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Critical Review

# Meta-Analysis of Fish Early Life Stage Tests—Association of Toxic Ratios and Acute-to-Chronic Ratios with Modes of Action

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**Abstract:** Fish early life stage (ELS) tests (Organisation for Economic Co-operation and Development test guideline 210) are widely conducted to estimate chronic fish toxicity. In these tests, fish are exposed from the embryonic to the juvenile life stages. To analyze whether certain modes of action are related to high toxic ratios (i.e., ratios between baseline toxicity and experimental effect) and/or acute-to-chronic ratios (ACRs) in the fish ELS test, effect concentrations (ECs) for 183 compounds were extracted from the US Environmental Protection Agency's ecotoxicity database. Analysis of ECs of narcotic compounds indicated that baseline toxicity could be observed in the fish ELS test at similar concentrations as in the acute fish toxicity test. All nonnarcotic modes of action were associated with higher toxic ratios, with median values ranging from 4 to $9.3 \times 10^4$ (uncoupling < reactivity < neuromuscular toxicity < methemoglobin formation < endocrine disruption < extracellular matrix formation inhibition). Four modes of action were also found to be associated with high ACRs: 1) lysyl oxidase inhibition leading to notochord distortion, 2) putative methemoglobin formation or hemolytic anemia, 3) endocrine disruption, and 4) compounds with neuromuscular toxicity. For the prediction of ECs in the fish ELS test with alternative test systems, endpoints targeted to the modes of action of compounds with enhanced toxic ratios or ACRs could be used to trigger fish ELS tests or even replace these tests. *Environ Toxicol Chem* 2018;37:955–969. © 2018 SETAC

**Keywords:** Fish early life stage test; Adverse outcome pathways; Baseline toxicity; Mode of action; Alternatives to animal testing; Fish embryo test

## INTRODUCTION

The assessment of chronic fish toxicity is an integral part of environmental risk assessment for the registration of industrial chemicals, pesticides, biocides, and pharmaceuticals around the globe (Scholz et al. 2013b). An early analysis of fish life cycle tests indicated that in most cases the embryo-larval and juvenile stages were the most sensitive in response to chemical exposure (McKim 1977). Therefore, the fish early life stage (ELS) test has been used ever since as a lower-tier test to detect chronic fish toxicity (Oris et al. 2012; Scholz et al. 2013b). In contrast, full–life cycle or multigeneration tests are only conducted in rare cases and for higher-tier testing. An appropriate Organisation for Economic Co-operation and Development (OECD) guideline, test guideline 210 (Organisation for Economic Co-operation and Development 2013), is available for regulatory testing, and endpoints measured include hatching success, pre- and post-hatch survival, growth (indicated by length and weight at the end of the test), and developmental abnormalities. The no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) are typically reported for each endpoint (Oris et al. 2012).

Given that the fish ELS test requires the highest number of vertebrates in environmental hazard assessment and that exposed animals may suffer from pain or distress, various

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approaches have been proposed to replace this test (Scholz et al. 2013b). For instance, Volz et al. (2011) described a 3-tiered testing strategy using cellular assays as an initial test (tier 1), embryo testing as an intermediate test (tier 2), and a fish ELS test as a confirmatory test (tier 3). In a subsequent publication it was suggested to identify mechanisms leading to fish ELS toxicity and develop an optimized fish embryo test (Villeneuve et al. 2014). Both suggestions relied on the use of the adverse outcome pathways (AOP) concept, that is, the assumption that any adverse effect of a chemical on an individual or a population is propagated from a molecular initiating event through perturbations of key events at the cellular, tissue, and organ levels (Ankley et al. 2010; Kramer et al. 2011; Ellison et al. 2011; Organisation for Economic Co-operation and Development 2012). Both Volz et al. (2011) and Villeneuve et al. (2014) suggested various potential molecular initiating events and key events as central to the development of fish ELS toxicity such as arylhydrocarbon receptor binding, acetylcholinesterase (AChE) inhibition, gill cell toxicity, and swim bladder inflation. For swim bladder inflation, a potential link of fish ELS toxicity to inhibition of the enzyme thyroid peroxidase and subsequent reduction of thyroid hormone levels was experimentally demonstrated by exposure of fathead minnow and zebrafish to 2-mercaptobenzothiazole (Nelson et al. 2016; Stinckens et al. 2016). However, a detailed analysis of existing fish ELS data aiming to identify major AOPs associated with chronic toxicity is lacking.

To identify relevant AOPs, fish ELS toxicity could be reviewed from 2 different angles: the identification of compounds with high toxic ratios or high acute-to-chronic ratios (ACRs). The toxic ratio represents the relationship of the experimental toxicity effect concentration (EC) versus the EC for baseline toxicity calculated with a quantitative structure–activity relationship (QSAR). Baseline toxicity or narcosis represents the unspecific interaction of chemicals, resulting in constant internal membrane concentrations that cause acute toxicity in aquatic organisms (see review in van Wezel and Opperhuizen 1995). Because internal concentrations in aquatic organisms are driven to a large extent by partitioning of the chemical between water and the organism, baseline toxicity is related to descriptors of the hydrophobicity of a compound, for example, the partition coefficient for octanol and water ($K_{OW}$) or lipid and water ($K_{lipw}$). Differences in the toxicokinetics of a compound may, however, interfere with the internal concentrations. The concept of baseline toxicity has been widely used for the identification of specific modes of action that cause acute toxicity at a considerably lower internal concentration (Escher and Hermens 2002). It has been applied to derive structural alerts or modes of action impacting on acute toxicity of various organisms (e.g., Verhaar et al. 1992; Russom et al. 1997; Maeder et al. 2004; Von der Ohe et al. 2005). To derive toxic ratios, typically a regression analysis of median lethal concentration (LC50) and the log $K_{OW}$ for compounds with a known narcotic mode of action is conducted to derive baseline toxicity levels. In contrast to acute toxicity, baseline toxicity regression of the fish ELS test has been analyzed for only a limited number of structurally related narcotic compounds (chlorobenzenes; Van Leeuwen et al. 1990b). Alternatively, specific modes of action may be identified by application of the constant toxic membrane concentration (Escher and Hermens 2002) or the chemical activity concept (Schmidt and Mayer 2015). The latter has already been applied to fish ELS toxicity for a limited number of hydrocarbons (Butler et al. 2016) and for a 14-d exposure of 50 industrial chemicals in guppy (Mayer and Reichenberg 2006).

