Given that many results were represented in more than one database duplicate entries were removed. Entries found in the US-EPA ECOTOX, OECD toolbox and EnviroTox databases were manually inspected for similarity to the OECD guidelines (OECD 210, 211, 202 and 201) by inspecting the original literature.

ECHA registration dossiers were searched via the OECD eChemPortal. Datasets were retrieved using the query provided in the supplementary material (Table S1). Data entries in the ECHA database are generated for the chemical registration under REACH by the registrant, therefore data provided by the registrants is partially confidential and thus the primary data source could not be evaluated. For quality control the search was limited to high quality data (reliability 1 - studies well documented and conducted according to or similar to international guidelines; reliability 2 - studies deviating from international guidelines but well documented and scientifically acceptable) and not assigned quality (reliability 4) as indicated by the provided Klimisch score (Klimisch et al., 1997). Studies with reliability level 3 (inappropriate method, insufficient documentation) were excluded from the database. For data obtained from ECHA registration dossiers the underlying data are not directly available from the online supplementary information, due to property and confidentiality reasons, all those data are available on the ECHA dissemination website (https://echa.europa.eu/home).

Data from the OpenFoodTox database (Bassan et al., 2018) was retrieved using a KNIME (Berthold et al., 2008) node that queries the AMBIT database (Jeliazkova and Jeliazkov, 2011) (https://ambitlri.ideaconsult.net/tool2). This node was developed by IdeaConsult and is freely available through GitHub (https://github.com/ideaconsult/ambit-knime).

For the comparative analyses, FELS, daphnia and algae tests were not considered if the purity of the test chemical was reported to be below < 90%. Furthermore, effect concentrations reported as “less than (<)” were also excluded from comparative analysis. Only mono constituent organic and organometallic compounds were included in the final FELS test database.

*Compilation of Fish Early-life Stage Toxicity Data*

In compliance with the existing FELS database (Scholz et al., 2018) the search for additional FELS data was restricted to studies that were conducted similar as described in the OECD TG 210. Furthermore, the search was limited to the fish species *Pimephales promelas, Oncorhynchus mykiss, Cyprinodon variegatus, Jordanella floridae, Danio rerio, Oryzias latipes*, and *Fundulus heteroclitus* which had been previously identified to represent the most abundant fish species used for FELS testing. FELS tests were identified by an initial collection of all studies that reported LOEC, NOEC, LOEL, and NOEL as endpoints. Only results based on laboratory tests (no field studies) were accepted. Furthermore, data sets with a mean observation duration < 60 days (for rainbow trout) or < 28 days for other species (minimum post-hatch test duration required by OECD TG 210) were excluded. If available, the original literature or report was reviewed to verify the FELS toxicity tests were conducted similar to the OECD TG 210. Data sets obtained under conditions strongly deviating from OECD requirements were not included in the subsequent correlation analysis. The lowest effect concentration that caused a statistically significant effect in comparison with control treatments was considered as the LOEC. Accordingly, the highest test concentration that did not provoke an effect was considered as the NOEC. The LOEC of the most sensitive endpoint (survival or growth) was used for a comparative assessment with EC50 values obtained for daphnia and algae. As has been shown previously the LOEC was close to the LC50 and EC50 in the FELS test (Scholz et al., 2018). Given that typically LOECs and NOEC rather than EC50s are reported for the FELS toxicity test, we used LOEC instead of EC50 or LC50 for the comparative assessment. However, in 2 cases no LOEC or NOEC was provided and the LC50 or EC50s were used.

Only chemicals (N=231) with available FELS studies were considered for subsequent identification of corresponding acute and chronic daphnid toxicity and algal toxicity. For this search the CAS registry numbers were used. Both ionic and neutral form CAS numbers were used to link effects of different taxa (list of CAS numbers is available as supplementary table S2).

The baseline FELS toxicity test LOEC was calculated based on a previously established regression for narcotic compounds (Scholz et al., 2018) using the following equation:

$$\text{Log LOEC survival (mM)} = 1.04 * \text{LogD} + 1.36$$

The Log D was used instead of the Log $K_{ow}$ to account for dissociation.

*Compilation of acute and chronic daphnia toxicity data*

For acute toxicity data only datasets generated from tests with various daphnia species and duration of 48 hours were considered. In case that an individual study reported EC50s separately for replicates, the geometric mean EC50 was calculated. Only results from laboratory tests were accepted. For details on endpoints and identifiers that were considered for retrieving EC50s in each database refer to supplementary table S3.

For chronic toxicity only data on reproductive effects obtained with *Daphnia magna* and test duration of 21 days were accepted. Studies identified via the OECD toolbox were inspected for assignment of the reproductive effect endpoints used in the US-EPA database (see supplementary table S3). Furthermore, after manual inspection of the original literature, reproductive effects were subdivided based on three commonly measured endpoints: (i) the total number of living offspring at the end of the test, (ii) the number of living offspring per day or brood, and (iii) the time required to production of first brood. From the data obtained via eChemPortal the endpoint “reproduction” was used for analysis. LOECs were used for analysis,

however in case no LOEC was reported, the EC50 value was used. The geometric mean of the LOEC or EC50 was calculated for studies that reported effect concentrations for replicates separately. Tests which did not allow deriving a LOEC or EC50 were excluded from the analysis.