The ACR is defined as the quotient of ECs for acute toxicity and chronic toxicity, typically the quotient of LC50 and NOEC. An ACR close to 1 would indicate compounds for which a similar range of ECs would be found in acute and chronic exposure scenarios. The toxicity of compounds with a high ACR would not be sufficiently described by acute toxicity tests. Often, ACRs have been calculated to provide assessment factors to extrapolate from acute to chronic toxicity (see Slooff et al. 1986; Elmegaard and Jagers op Akkerhuis 2000; Forbes and Calow 2002; European Centre for Ecotoxicology and Toxicology of Chemicals 2003). A more significant use of ACRs might, however, be seen in the possibility of providing information on compound characteristics and mechanisms leading to chronic toxicity. For instance, a study by Kenaga (1982) revealed particularly high ACRs for insecticides, herbicides, and benzene substitutes in fish and invertebrates. Ahlers et al. (2006) detected a tendency of narcotic compounds (i.e., baseline toxicants) to show low ACRs. Various structural alerts indicating a higher probability to provoke increased chronic toxicity (i.e., high ACRs) were identified (Ahlers et al. 2006). A study by Roex et al. (2000) showed that nonpolar narcotics exhibited the smallest variation in ACRs. It was concluded that for this group of compounds chronic effects could be predicted using acute toxicity tests. A mechanistic explanation would be that narcosis occurs at a constant membrane concentration or membrane volume fraction (Warne et al. 1991), is independent or only slightly dependent on the exposure duration (Chaisuksant et al. 1997), and is reversible (i.e., disappears completely once the chemicals are depurated; van Wezel and Opperhuizen 1995).

Higher ACRs were observed particularly for compounds with specific modes of action (herbicides, central nervous system seizure agents, and AChE inhibitors) in fish and invertebrates (European Centre for Ecotoxicology and Toxicology of Chemicals 2003). Both Kenaga (1982) and the European Centre for Ecotoxicology and Toxicology of Chemicals (2003) suggested that modes of action for which the toxicity could be expected to be established at the acute exposure levels may exhibit high ACRs. However, a systematic evaluation of whether a potential cumulative damage, increasing susceptibility, toxicokinetics, and/or potential differences in the mode of action leads to high ACRs is lacking.

The main aim of the present study was to conduct a meta-analysis on the fish ELS test to identify potential modes of action that could be used to design mode of action– and AOP–targeted assays. From many available analyses of chronic fish toxicity data, however, it is difficult to derive indicators for modes of action or mechanisms of action that lead to high toxic ratios or ACRs in the fish ELS test because they had different goals. In many of these studies, the identity of the compounds has not been revealed in the publication; data from different species, animal classes (e.g., invertebrates and fish), or types of tests

(e.g., full life cycle, reproduction, early life stage) were combined; the analysis was limited to a low number of compounds; or the evaluations were focused on experimental design and statistics (Suter and Rosen 1988; Slooff et al. 1986; Van Leeuwen et al. 1990b; Elmegaard and Jagers op Akkerhuis 2000; Roex et al. 2000; European Centre for Ecotoxicology and Toxicology of Chemicals 2003; Ahlers et al. 2006; Oris et al. 2012). In comparison with earlier analyses (see Kenaga 1982), the database was extended and included a wider and more systematic approach (regression-based ACRs, baseline toxicity analysis). Given that principally an unlimited number of organic compounds could be expected for future chemical development, the present analysis was restricted to organic compounds. We compared fish ELS ECs from publically available studies with corresponding acute fish and fish embryo LC50 data. A particular focus was on the embryonic stages of the fish ELS test and the fish embryo test because the embryonic stage may provide a key to predict fish ELS toxicity and to develop alternative test systems.

## MATERIALS AND METHODS

### Database search

We searched the US Environmental Protection Agency's (USEPA's) ECOTOX database for fish ELS toxicity studies with organic compounds reported until December 2015. This was done by an initial search for fish studies that reported NOEC and/or LOEC values (which are typically reported for fish ELS tests) for a minimum of a 32-d exposure in warm-water species and 60 d for rainbow trout. The search was limited to studies using one of the 7 major species for which fish ELS tests are mainly conducted: fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), sheepshead minnow (*Cyprinodon variegatus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), flagfish (*Jordanella floridae*), and mummichog (*Fundulus heteroclitus*). These species are commonly used in ecotoxicity studies. Nevertheless, for some of them, such as mummichog, only 4 studies/compounds with fish ELS tests were identified. Therefore, to avoid a too heterogeneous data set with respect to species, no species other than those just indicated were considered in the data search. Subsequently, studies that referred to fish ELS tests conducted as described in the OECD 210 guideline were manually selected. As a minimal criterion, it was required that exposure was initiated within 24 or 48 h (fathead minnow) post fertilization. In addition, we searched the open literature (the Institute for Scientific Information's Web of Science, Google Scholar) for fish ELS studies. Any fish ELS studies that indicated purities of the test compound $<$90% were excluded. Wherever possible (i.e., in all cases where the data stemmed from a published journal article or publically available report), the original study was used to extract the EC (indicated in Supplemental Data, Table S1). In cases where original data were not publically available (22%), the USEPA database entry was used. For correlation analysis, only studies that reported both a NOEC and a LOEC for either survival or growth (length and weight of fish at the end of the test) were considered.

Growth LOECs (length and weight at the end of the test) exhibited a strong correlation with survival LOECs, occurring on average at 2- to 3-fold lower concentrations. Therefore, in cases where no survival rates (LOEC/NOEC pairs) were available, these were predicted from the LOEC for growth or vice versa based on the regression equations (Supplemental Data, Figure S5). This was done to combine studies for which LOEC/NOEC pairs were only available for either survival or growth and to increase the number of data points for the subsequent comparison with acute fish or fish embryo toxicity data. However, it must be noted that a few compounds, such as 17$\alpha$-methyltestosterone, exhibited a significantly higher sensitivity for growth and that, hence, ECs for survival rates might be underestimated in a few cases.

After retrieval of fish ELS toxicity data, corresponding acute 96-h fish toxicity data were identified for the same species that was used in the fish ELS study. For fathead minnow, most acute fish toxicity data were obtained from the Duluth database (Russom et al. 1997). The acute toxicity data of other species were retrieved from the USEPA ECOTOX database. Corresponding zebrafish embryo LC50 values stemming from studies using diverse protocols and variable exposure times were identified from a previously established database (Scholz et al. 2014; Sobanska et al. 2018). In case of multiple studies conducted for one compound and species, the geometric mean of the LC50 was used. Physicochemical properties (molecular weight, log $K_{OW}$, water solubility) were calculated from SMILES codes using EPI Suite, Ver 4.11 and ACD Labs, Ver 12.5 (Build 39480). All values from the EPI Suite program were assumed to represent the neutral form of the chemical. The degree of ionization was calculated by treating all chemicals as monoprotic acids or bases and using the most influential p$K_a$ (predicted by ACD Labs) to calculate the degree of ionization at pH 7. Compounds with a degree of $>$50% ionization were labeled and excluded from the regression analysis for baseline toxicity (narcotics). The 50% ionization level was chosen to highlight compounds for which the ionization may impact on uptake and, if not considered, lead to an underestimation of baseline LC50 and toxic ratios. Ionization could principally be considered by replacing the log $K_{OW}$ with the log membrane–water partition coefficient ($K_{lipw}$) and calculating the $K_{lipw}$ for the charged species. However, for neutral compounds the $K_{OW}$ is very similar to the $K_{lipw}$ (Endo et al. 2011), and the partitioning of charged species is often within a factor of approximately 10 compared with the corresponding neutral species for most classes of ionic organic chemicals (Bittermann et al. 2016). Hence, the impact on baseline toxicity and/or toxic ratio calculation of ionizable compounds is limited and may only be observed with higher degrees of ionization.

### Correlation analysis

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 12.0 (Systat Software) or the R-package mrc (R Development Core Team 2017). Statistically significant deviation of the regression slope

from 1 or –1 was calculated with the *F* test in Sigma Plot 12.0 ($p < 0.05$). Compounds deviating from the regressions were identified with a box plot analysis of the regression residuals using the software IBM SPSS Statistics, Ver 21, based on a deviation from the regression by >1.5-fold of the interquartile distance below or above the 25th or 75th percentile. After the box plot analysis, the regression analysis was repeated, excluding the previously identified deviating values to avoid a distortion of the regression by individual values deviating from the regression.

## Calculation of toxic ratios and ACRs

Values of LC50 are typically obtained from modeled concentration–response curves to describe acute toxicity. They are used for describing the relationship between physicochemical and structural characteristics for acute aquatic toxicity and to derive toxic ratios from the comparison of predicted LC50 for narcotic compounds versus observed LC50s (e.g., Maeder et al. 2004). In contrast, NOEC and LOEC values are used to describe chronic fish toxicity based on concentrations where a statistically significant difference in the effects (e.g., survival, growth) is observed. The disadvantage of NOEC and LOEC is their dependency on the sample size and variability of the effect. Hence, ideally modeled ECs would also be used to analyze fish ELS toxicity. Unfortunately, for many of the publically available chronic toxicity studies, the raw data are not provided and it is difficult to reanalyze data for modeling of ECs. However, if NOEC and/or LOEC data would correlate to modeled ECs, they could be used as a surrogate. To demonstrate the relationship of the NOEC/LOEC to the LC10 and LC50 for survival, we analyzed the data of one study with a larger compound set including compounds with different modes of action and availability of all pertinent raw data (Call and Geiger 1992). Concentration–response curves for survival in the fish ELS test were fitted to the data using the Hill-slope equation (Equation 1) and used to estimate LC10 and LC50 values.

$$y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{x}{\text{LC50}}\right)^{-p}} \quad (1)$$

The parameter Max was set to 100%, and the slope (*p*) was estimated. Given the relatively high background mortality in some replicates of the compound tested, the parameter Min was estimated as well. This means that LC10 or LC50 may in some cases not represent 10 or 50% lethality levels, respectively, but different percentages of mortality. However, this approach was required because 10% effect levels would otherwise be difficult to calculate, in part because of the limited number of compounds ($n = 24$) for which concentration-dependent mortality data were available. The independent variable *x* represents the measured exposure concentration (micrometers), and *y* represents the percentage of survival. The software R and the package drc (R Development Core Team 2017) embedded into a KNIME workflow were used to model concentration–response curves (Berthold et al. 2008; see Supplemental Data, Figure S3, for concentration–response modeling).

A strong correlation (Pearson's correlation coefficient = 0.99) and similar sensitivity (slope between 0.97 and 0.99 and intercept of –0.09 and 0.01, respectively) were found for both the comparison of log-transformed $\text{NOEC}_{\text{survival}}$ with the LC10 and $\text{LOEC}_{\text{survival}}$ with the LC50 (Supplemental Data, Figure S4). Regression analysis indicated a slope not significantly different from 1 for both comparisons, and the regression lines were very close to the line of unity. Maximum differences of 4.7 (LC10 versus NOEC) and 2.8 (LC50 versus LOEC) were observed. Given the high correlation of LOECs and LC50s, we used LOECs for the calculation of fish ELS test toxic ratios ($\text{TR}_{\text{FELST}}$; Equation 2), with the $\text{LOEC}_{\text{baseline toxicity}}$ representing the predicted baseline toxicity LOEC for narcotic compounds and the $\text{LOEC}_{\text{exp}}$ representing the statistically significant different LOEC from an experimental study. Baseline toxicity is believed to reflect the nonspecific disturbance of the integrity and functioning of cell membranes by chemicals (Escher et al. 2011). At log $K_{OW} < 0$, the internal cellular bioavailable concentrations will equal the external exposure concentration and, hence, membrane accumulation will be relatively low and potentially less important for unspecific toxicity. Hence, to avoid overestimation of toxic ratios, a minimum log $K_{OW}$ of 1 was used for baseline toxicity and toxic ratio calculation.

$$\text{TR}_{\text{FELST}} = \frac{\text{LOEC}_{\text{baseline toxicity}}}{\text{LOEC}_{\text{exp}}} \quad (2)$$

Data derived from the marine species *C. variegatus* and *F. heteroclitus* were not corrected for the impact of ionic strength on solubility and partitioning, which might impact ECs for baseline toxicity and possibly other types of toxicity. As indicated in previous publications (Gouliarmou et al. 2012; Escher et al. 2017), the salting-out effect on partitioning and solubility was of limited significance at least when assessing LOEC values covering more than 8 orders of magnitude and presenting these values in log–log plots.

The ACR represents the ratio of acute versus chronic toxicity. There is no agreement in the literature with regard to the ECs to be used for the calculation of ACR, but usually the LC50 for acute toxicity is compared with the NOEC of chronic toxicity (e.g., Ahlers et al. 2006). Given the strong correlation of LC50s and LOECs for the fish ELS test, we calculated the ACR based on the LOECs for survival. We derived 3 different ACRs depending on the acute toxicity data that were used as reference (Equations 3–5).

$$\text{ACR}_{\text{int}} = \frac{\text{LOEC}_{\text{embryo toxicity}}}{\text{LOEC}_{\text{FELST,survival}}} \quad (3)$$

$$\text{ACR}_{\text{AFT}} = \frac{\text{LC 50}_{\text{AFT}}}{\text{LOEC}_{\text{FELST,survival}}} \quad (4)$$

$$\text{ACR}_{\text{ZFET}} = \frac{\text{LC50}_{\text{ZFET}}}{\text{LOEC}_{\text{FELST, survivsal}}} \quad (5)$$

The intrinsic ACR value ($\text{ACR}_{\text{int}}$) describes the ratio of the observed toxicity in the embryonic phase of the fish ELS test

($LOEC_{embryo\ toxicity}$) to the LOEC of the entire fish ELS test ($LOEC_{FELST,\ survival}$, Equation 3). Some studies do not distinguish between teratogenic effects and mortality and, hence, the $LOEC_{embryo\ toxicity}$ may in some cases include sublethal effects as well. A high $ACR_{int}$ would indicate that the mortality would increase after the embryonic phase. Given the lack of LC50 data for embryo toxicity in fish ELS tests, the LOEC was used. The acute fish toxicity ACR value ($ACR_{AFT}$) is based on the comparison of acute fish 96-h toxicity LC50s ($LC50_{AFT}$) and the $LOEC_{FELST,\ survival}$ of the same species. This type of ACR (i.e., comparison with the acute toxicity of juvenile or adult fish) is most commonly used for deriving ACRs. The LC50s and LOECs for the $LOEC_{FELST,\ survival}$ value may be derived from different studies. The $ACR_{ZFET}$ value compares acute zebrafish embryo toxicities ($LC50_{ZFET}$) with the $LOEC_{FELS,\ survival}$ test.