*Compilation of algae chronic toxicity data*

Only tests with a duration of 72 h and 96 h and in accordance with OECD TG 201 ("Freshwater Alga and Cyanobacteria, Growth Inhibition Test") or other guidelines (see query search, table S3) were included in the final dataset. Checked endpoints included EC50 and IC50, LOEC and NOEC obtained under laboratory conditions. Effect measures were limited to direct measures of algal growth (according to OECD TG 201), for details on species and the effect measurement identifiers that were considered as indicators for growth in the different databases please refer to supplement table S3 and S4.

In case a reference reported more than one effect concentration for a certain compound, the geometric mean was calculated and included in the final database. The data selection for sensitivity comparison was conducted as follows: (1.) the most sensitive EC50 was selected from the algal endpoints (supplementary table S4), (2.) if no EC50 but a pair of NOEC and LOEC values was available, the most sensitive LOEC was used.

*Fish juvenile/adult and embryo acute toxicity data*

Fish juvenile/adult and embryo toxicity data were collected for those chemicals for which the FELS test was 10 fold or more sensitive than daphnia (acute or chronic) and algae chronic toxicity tests. Acute fish toxicity was identified from the aforementioned databases (ECOTOX,

QSAR-Toolbox, EChemPortal, EnviroTox and OpenFoodTox databases), from Klüver et al. (2015) and Sobanska et al. 2018). Tests following OECD guideline 203 or other ISO and EPA guidelines were used. Fish embryo acute toxicity data, LC50 and EC50 for sub-lethal and additional endpoints, were identified from Sobanska et al. (Sobanska et al., 2018) and from the open literature (www.pubmed.com). Preferentially LC50 values recorded after 96 h or 120 h of exposure were used. If no values for 96 or 120 h exposure duration were available, the LC50 values recorded after 72 h or 48 h were used.

*Physicochemical properties*

The physicochemical properties of compounds tested in the FELS were estimated using ACD/PERCEPTA (ACD/Labs, v. 14.0.0.2726; molecular weight, LogP, LogSW, LogD at pH 7, pKa first strongest acid and first strongest base) and EPIWEB 4.1 (Log Henry's low coefficient, bond method). The following prediction models of ACD/Percepta were used: Consensus LogP, LogS, LogD, ACD/pKa Classic GALAS and classical ACD consensus model.

*Identification of modes of action*

Modes of action (MoA) were assigned by searching databases (e.g. Drugbank, IRAC), a recently established database for predictive model development (Barron et al., 2015) and available literature for the primary MoA of the chemical. If no data on the primary MoA was available, the potential MoA for acute fish toxicity was identified using a structural alert QSAR based on algorithm of Russom et al. (Russom et al., 1997) and Verhaar et al. (Verhaar et al., 1992). This analysis was conducted using the software ChemProp version 6.8 (UFZ, 2018).

*Correlation analysis*

The compiling and grouping and statistical analysis of data in the different databases was conducted using the analytical workflow program KNIME (version 3.7, www.knime.org).

Regression analysis of molar ECs was conducted using a Deming (type II) regression to consider variability for both the independent and dependent variables. The regression analysis was performed using the software Sigma Plot 13.0 (Systat Software) or the R-package mrc (R Core Team, 2014). Statistically significant deviation of the regression slope from 1 or –1 was calculated with the *F* test in Sigma Plot 13.0 ($p<0.05$).

# RESULTS

*Availability of data*

The search for newly published FELS tests not included in an existing dataset (Scholz et al., 2018) revealed 83 additional studies representing 73 chemicals. Since four of these chemicals have been already tested in other studies included in the existing dataset, the update added a total number of 69 chemicals to the existing FELS test database. One chemical (polymeric ethylenethiuramdisulfide (CAS# 30394140)) from the existing dataset without specified molecular weight was not included in the sensitivity comparison because the analysis was conducted based on molar concentrations (µmol/L), resulting in a final number of 231 chemicals with FELS data that could be used for comparative analyses (detailed tables with Chemical Abstracts Service reference numbers, chemical properties, ECs, MoAs and references for each compound are given in the Supplementary tables S5-S10). These chemicals represent 313 entries because some compounds have been studied in more than one species or study. We found FELS toxicity data for 168 (*P. promelas*), 45 (*O. mykiss*), 36 (*D. rerio*), 26 (*C. variegatus*), 25 (*O. latipes*), 10 (*J. floridae*), and 3 (*F. heteroclitus*) compounds. For around 19% of the FELS data entries with available NOEC/LOEC pairs, nominal exposure concentrations were not verified by chemical analysis. Information on the purity of the test chemical was lacking for 32.6% of the data entries. For 190 of 231 compounds, toxicity data on at least one of the investigated

alternative test systems (daphnia acute toxicity –DAT–, daphnia chronic toxicity – DCT– or algae chronic toxicity –ACT–) which fulfilled the quality criteria, was available (supplementary figure S1). The greatest compound overlap was observed between FELS and acute daphnia toxicity test data (Table 1).

*Comparison of daphnia chronic and acute toxicity*

Daphnia toxicity data used for long-term aquatic hazard PNEC calculations are typically derived from chronic toxicity tests. However, chronic daphnia toxicity data were not available for many compounds and restriction to compounds for which chronic toxicity was available would have reduced the number of compounds for comparative assessment to 95 compounds. Therefore, we explored if chronic Daphnia test on average exhibit a higher sensitivity or whether acute tests may be used in case of unavailability of chronic data. From the overall compiled data, there were 99 compounds for which chronic and acute daphnia toxicity data was available. The regression analysis of logarithmic values indicated a high correlation of acute and chronic daphnia toxicity with a data correlation coefficient (R) of 0.87 (Figure 1). The slope of the regression was not significantly different from 1 and the average difference in sensitivity was around 4-fold higher for reproduction in the daphnia chronic toxicity test. Compounds with larger deviation (>100 fold difference) included chlorotetracycline (CTC), fenitrothion (FNT), afidopyropen (AFI), dimethyl disulfide (DDS) and chloroacetic acid (CAA) (5% of all compounds, total number of test compounds = 99). Despite the difference in sensitivity, acute daphnia data were used as a surrogate to increase the database. Hence, in some cases where the FELS test represents the test with the highest sensitivity the lack of chronic daphnia toxicity should be considered as a potential bias.