### Calculation of reference lines for effective chemical activity and the constant toxic membrane concentration

To estimate whether baseline toxicity in the fish ELS test is within the range of baseline toxicity typically observed for narcotic compounds in acute toxicity, reference lines for effective chemical activities (*a*) of 0.01 and 0.1 and a constant toxic membrane concentration of 100 mM were calculated. For the calculation of effective chemical activities, we used the generalized solubility equation of Ran and Yalkowsky (2001) to derive the water solubility of the corresponding subcooled liquids ($S_w^{liquid}$, Equation 6) and to calculate the corresponding EC for a given log $K_{OW}$ (Equation 7).

$$\log S_{W^{liquid}}(\mathrm{mol/L}) = 0.5 - \log K_{OW} \tag{6}$$

$$a = \log \frac{EC\left[\frac{mmol}{L}\right]}{S_{w^{liquid}}\left[\frac{mmol}{L}\right]} \tag{7}$$

The constant toxic membrane concentration reference line (Equation 8) for exposure concentrations causing narcosis ($C_{water,narcosis}$) at equilibrium conditions (Goss and Endo 2016) was calculated using the $K_{OW}$ instead of the membrane partition coefficient, given the strong concordance of both partition coefficients for neutral compounds (Endo et al. 2011).

$$C_{water,\ narcosis} = \frac{100\ \mathrm{mmol/L}}{K_{OW}} \tag{8}$$

### Identification of modes of action

The modes of action of all compounds included in the analysis were identified primarily according to the intentional use and by considering whether the appropriate target would be present in fish. For instance, an AChE-inhibiting insecticide would target the synapse of cholinergic nerve cells, which are abundant in fish, although it may be less important because of metabolic degradation or weaker receptor/enzyme affinity. In contrast, photosystem II inhibition caused by an herbicide represents the primary intended mode of action, but this mode of action is not relevant for heterotrophic organisms. Hence, in cases where no primary mode of action was available for fish or at least vertebrates, publically available literature or reports from governmental agencies were searched to deduce a presumable mode of action. In cases where no data on the primary or presumable mode of action were available, they were predicted for acute toxicity based on a modified structural alert approach (Ellison et al. 2015) of Verhaar et al. (1992) using the software Toxtree 2.6.13. Oxidative phosphorylation uncouplers were identified using a combination of structural alerts based on Russom et al. (1997) and Schultz and Cronin (1997) conducted with the software ChemProp (UFZ–Helmholtz Centre for Environmental Research 2016) and/or experimental data summarized by Escher and Schwarzenbach (2002). Experimental evidence provided by Escher and Schwarzenbach (2002) was preferred in case of contradictory classifications. Furthermore, the classifications were checked for agreement with structural requirements for uncoupling (Terada 1990).

## RESULTS

### Establishment of a fish ELS test data collection

Effect concentrations (NOEC and/or LOEC) were identified for 183 compounds from the USEPA ECOTOX database and the open literature (detailed tables with Chemical Abstracts Service reference numbers, chemical properties, ECs, modes of action, and references for each compound are given in the Supplemental Data, Tables S1 and S2). These data represent 258 database entries because some compounds have been studied in more than one species or study. We found fish ELS data for 131 (*P. promelas*), 50 (*O. mykiss*), 22 (*C. variegatus*), 24 (*D. rerio*), 17 (*O. latipes*), 10 (*J. floridae*), and 4 (*F. heteroclitus*) compounds. In addition, 41 compounds/studies were not included in subsequent analyses because of a test compound purity of <90% or obvious deviations from the OECD 210 guideline (exposure was started after 48 h post fertilization or after hatching). For 14% of the fish ELS 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 27% of the data entries. The comparative analyses were limited to compounds and studies, respectively, that derived both a LOEC and a NOEC (164 compounds and 231 study entries, respectively).

### Compounds with high toxic ratios in the fish ELS test

Using the data set established for the present study, it was possible to establish a baseline toxicity regression and to calculate the corresponding toxic ratios for a wider set of compounds. The baseline toxicity regression was obtained by comparing the $LOEC_{FELST}$ of all neutral narcotic compounds with the corresponding log $K_{OW}$. A narcotic mode of action was assigned to all neutral compounds that were not known or not

hypothesized to exhibit a specific or reactive mode of action and/or were predicted to be acute nonpolar narcotic chemicals using structural alerts defined by Russom et al. (1997) and Verhaar et al. (1992). The LOEC was used for this analysis given the high concordance with the LC50 (see *Calculation of toxic ratios and ACRs*). The regression analysis revealed a slope not significantly different from –1 and an intercept of 1.73 (Figure 1).

We then used baseline regression to calculate the toxic ratio. Compounds that could not be associated with a mode of action or with a presumed uncoupling mode of action in acute toxicity tests exhibited the lowest toxic ratio with median values ≤4.3 (Figure 2). Increasing toxic ratios were observed for neurotoxic compounds, compounds potentially provoking methemoglobin formation, potential endocrine disrupters, and compounds with other specific modes of action (e.g., mutagenic compounds, alcohol dehydrogenase inhibitors). The highest toxic ratio ranges were obtained for dithiocarbamate fungicides interfering with extracellular matrix formation as potential mode of action (toxic ratios ranging from 7.2 to $3 \times 10^9$). However, in all groups compounds with a low toxic ratio were found as well. Toxic ratios well below 1 indicate that exposure concentrations may have been underestimated because of experimental limitations or a reduced internal bioavailability.

The observed fish ELS test ECs were compared with chemical activities of 0.1 and 0.01 and a constant toxic membrane concentration of 100 mmol/$kg_{lip}$, both of which have been shown to characterize the acute baseline toxicity (narcosis) of neutral organic compounds. The fish ELS toxicity data were found around chemical activities of 0.01 for narcotic compounds and were close to the reference line of a constant toxic membrane concentration of 100 mM (Figure 1).