*Compounds with high sensitivity in the FELS test*

In order to study the potential impact of the FELS test on PNEC determination the effect concentrations of the FELS test were compared to the most sensitive effect concentration between acute or chronic daphnia toxicities and algae chronic toxicity (ACT). Acute toxicity data for Daphnia was used in order to increase the size of the dataset (see previous section for the comparative analysis between acute and chronic daphnia toxicity test). FELS test data was compared with the most sensitive effect concentration of any of the other tests. The line of unity and a threshold value of 10 were used to compare the overall sensitivity of the FELS test with respect to the most sensitive endpoint of daphnia or algae tests.

A total number of 129 compounds were used for comparative analysis, i.e. for these compounds data for FELST, algae chronic toxicity and daphnia toxicity test (chronic or acute) were available (Figure 2). There was a total of 16 chemicals (12%) that showed a higher sensitivity in the FELS test (effect concentration <10 fold lower) compared to daphnids or algae. This included also compounds that did not provoke any toxicity in daphnids or algae, if the highest tested concentration was at least 10 fold above the FELS LOAEC. Six out of the 16 compounds with higher FELS sensitivity (4.6%) showed effect concentration <100-fold lower (Table 2). Four compounds did not provoke any toxicity to algae and are not represented in figure 2 (total n=125). For 25 compounds (19%) algae or daphnia toxicity tests displayed a 10 fold or higher sensitivity. Ten of those compounds exhibited more than 100-fold higher sensitivity (supplementary table S11). All compounds with higher sensitivity in the FELS test were associated with high toxic ratios in the FELS test (TR = Baseline $toxicity_{FELST}$ $/FELST_{LOEC}$), for some compounds reaching levels of $10^5$ to $10^6$.

*Relation of high sensitivity in the FELS test with modes of action*

Table 2 indicates MoAs that were associated with compounds of 10 fold higher sensitivity in the FELS test. In order to link FELS sensitivity to specific MoA one can compare the range of sensitivities for each MoA. Previous analyses have indicated that there are certain MoAs associated with high toxic ratio or ACR in the FELS test (e.g. neurotoxicity, methemoglobin formation, extracellular matrix formation inhibition and endocrine disruption). If FELS test sensitivity is compared to modes of action, particularly 2 MoAs, endocrine disruption and inhibition of extracellular matrix formation appeared to be associated with higher FELS sensitivity ratios, with median values between 1 and 10 and peak values of 8100 and 37, respectively (Figure 3, see supplementary table S12).

*Fish juvenile/adult and embryo toxicity of compounds with high sensitivity in the FELS test*

In many cases FELS test concentrations relate to acute effect concentrations (Scholz et al. 2018). Furthermore, the FELS toxicity test includes embryonic stages and hence, chronic effects may be indicated already by (sub-lethal) effects of compounds in fish embryos. Therefore, we investigated, how reported effect concentrations for acute fish toxicity and sub-lethal and lethal effect concentrations of fish embryos relate to effect concentrations in the FELS test, with focus on compounds that exhibit highest sensitivity in the FELS test (see section "Compound with high sensitivity in the FELS test", n= 16). Figure 4 shows the $EC_{50}$ or LOEC values, for fish, algae, daphnia and fish embryo toxicity tests. Fish embryo toxicity data were available for 8 out of 16 compounds. For three compounds (azinphos-methyl, benzovindiflupyr and peracetic acid) the effect concentrations of acute fish toxicity were already close (i.e. in the range of 10 fold difference) to the FELS toxicity test (Figure 4). Thiram, a dithiocarbamate associated with inhibition of cellular matrix formation, showed similar sensitivity for the fish embryo test and the

chronic FELS test when the $EC_{50}$ for sub-lethal effects was compared with the chronic fish LOEC.

A large proportion of the compounds (5 out of 16) with high sensitivity to the FELS test were endocrine disruptors. Therefore, an endpoint related to estrogenic effects, i.e. induction of cyp19a1b measured with reporter gene fluorescence in embryos of a transgenic zebrafish strain was included (Brion et al., 2012). Figure 4 shows the sensitivity ratios using the most sensitive EC50 for malformations or cyp19a1b induction in the fish embryo test. Three compounds (4-tert-butylphenol, estradiol and 17alpha-ethynilestradiol) showed an effect concentration close to the FELS test (Figure 4 and supplement table S13) if the induction of cyp19a1b was considered.

## DISCUSSION

The FELS test is the most demanding vertebrate animal test routinely conducted for environmental hazard and risk assessment and is requested by many different regulations (Oris et al., 2012; Scholz et al., 2013, 2018). Similarly, as observed for acute fish toxicity, FELS toxicity for many compounds is described by an intrinsic baseline or minimal toxicity, driven by the hydrophobicity of the test compound (Scholz et al., 2018). Only compounds with a specific non-narcotic mode of action are more likely to show an enhanced toxicity, i.e. a higher TR and/or higher ACR. Therefore, it has been proposed that development of alternative approaches could target these MoAs and relate to key events of an AOP. The use of such targeted assays would enable to reduce or replace FELS tests, by either predicting effect concentrations or indicating for which compounds the conduction of an FELS test may finally be required (Scholz et al., 2018). However, given that FELS tests are used in the context and comparison to endpoints of other taxonomic levels (typically represented by algae and daphnia toxicity), even for compounds with a specific MoA the FELS toxicity may not be relevant for risk assessment,

classification and labeling. Therefore, it is also critical to understand how FELS toxicity relates to algae and daphnia toxicity and for which type of compounds or MoA the higher sensitivities for the FELS test are observed. Such a comparative assessment represents the rationale for the development of the threshold approaches for reduction of acute fish toxicity tests (Hoeger et al., 2003; OECD, 2010; Creton et al., 2014). The threshold approach acknowledges that algae and daphnia represent in many cases the most sensitive models. Hence, it was proposed that chemicals are first tested in daphnia and algae and the lowest effect concentration of these models is used for a limit test of acute fish toxicity. A full range of concentrations would only be tested for acute fish toxicity if mortality would occur in the limit test (Rawlings et al., 2019).

In order to compare effect concentration of the FELS test to algae and daphnia we made use of a previously established database with effect concentrations of the FELS toxicity test for 183 compounds (Scholz et al., 2018). However, given that for these compounds only a limited number of algae and daphnia effect concentrations were available and in order to increase the data basis for a comparative assessment, the database was extended by searching additional databases. This search was leading to the identification of 73 additional compounds and also an increased number of corresponding algae and daphnia effect concentrations. While our database content was increased it may also contain a higher degree of uncertainty as the previously established data set. The reason is that our assessment was based on data that were retrieved from other database without an accompanying publication and/or limited availability of the original studies or raw data (e.g. from dossiers submitted to ECHA) and it is difficult to assess the quality and reliability of data in detail. Hence, for individual compounds there might be a bias with regard to experimental protocols and data quality. However, restricting the analysis to data for which original data sources were available would result in a very limited dataset that

would compromise a quantitative comparative assessment. Thus, for individual dataset a subsequent analysis of the original study data or replication of the study may be required to confirm the reliability. Detailed dossiers submitted to agencies may represent a potential source of data associated with sufficient details for quality assessment. However, for this kind of data it is often required to keep the compound identity confidential (e.g.(Ahlers et al., 2006)). This would hamper to release the MoAs related to high sensitivity in the FELS test and was hence, not considered as an option for obtaining more data on FELS toxicity tests.

The present study demonstrated that in many cases daphnia and algae chronic toxicity tests revealed similar effect concentration as the FELS test. Hence, for these compounds the FELS test would have a weak or no impact on the determination of PNECs. For around 12 % of the test compounds the FELS test revealed an at least 10 fold higher sensitivity. The compounds for which the higher FELS toxicity was observed appeared to exhibit a specific mode of action, indicated by their TRs and the known MoA, such as endocrine disruption or extracellular matrix inhibition. For some of the compounds with high FELS sensitivity, however, only acute daphnia studies were available. Availability of chronic effect concentration may change the classification of these compounds, i.e. FELS tests may no longer represent the test with highest sensitivity.

As indicated by the previous assessment of TRs and ACRs of the FELS test, typically compounds with a specific MoA provoke enhanced toxicity. Similarly, sensitivity (if compared to algae and daphnia) in the FELS test appears to be associated with a non-narcotic and specific MoA. Partially the same MoAs were identified that also lead to high TR or ACRs in the FELS test, such as extracellular matrix synthesis inhibition and endocrine disruption (Figure 3). Some of the MoA displayed a wide range of sensitivity ratios with the FELS test (supplementary table S11) which could be due to, e.g experimental variability, species specific sensitivities in the

FELS test and other (unknown) MoA as assigned in this study. While the inhibition of extracellular matrix synthesis inhibition was linked to enhanced FELS toxicity via impact on the embryonic development and impaired swimming and feeding, the relation of endocrine disruption to growth is not well understood (Scholz et al., 2018). However, there is some evidence that environmental estrogens can affect post-embryonic growth in fishes through impact on the growth hormone-insulin-like-growth factor (GH-IGF) system (Hanson et al., 2012; Reindl and Sheridan, 2012). For compounds with other MOAs, such as benzovindiflupyr, a fungicide that inhibits succinate dehydrogenase, there were also concerns that this compound may exhibit endocrine disrupting properties (Food Safety Authority, 2015). We also identified one COX inhibitor (diclofenac) with high sensitivity in the FELS test. Prostaglandins have been discussed to play an important role in fish reproduction (Martinović-Weigelt et al., 2017), but the relation to the higher sensitivity in FELS test observed especially for diclofenac is not known. Furthermore, two other COX inhibitors included in the comparative assessment did not exhibit higher FELS sensitivity. For some of the other additional MoAs (e.g. reactive electrophile) that were identified in the present study, appropriate knowledge why they are related to higher sensitivity in the FELS test is lacking and may require research to provide data for a mechanistic process understanding. There were three compounds for which no specific or reactive mode of action was identified, all of them out of the structural alert domain for prediction of MoAs (dibutyl thiourea, 4,4'-oxydi(benzenesulphonohydrazide) and peracetic acid). However, the high toxic ratios found for the FELS test indicate that these compounds are likely to exhibit an unknown specific or reactive MoA. Interestingly, although known to be associated with high TRs (Scholz et al., 2018), the comparative analysis did not reveal neurotoxicity as a MoA that leads to high sensitivity in the FELS test. It is likely that many neuroactive compounds impact

primarily on invertebrates. Hence, Daphnia represented the most sensitive species for neuroactive compounds (around 34.6% –9 out of 26– of the neuroactive chemicals displayed a 10fold higher sensitivity in acute or 3chronic daphnia tests). This has also been observed for the comparison of acute toxicity data (Rawlings et al., 2019).