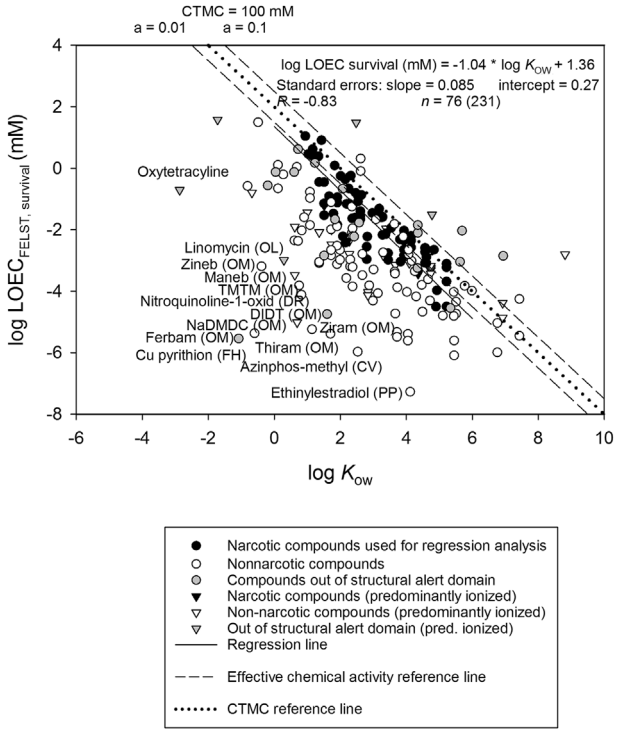



**FIGURE 1:** Determination of baseline toxicity for fish early–life stage test toxicity data (lowest-observed-effect concentration survival) of different species. Compounds indicated by name exhibited a toxic ratio of more than 1000-fold. Ionized chemicals have also been plotted against the log $K_{OW}$ of the neutral form, although in reality the actual hydrophobicity will be shifted to the left of the plot depending on the acidity constant p$K_a$. "Out of structural alert domain" refers to compounds for which the structures have not been included in the training set for fish acute toxicity structural alerts. For better visualization the reference lines for chemical activity and the constant toxic membrane concentration have been drawn over the entire area of the plot which does not represent the valid range (for details on the calculation, refer to *Materials and Methods*). CTMC = constant toxic membrane concentration; CV = *Cyprinodon variegatus*; DIDT = 5,6-Dihydroimidazo[2,1-c][1,2,4]dithiazol-3-thion; DR = *Danio rerio*; FELST = fish early life stage test; $K_{OW}$ = octanol–water partition coefficient; LOEC = lowest-observed-effect concentration; NaDMDC = sodium dimethyldithiocarbamate; OL = *Oryzias latipes*; OM = *Oncorhynchus mykiss*; PP = *Pimephales promelas*; TMTM = tetramethylthiuram monosulfide.

## ACR: Comparison of acute fish and fish ELS toxicity

The toxic ratio analysis of the fish ELS test revealed diverse modes of action with ECs well below the baseline toxicity. Several of the described modes of action (uncoupling, reactivity, neurotoxicity) had been identified in previous analyses (e.g.,

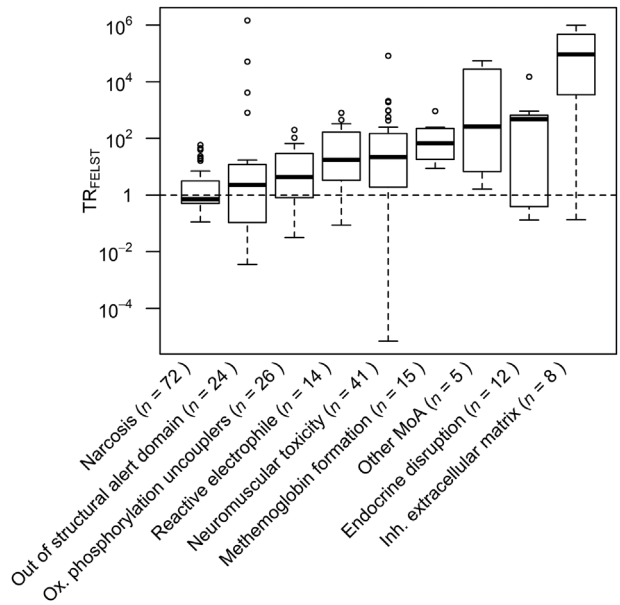



**FIGURE 2:** Distribution of toxic ratios for different modes of action in the fish early life stage test (details on compound identity, hydrophobicity, lowest-observed-effect concentration values and the corresponding mode of action can be found in Supplemental Data, Table S1). Boxes represent the median and the 25th and 75th percentiles. Whiskers represent 1.5 times the interquartile range, and dots refer to any data outside of the interquartile range. Numbers (*n*) indicate the number of studies. Some compounds could be represented multiple times. Groups were sorted according to the median toxic ratio. Surfactants ($n = 3$) were excluded from the analysis. FELST = fish early life stage test; Inh. extracellular matrix = inhibition of extracellular matrix formation by lysyl oxidase inhibition; MoA = mode of action; Out of structural alert domain = compounds with structures that have not been included in the training set for fish acute toxicity structural alert; Ox. = oxidative; TR = toxic ratio.

Verhaar et al. 1992; Russom et al. 1997) with high toxic ratios for acute toxicity. Hence, the low ACR of these compounds may indicate that fish ELS toxicity is already established during an acute exposure. We compared 3 different types of ACR: the LOEC for the embryonic phase of the fish ELS test ($LOEC_{FELST,\ embryo\ toxicity}$), the LC50 for acute 96-h fish toxicity ($LC50_{AFT}$), and the acute zebrafish embryo toxicity ($LC50_{ZFET}$). These reference values were used to calculate corresponding ACRs ($ACR_{int}$, $ACR_{AFT}$, $ACR_{ZFET}$) with Equations 3 to 5.

***$ACR_{int}$.*** A fish ELS test includes the exposure of embryonic stages, and the toxicity of many test compounds might already be fully established during acute exposure in the embryonic period. A low $ACR_{int}$ would indicate that continuation of exposure beyond the embryonic stage would not result in increased toxicity. Furthermore, $ACR_{int}$ was derived from the same experiment and, hence, is not vulnerable to experimental variability between different studies. Therefore, we compared the LOECs for embryo toxicity (cumulative teratogenicity and survival) with the LOECs obtained for overall survival and growth of the entire test duration. A high concordance was observed for 36 data pairs for which LOECs for embryo toxicity and the entire test duration were available (Figure 3A). The regression slope of 0.99 and intercept of 0.077 (for comparison of logarithmic values) were close to the line of unity and indicated nearly equal sensitivity of survival LOECs for the embryonic and entire test periods. The slightly higher sensitivity for some compounds in the embryonic phase results from the cumulative assessment of teratogenicity and mortality because separate LOECs for these endpoints were not available in some studies (Van Leeuwen et al. 1990a, 1986). However, no effect on survival was observed for 31 compounds in the tested concentration range during embryonic development. For most of these compounds ($n = 29$) the maximum test concentration was close to the LOEC for overall survival or growth. Hence, to estimate whether these compounds exhibit a considerably increased ACR, higher test concentrations would need to be considered for the embryonic period. For only 3 compounds (with different modes of action)—triethylamine, 2,4-dichlorophenol, and 17-methyltestosterone—the tested range of concentrations indicated that chronic effects in the fish ELS test were at least 10-fold below the concentrations that caused mortality in the embryonic period (Supplemental Data, Table S6).

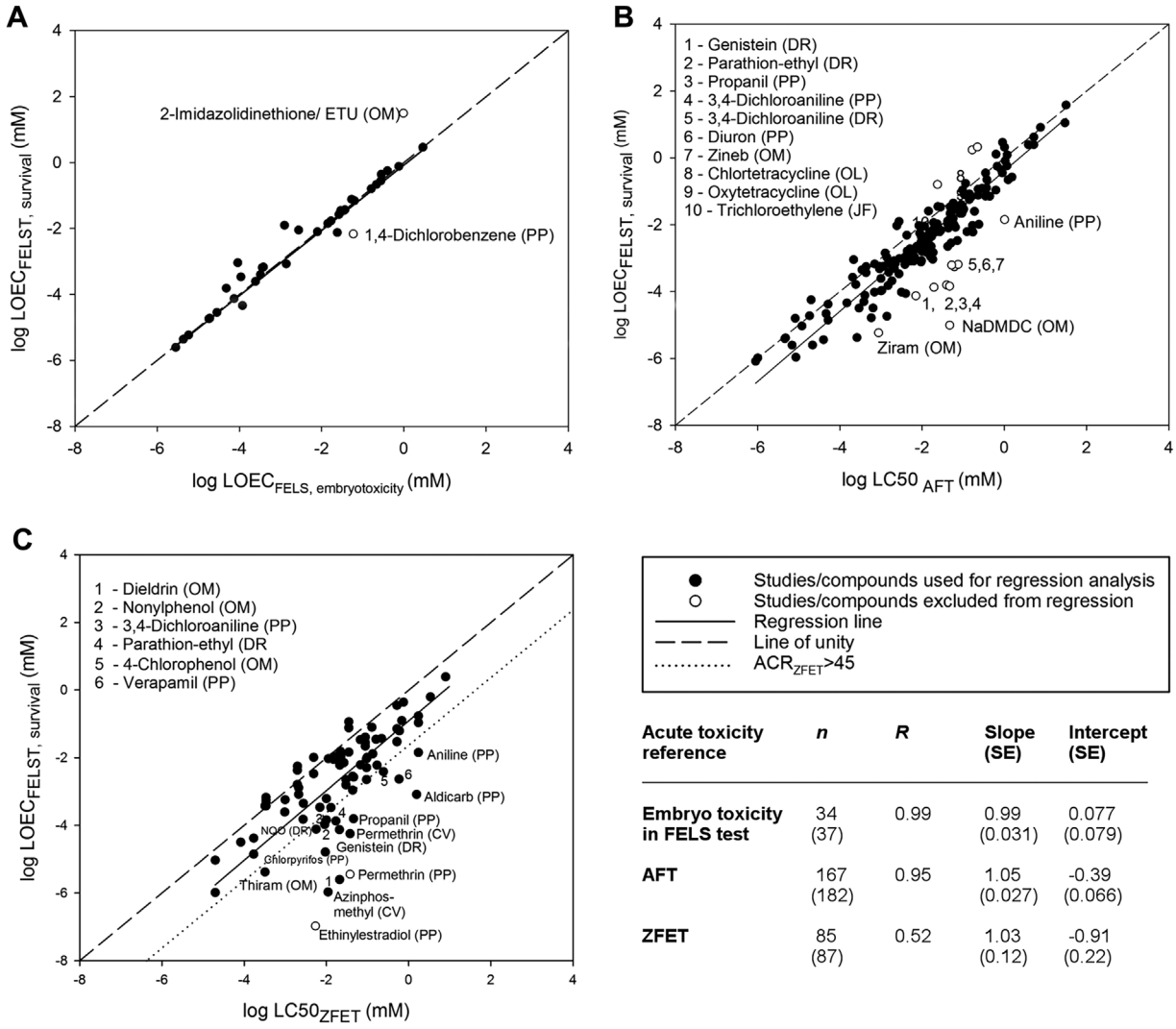
***$ACR_{AFT}$.*** The analysis of $ACR_{AFT}$ was based on a comparison of ECs for the same species. It was hypothesized that for the majority of compounds the ECs in both tests would be similar. Only for some compounds, presumably those with a specific mode of action, might a high $ACR_{AFT}$ be observed. In contrast to the comparison of survival rates of the embryonic period and the overall test (see *ACR: Comparison of acute fish and fish ELS toxicity*), the ECs of this comparison stem from different tests or studies introducing additional variability. In cases where, for the fish ELS test, only a pair of LOEC/NOEC values for growth was available, the corresponding ECs for survival were estimated using a linear regression equation (Supplemental Data, Figure S5).

For the correlation analysis, 182 data pairs (LOEC/LC50) referring to 128 different compounds were identified. Of these compounds 81 were tested in fathead minnow (*P. promelas*), 28 in rainbow trout (*O. mykiss*), 16 in sheepshead minnow (*C. variegatus*), 8 in flagfish (*J. floridae*), 10 in zebrafish (*D. rerio*), 8 in medaka (*O. latipes*), and 1 in mummichog (*F. heteroclitus*; see Supplemental Data, Tables S1 and S2, for the complete data set including physicochemical characteristics of the test compounds and references to original studies).

The regression analysis of logarithmic values indicated a high correlation of acute fish toxicity and fish ELS test data with a data correlation coefficient ($R$) of 0.95 (Figure 3B). The slope of the regression was not significantly different from 1, and the intercepts indicate a similar overall sensitivity (2.5-fold higher sensitivity for survival in the fish ELS test). Twelve compounds (represented by 13 studies; Figure 3B) deviated for the acute fish toxicity–fish ELS test survival regression. The ACRs of these compounds ranged from 45 to 8318 (Supplemental Data, Table S6). Five compounds did not provoke any acute toxicity in the tested range of concentrations. For these compounds ACRs $>1.7$ to 2570 can be expected based on the comparison of the fish ELS test with the maximum tested concentration in the acute fish toxicity test (Supplemental Data, Table S6). Four compounds (trichloroethylene, neodol, aldicarb, permethrin) with higher toxicity in the acute fish toxicity test were detected. The higher sensitivity is likely to represent an artifact or experimental variability, respectively, given that data stem from different experiments and studies.

***$ACR_{ZFET}$.*** Zebrafish embryo acute toxicity is known to exhibit on average a high correlation to the acute fish toxicity with a similar sensitivity (Lammer et al. 2009; Knöbel et al. 2012; Belanger et al. 2013; Klüver et al. 2015). Hence, as found for the comparison of acute fish toxicity with fish ELS toxicity, a high correlation would be expected if the zebrafish acute embryo LC50 were compared with fish ELS LOECs (Figure 3C). Corresponding zebrafish embryo LC50 values were available for 57 compounds of the fish ELS test database (obtained from a previously established database [Sobanska et al. 2018]). A regression analysis (based on logarithmic values) indicated a weaker correlation ($R = 0.52$) compared with $ACR_{int}$ and $ACR_{AFT}$ analyses. Apparently, the data were more scattered and a higher number of compounds with lower toxicity in the fish embryo was observed. Because of the more scattered data, it was not possible to identify compounds with a lower EC in the fish ELS test by a statistical analysis. Therefore, we used an $ACR_{ZFET} > 45$ as a threshold to identify compounds with increased chronic toxicity (Figure 3C). The factor of 45 was useful to describe outliers from the regression of acute 96-h fish toxicity and fish ELS correlation (see paragraph on $ACR_{AFT}$ analysis and Figure 3). If this threshold were applied to the comparison of zebrafish embryo LC50 and fish ELS data, 20% of all studies would show a weaker toxicity in fish embryos (in contrast to only 6.6% of the studies in the comparison of 96-h acute fish toxicity with fish ELS toxicity). Fourteen compounds tested in zebrafish embryos did not provoke mortality in the tested range of concentrations. For these compounds, a minimal expected toxic ratio based on the




| Acute toxicity reference | *n* | *R* | Slope (SE) | Intercept (SE) |
|---|---|---|---|---|
| **Embryo toxicity in FELS test** | 34 (37) | 0.99 | 0.99 (0.031) | 0.077 (0.079) |
| **AFT** | 167 (182) | 0.95 | 1.05 (0.027) | -0.39 (0.066) |
| **ZFET** | 85 (87) | 0.52 | 1.03 (0.12) | -0.91 (0.22) |