Overall, the comparative assessment of FELS toxicity provides the perspective that measurement of endpoints related to key events (KEs) and AOPs in alternative test system could contribute to reduce animal test numbers. With respect to the compounds with high FELS sensitivity, the analysis of malformations and markers for endocrine disruption in fish embryos could provide an endpoint with similar sensitivity and related to or representing KE of AOPs leading to high FELS toxicity. This was indicated for 4 compounds, for which fish embryo EC50 concentrations were found in the range of the FELS test LOEC. However, appropriate corresponding data in fish embryo are still lacking for most of the other compounds and a systematic experimental analysis would support to develop alternative endpoints. If not for the prediction of the effect concentration, the assessment of alternative endpoints in fish embryos or other alternative test systems may at least lead to the identification of compounds for which a FELS test should be conducted. Such an approach may be combined with a threshold approach proposed for the reduction of acute toxicity tests and help to reduce the need for conducting FELS toxicity tests to assess long-term aquatic hazard of chemicals.

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# Tables and Figures

**Table 1.** Results of the literature search for available toxicity data in the FELS toxicity test, daphnia acute and chronic toxicity test and algae chronic toxicity test[a]

| Test | Database | Number of study entries | | Number of chemicals | | FELST test compound overlap |
|---|---|---|---|---|---|---|
| | | Total entries | Entries after application of quality criteria/ removal of duplicates | Total chemicals | Entries after application of quality criteria/ removal of duplicates | |
| FELS test | ECOTOX-QSAR Toolbox | 328 | 239 | 206 | 169 | n/a |
| | eChem Portal | 381 | 68 | 265 | 61 | |
| | EnviroTox db | 717 | 5 | 317 | 5 | |
| | OpenFoodTox | 16 | 0 | 14 | 0 | |
| Daphnia acute toxicity | ECOTOX-QSAR Toolbox | 422 | 344 | 140 | 134 | 162 |
| | eChem Portal | 146 | 116 | 99 | 83 | |
| | EnviroTox db | 272 | 27 | 85 | 19 | |
| | OpenFoodTox | 21 | 3 | 16 | 2 | |
| Daphnia chronic toxicity | ECOTOX-QSAR Toolbox | 179 | 131 | 97 | 81 | 119 |
| | eChem Portal | 92 | 64 | 79 | 61 | |
| | EnviroTox db | 377 | 12 | 95 | 8 | |
| | OpenFoodTox | 16 | 0 | 13 | 0 | |
| Algae chronic toxicity | ECOTOX-QSAR Toolbox | 339 | 235 | 124 | 99 | 136 |
| | eChem Portal | 198 | 119 | 102 | 87 | |
| | EnviroTox db | 669 | 6 | 86 | 6 | |
| | OpenFoodTox | 18 | 1 | 8 | 1 | |

[a] The search was limited to chemicals for which FELS data were available. Search was conducted subsequently, i.e. first via the ECOTOX QSAR Toolbox, followed by echemPortal and EnviroToxDB. This resulted in a decreasing number of newly identified chemicals in the subsequent searches. Quality criteria refer to purity of the test chemical (below < 90%) or deviation from the test guidelines. The search for daphnia and algae data was limited to compounds for which FELS data were available.

**Table 2.** Compounds with >10 fold higher sensitivity in fish early-life stage test compared to daphnia acute toxicity or Daphnia magna chronic toxicity, algae chronic toxicity and the FELS test baseline toxicity[a]

| CAS Number | Common Chemical Name | 3-Letter Abbrv. | Mode of action | Sensitivity Daphnia acute/ FELST | Sensitivity Daphnia chronic/ FELST | Sensitivity Algae chronic/ FELST | TR[b] |
|---|---|---|---|---|---|---|---|
| 57-63-6 | 17alpha-Ethinylestradiol | AEE | Endocrine disruption | | 8101 | 66408 | 73166 |
| 613-62-7 | 2-(phenylmethoxy)naphthalene | 2NP | Narcosis | | 28 | >31 | 26 |
| 80-51-3 | 4,4'-oxydi(benzenesulphonohydrazide) | OBH | Out of structural alert domain | 22 | 15 | 15 | 66194 |
| 98-54-4 | 4-tert-butylphenol | 4TB | Endocrine disruption | 160 | 85 | 89 | 61 |
| 86-50-0 | Azinphos-methyl | APM | AChE inhibition | 176 | 1176 | >2940 | 24395 |
| 1072957-71-1 | Benzovindiflupyr | BVF | succinate dehydrogenase inhibitor | 47 | 19 | >494 | 264 |
| 80-09-1 | Bisphenol S | BPS | Endocrine disruption | 1000 | 140 | 160 | 1366 |
| 109-46-6 | Dibutyl thiourea | DBT | Out of structural alert domain | 38 | | 69 | 196 |
| 15307-79-6 | Diclofenac | DCF | COX inhibition | 427 | 255 | 308 | 1684 |
| 105-53-3 | Diethyl malonate | DEM | reactive electrophiles/pro-electrophiles | 205 | 28 | 544 | 1055 |
| 50-28-2 | Estradiol | ETD | Endocrine disruption | 10630 | >513 | >9180 | 5842 |
| 79-21-0 | Peracetic acid | PAA | Out of structural alert domain | 117 | | 106 | 590293 |
| 1918-02-1 | Picloram | PCL | Methemoglobin formation | 78 | | 55 | 23465 |
| 835621-07-3 | Regorafenib | RGF | Other MoA | | 2304 | 127 | 15882 |
| 137-26-8 | Thiram | THI | Extracellular matrix formation | 37 | 47 | 38 | 51235 |
| 76-87-9 | Triphenylstannanol | TPS | Endocrine disruption | 56 | | 61 | 872109 |

[a] Color code: blue = most sensitive, red = lowest sensitive, grey = either acute or chronic toxicity data were available for daphnids. Sensitivity values represent the ratio of effect concentrations (daphnia or algae toxicity versus FELST). A ">" indicates that no toxicity was observed, in these cases the highest tested concentration was used to calculate the effect ratio.

[b]The toxic ratio (TR = Baseline toxicity$_{FELST}$/ LOEC$_{FELST}$) was calculated for the fish early life stage test.

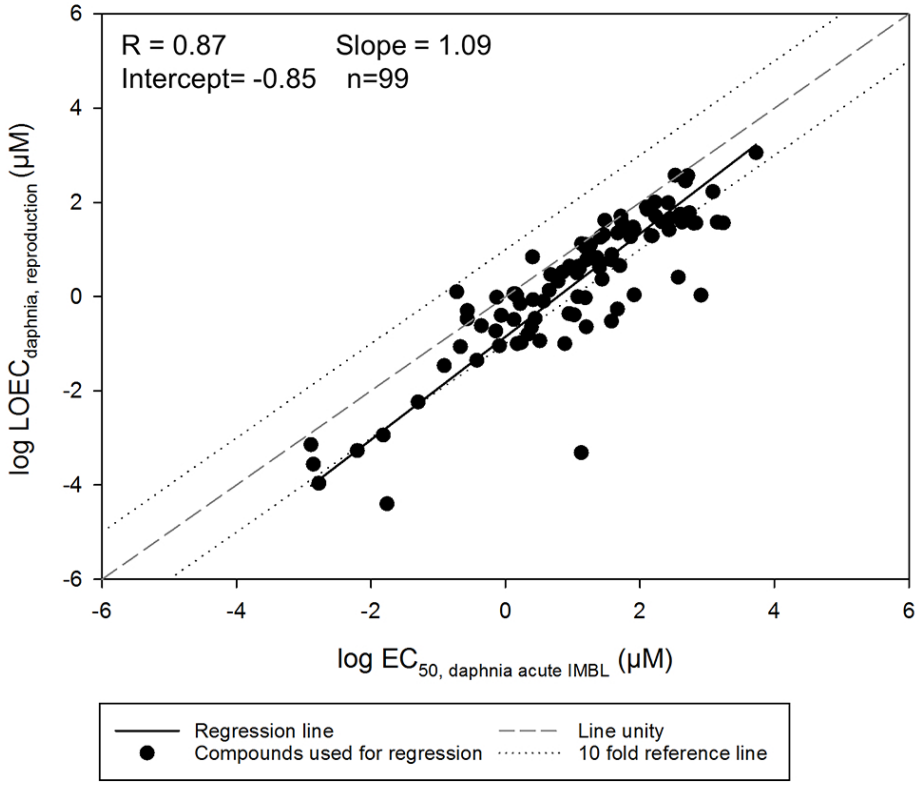
FELST= fish early-life stage test; AChE = Achetylcholinesterase; COX= cyclooxygenase; MoA = mode of action

**Figure 1.** Correlation of daphnia chronic toxicity and daphnia acute toxicity. The indicated sample numbers (n) refer to the number of compounds used for regression analysis. For details on compounds and data sources, refer to Supplemental Data. The table summarizes the parameters of the linear regression. EC50, median effective concentration; LOEC, lowest-observed-effect concentration; IMBL, immobile endpoint.

**Figure 2.** Comparison of effect concentrations in fish early-life stage tests (FELST) and the most sensitive test concentration between Daphnia sp. (chronic (DCT) or acute (DAT) toxicity), and algae chronic toxicity (ACT). Toxicity data are given in µmol/L. Comparison of all data for which at least a chronic algae toxicity test and one daphnia (acute or chronic) test – in addition to the FELS test – was available (n=125). The type of test yielding the most sensitive effect concentration can be identified from the graph by the symbol preceding the abbreviation of the compound name. (§) - DAT, (*) - DCT, (~) – ACT. Compound name abbreviation can be found in table 2. Dashed lines represent the line of unity ± 10 fold difference (1 log).

**Figure 3.** Relation of FELS test sensitivity to the MoA. The FELS sensitivity is described by the ratios of the lowest effect concentration found in the chronic algae, acute or chronic daphnia test to the effect concentration of the FELS test. The dashed line represents a ratio of 10. The number inside the parenthesis indicates the number of chemicals present in each class. For details on the compounds and data sources, refer to the Supplement (table S5-S10 and S12). Inh. extracellular matrix - inhibition of extracellular matrix formation by lysyl oxidase inhibition; LOEC - lowest effect concentration; MoA - mode of action; Ox. - oxidative.