**FIGURE 3:** Correlation of acute fish toxicity and fish early life stage (ELS) toxicity. Three different endpoints were used for the acute fish toxicity comparison: embryo toxicity in the fish ELS test (**A**), acute 96-h fish toxicity (juvenile/adult fish) (**B**), and zebrafish embryo test (**C**). For (**A**) individual data pairs were derived from the same experiment; for (**B**) all data pairs referred to the same species. The indicated sample numbers (*n*) refer to the number of studies used for regression analysis (the total number of studies is given in parentheses). For details on compounds and data sources, refer to Supplemental Data, Tables S1 and S6. Indicated compounds represent outliers from the regression analysis (**A**, **B**) or compounds with an $ACR_{ZFET}$ >45 (**C**). The table summarizes the parameters of the linear regressions shown in (**A**–**C**). FELS = fish early life stage; $ACR_{ZFET}$ = ratio of acute versus chronic toxicity in zebrafish embryo toxicity test; AFT = acute fish toxicity; CV = *Cyprinodon variegatus*; DR = *Danio rerio*; ETU = ethylene thiourea; JF = *Jordanella floridae*; LC50 = median lethal concentration; LOEC = lowest-observed-effect concentration; NaDMDC = sodium dimethyldithiocarbamate; NQO = 4-nitroquinoline N-oxide; OL = *Oryzias latipes*; OM = *Oncorhynchus mykiss*; PP = *Pimephales promelas*; SE = standard error.

highest concentration tested in the zebrafish embryo was calculated (Supplemental Data, Table S7). Three compounds exhibited a minimal toxic ratio above 100 (pentachlorobenzene, methomyl, ethoprop).

## Relation of high ACRs with modes of action

As indicated by the comparison of acute fish toxicity (adult/juvenile) and zebrafish embryo toxicity (Figure 3B), similarity of ECs in acute fish toxicity and fish ELS toxicity may not apply for all modes of action. Therefore, the ACRs were compared also with respect to their different modes of action. Most of the modes of action exhibited ACRs close to that of narcotic compounds and ranged between 1 and 10 (Figure 4A). Only 2 modes of action, methemoglobin formation and extracellular matrix formation inhibition, appeared to exhibit a substantially higher ACR range, with median values between 10 and 100 and peak values of 1066 and 4786, respectively. However, a few compounds with ACRs >10 were also observed for most of the other modes of action.

Therefore, the transduction to changes at the higher organism level and adverse effects for these modes of action (lysyl oxidase inhibition and methemoglobin formation) were graphically summarized according to the AOP concept (Supplemental Data, Figure S9). The putative AOP for lysyl oxidase inhibition leading to enhanced chronic fish toxicity has also been submitted to the AOP wiki (https://aopwiki.org/aops/242).

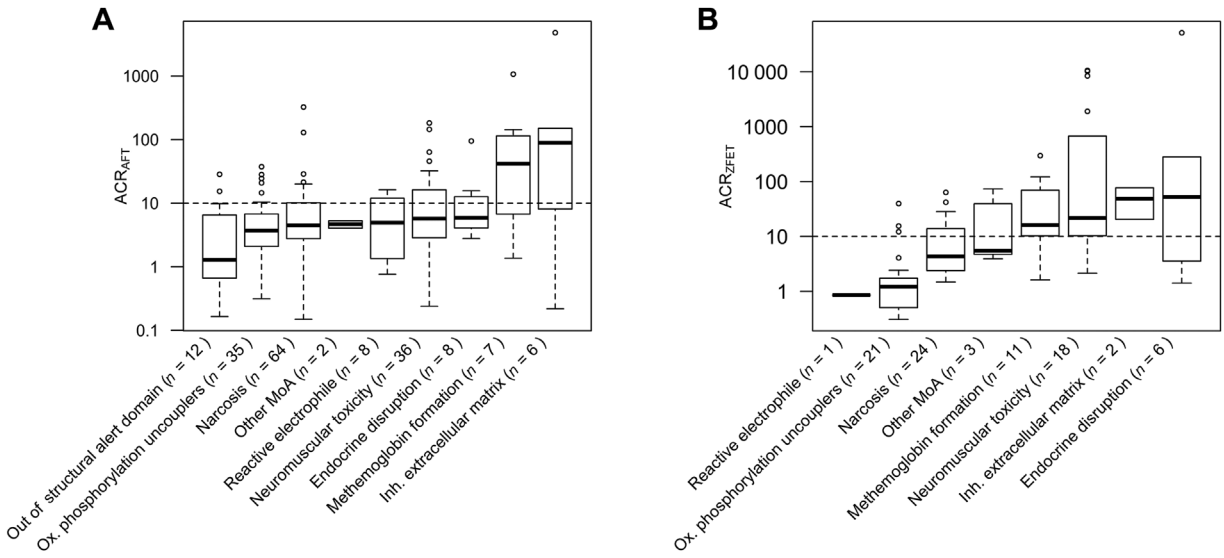



**FIGURE 4:** Relation of the acute-to-chronic ratio (ACR) to the mode of action. The ACRs were calculated using acute 96-h fish toxicity (**A**) or acute zebrafish embryo toxicity (**B**) as references. Dashed line represents an ACR of 10. For details on the compounds and data sources, refer to Supplemental Data, Tables S1, S2, and S6. AFT = acute fish toxicity; Inh. extracellular matrix = inhibition of extracellular matrix formation by lysyl oxidase inhibition; LC50 = median lethal concentration; MoA = mode of action; Ox. = oxidative; ZFET = zebrafish embryo toxicity.

Given that evidence for the link between the different levels of the AOPs is only partially available for mammals but lacking for fish or only a limited amount of data supporting these links is available, these AOPs must be considered putative at present and may require further experimental data for confirmation.

Similar to the comparison of acute fish and fish ELS toxicity, potential methemoglobin formation and extracellular matrix inhibition were found to represent modes of action with the highest $ACR_{ZFET}$. In contrast to the comparison with acute fish toxicity, neuromuscular toxicity and endocrine disruption were also identified as modes of action with high $ACR_{ZFET}$. The analysis of $ACR_{int}$ versus mode of action was not conducted for embryo toxicity in the fish ELS test given the high concordance of embryo and fish ELS toxicity (Figure 3A).

For a few compounds and studies, ACRs <1 were observed. These low ACRs could result from variability between different species and stages, for example, because of a different degree of metabolic degradation and clearance. Furthermore, ACRs <1 may represent artifacts related to the solubility of the compounds or other experimental limitations. It must be noted that the result could also be biased because of the partially low number of chemicals representing a certain mode of action.

### *No relationship of high ACRs with hydrophobicity*

Compounds may not reach equilibrium of internal bioavailable concentration within short-term exposure. Indeed, the time to reach equilibrium would be particularly dependent on the hydrophobicity of the compounds, and high ACRs would reflect differences in the internal bioavailable concentrations. Hence, to estimate whether the level of ACR may also be driven by the hydrophobicity of compounds, the ACR of narcotic compounds was compared with the log $K_{OW}$. No dependency of the ACR on the log $K_{OW}$ was observed for both $ACR_{AFT}$ and $ACR_{ZFET}$ (Supplemental Data, Figure S7). The comparison was restricted to narcotic compounds because for other modes of action the different reactivity or affinity to the target site may override the influence of hydrophobicity. The mean ACRs of 5 and 5.8 reflect the slightly lower ECs obtained, on average, for the fish ELS test (Figure 3B and C).