**Figure 4.** Differential sensitivity of 16 chemicals to six toxicity tests (chronic FELS test, Daphnia acute and chronic test, algae chronic test and acute and embryo fish toxicity test). In the case of the fish embryo test, two type of endpoints are displayed, the lethal concentration ($LC_{50}$) and the effect concentration ($EC_{50}$) for sub-lethal effects (malformations, locomotor response or cyp19a1b induction, see supplementary table S13 for details). The dashed line indicates 10 fold sensitivity difference from FELS toxicity test. In case more than one study was available the bars represent median values and the range of values for the toxicity studies is represented by error bars. No bars indicate lack of data or no toxicity was observed (denoted by a #). FELST, chronic fish early-life stage toxicity test; DAT, daphnia acute test; DCT, daphnia chronic test; ACT, algae chronic test; FET; fish embryo test; baseline chronic fish toxicity; FELS baseline toxicity.




Correlation of daphnia chronic toxicity and daphnia acute toxicity. The indicated sample numbers (n) refer to the number of compounds used for regression analysis. For details on compounds and data sources, refer to Supplemental Data. The table summarizes the parameters of the linear regression. EC50, median effective concentration; LOEC, lowest-observed-effect concentration; IMBL, immobile endpoint.

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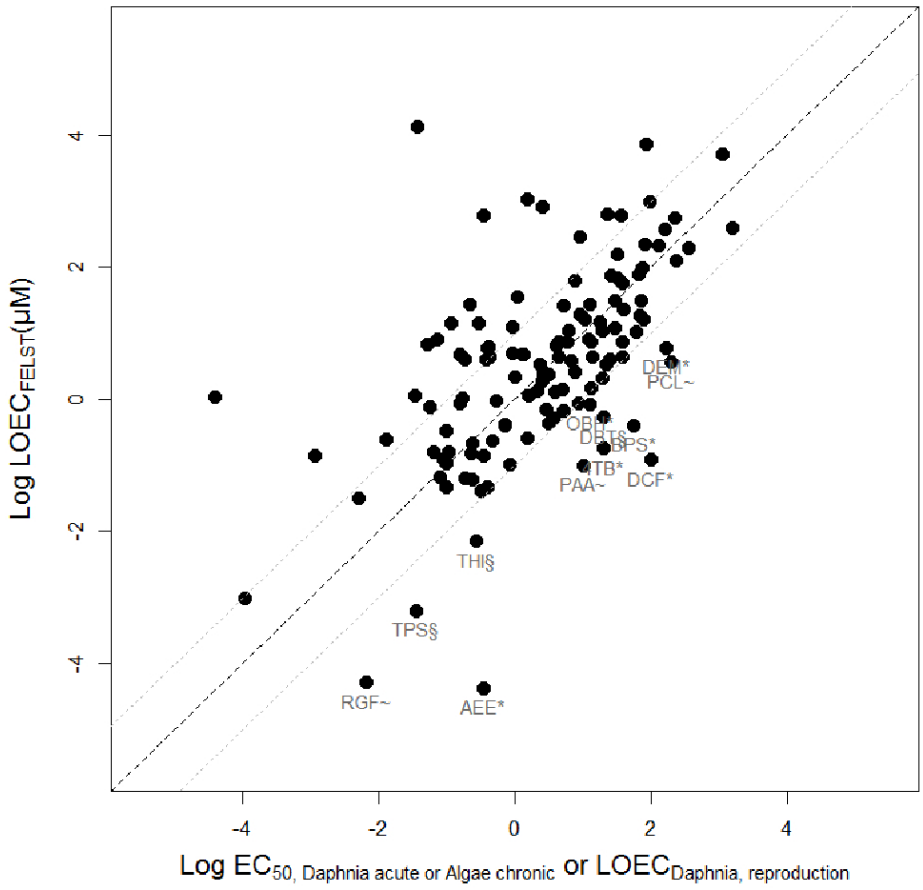


Comparison of effect concentrations in fish early-life stage tests (FELST) and the most sensitive test concentration between Daphnia sp. (chronic (DCT) or acute (DAT) toxicity), and algae chronic toxicity (ACT). Toxicity data are given in µmol/L. Comparison of all data for which at least a chronic algae toxicity test and one daphnia (acute or chronic) test – in addition to the FELS test – was available (n=125). The type of test yielding the most sensitive effect concentration can be identified from the graph by the symbol preceding the abbreviation of the compound name. (§) - DAT, (*) - DCT, (~) – ACT. Compound name abbreviation can be found in table 2. Dashed lines represent the line of unity ± 10 fold difference (1 log).

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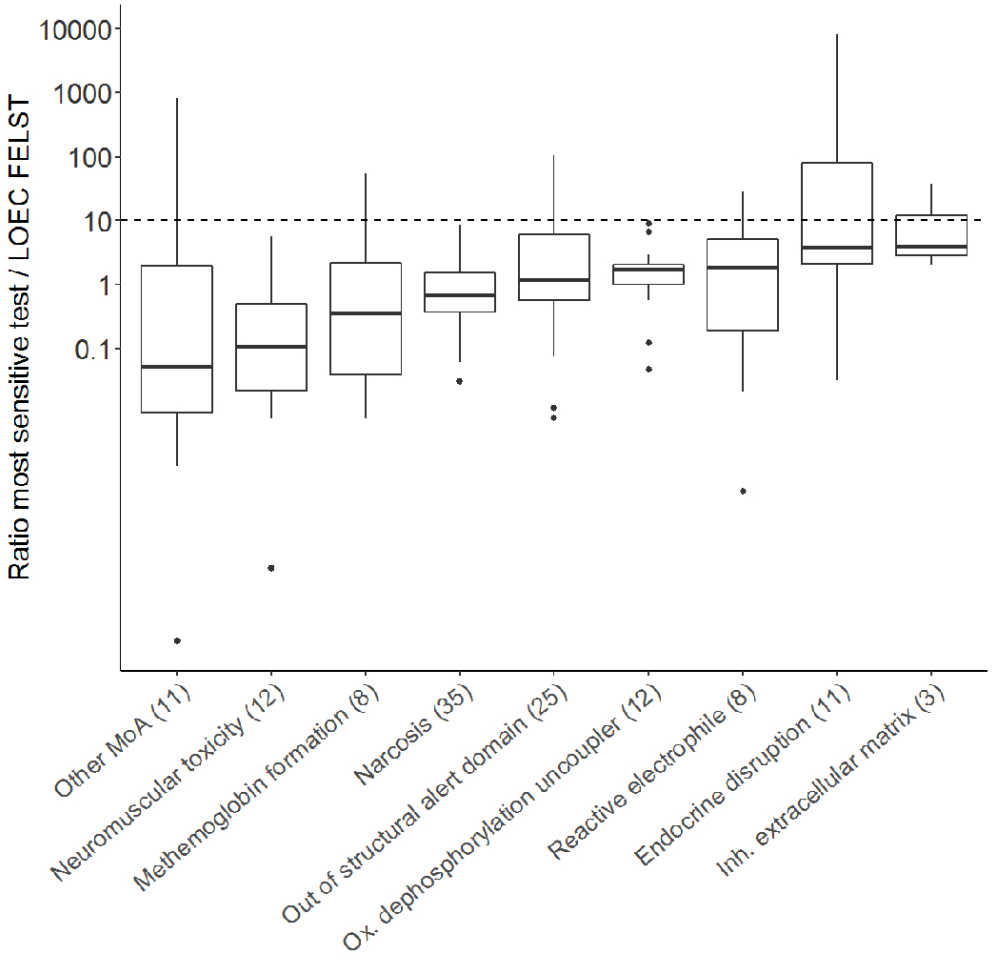


Relation of FELS test sensitivity to the MoA. The FELS sensitivity is described by the ratios of the lowest effect concentration found in the chronic algae, acute or chronic daphnia test to the effect concentration of the FELS test. The dashed line represents a ratio of 10. The number inside the parenthesis indicates the number of chemicals present in each class. For details on the compounds and data sources, refer to the Supplement (table S5-S10 and S12). Inh. extracellular matrix - inhibition of extracellular matrix formation by lysyl oxidase inhibition; LOEC - lowest effect concentration; MoA - mode of action; Ox. - oxidative.

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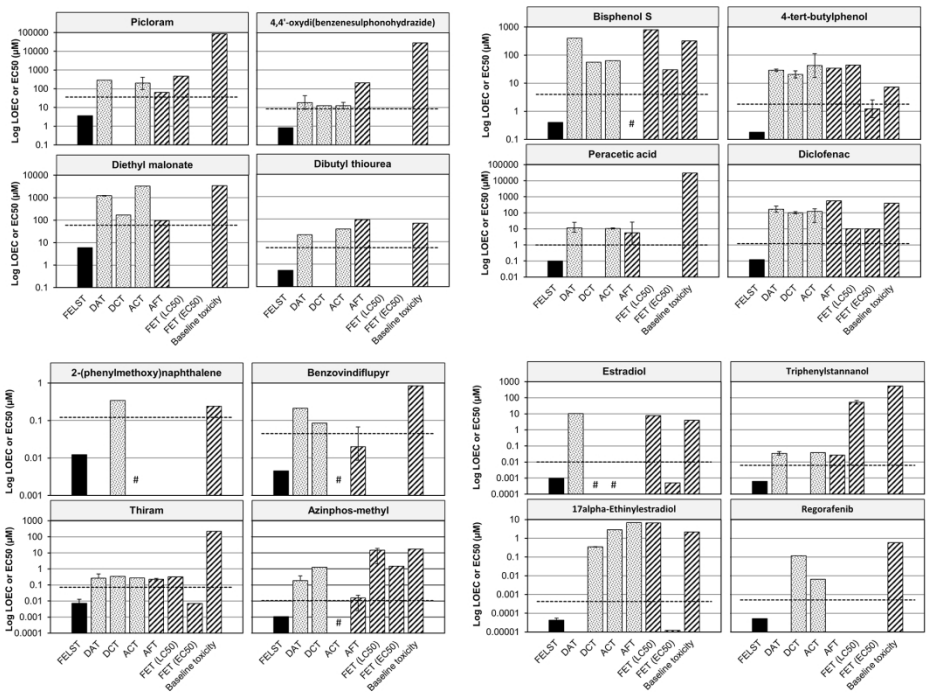


Differential sensitivity of 16 chemicals to six toxicity tests (chronic FELS test, Daphnia acute and chronic test, algae chronic test and acute and embryo fish toxicity test). In the case of the fish embryo test, two type of endpoints are displayed, the lethal concentration (LC50) and the effect concentration (EC50) for sub-lethal effects (malformations, locomotor response or cyp19a1b induction, see supplementary table S13 for details). The dashed line indicates 10 fold sensitivity difference from FELS toxicity test. In case more than one study was available the bars represent median values and the range of values for the toxicity studies is represented by error bars. No bars indicate lack of data or no toxicity was observed (denoted by a #). FELST, chronic fish early-life stage toxicity test; DAT, daphnia acute test; DCT, daphnia chronic test; ACT, algae chronic test; FET; fish embryo test; baseline chronic fish toxicity; FELS baseline toxicity.

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