## DISCUSSION

Using a database with 183 compounds, fish ELS data were analyzed with respect to whether certain modes of action may be of particular concern for chronic fish toxicity. Despite the heterogenous data sources and relaxed quality criteria, it was possible to conduct regression analyses and to determine toxic ratios and ACRs for diverse modes of action and relate chronic toxicity in the fish ELS test to baseline toxicity.

### *Association of high toxic ratios with modes of action*

Determination of the toxic ratio (i.e., the relationship of calculated baseline versus observed toxicity) can provide evidence for reactivity, a specific mode of action, or other interactions leading to high toxic ratios. To derive toxic ratios, a regression analysis for the baseline survival LOEC using the narcotic compounds of the data set was conducted. The obtained regression was very close to a previous baseline toxicity analysis of the fish ELS test that was based on NOECs of a limited number of structurally related narcotic compounds in zebrafish (chlorobenzenes, log NOEC [millimoles] = $-1.09 \times \log K_{OW} + 1.78$; Van Leeuwen et al. 1990b). The similarity to this data set indicates that even heterogeneous data originating from different species and studies can be combined to analyze the role of mode of action in toxicity in fish. The baseline toxicity of

the fish ELS was also very close to the baseline toxicity described for acute fish toxicity (log LC50 [millimoles] = –0.94 log $K_{OW}$ + 1.75 [Russom et al. 1997]) and fish embryo acute toxicity (log LC50 [millimoles] = –0.99 log $K_{OW}$ + 2.02 [Klüver et al. 2016]), providing evidence that a nonspecific (narcosis) mode of action can be used to link hydrophobicity and effect universally across species and life stages and that narcotic compounds are generally not associated with high ACRs. The slightly lower ECs in the fish ELS test, on average, could be associated with higher internal concentrations or cumulative damage. However, given the lack of experimental internal concentration data and detailed time-resolved survival data, this cannot be concluded at present.

The analysis of the mode of action–specific range of toxic ratios indicated that many of the compounds with nonnarcotic modes of action exhibited an increased toxic ratio, with putative extracellular matrix inhibition and endocrine disruption as the mode of action groups with the strongest deviation from the baseline. Within the specific mode of action groups, compounds with a low toxic ratio were identified. These compounds may indicate that the mode of action has not been assigned correctly, the intrinsic potency is low, or a strong metabolic degradation may result in a reduced internal bioavailability, confounding the analysis of exposure–concentration-based toxic ratios. It must be noted that analysis of modes of action leading to high chronic fish toxic ratios could be also skewed because of a bias of compounds available for the present study. For instance, relatively few data were available for pharmaceuticals at the time of analysis. Given their biological activities, these compounds may provide additional modes of action not represented in the database and, hence, no conclusion on the potency to provoke high toxic ratios can be made for this group of compounds at present. However, the analyses gave a first strong indication of modes of action associated with high toxic ratios in the fish ELS test.

## Chemical activity and constant toxic membrane concentrations

Baseline toxicity is frequently also analyzed with respect to chemical activity (Schmidt and Mayer 2015) or the critical membrane concentration leading to acute toxicity (Escher et al. 2002). In the chemical activity concept the toxicity of a chemical is expressed as the fraction of water solubility that leads to toxicity, where water solubility refers to the liquid state of the chemical (i.e., normal solubility for liquids and subcooled liquid solubility for solids; Schmidt and Mayer 2015). This results in lethal chemical activities typically between 0.1 and 0.01 for baseline toxicants and <0.01 for compounds with a reactive or specific mode of action. The constant toxic membrane concentration concept relates the baseline toxicity of a chemical to a constant membrane concentration resulting from equilibrium partitioning. For instance, acute toxicity of narcotic compounds is related to a membrane concentration (at equilibrium) of 100 $mmol/kg_{lip}$ (Escher and Hermens 2002). There is some controversial discussion on the relevance of the chemical activity concept (Goss and Endo 2016; Thomas et al. 2016), but it is beyond the scope of the present article to contribute to this discussion. However, both approaches are useful to compare the range of baseline toxicity of acute and chronic toxicity for neutral compounds. This comparison confirmed that the survival in the fish ELS test for narcotic compounds is driven mainly by their acute baseline toxicity.

Toxicity observations above the baseline toxicity range in terms of chemical activities or constant toxic membrane concentrations could indicate a reduced uptake or metabolic transformation of compounds leading to a reduced internal bioavailability. Experimental limitations (e.g., overestimated ECs or water solubility) may also result in high lethal chemical activities or constant toxic membrane concentrations. Toxicity below the baseline toxicity range in terms of chemical activities or constant toxic membrane concentrations could indicate a specific mode of action, reactivity, or other interactions leading to high toxic ratios in a chronic exposure. Lethal chemical activities that are slightly below 0.01 may also apply to some narcotic compounds because the narcotic mode of action has been assigned based on acute toxicity QSARs. It must be noted that 28 of the compounds in our database were estimated to be in a predominantly ionized state at pH 7. For these compounds the comparison of the toxicity with the log $K_{OW}$, constant toxic membrane concentration, and chemical activity may not be valid because it is biased by a potentially limited uptake and different partitioning of the predominant ionized chemical (Bittermann et al. 2016). Therefore, the corresponding compounds have been labeled in Figure 1 and are indicated in Supplemental Data, Tables S1 and S6.

## Association of ACRs with modes of action and hydrophobicity

Although the toxic ratio analysis clearly indicates that reactivity or specific modes of action result in fish ELS ECs well below the baseline toxicity, it may not indicate modes of action that are specifically relevant for a chronic exposure scenario or that become relevant during the development from embryo to juvenile stage. The comparison of the LOECs for embryotoxicity and overall survival in the same fish ELS test did not reveal any information regarding modes of action specifically relevant for chronic toxicity. This was because of the lack of LOEC data for the embryonic phase of the test, a limited exposure concentration range (46% of the compounds did not provoke toxicity in the embryonic phase), and the high correlation for the compounds for which this information was available. A high correlation and similar sensitivity were also found for the comparison of acute fish toxicity and the fish ELS test, confirming previous observations (Suter and Rosen 1988). However, the acute fish toxicity–fish ELS test comparison indicated putative methemoglobin formation or extracellular matrix inhibition with ACRs increased by more than a factor of 10, on average. Hence, all other modes of action that exhibited high toxic ratios for the fish ELS test apparently resulted in high toxic ratios already in a short-term exposure setup. That the ACRs were, on average, low (i.e., close to 1) is probably also determined by the similarity of baseline toxicity for acute fish

toxicity and fish ELS tests, because approximately 30% of the compounds of the comparative analysis were assigned a narcotic mode of action. Interestingly, additional modes of action—neuromuscular toxicity and endocrine disruption—were found to lead to high ACRs when they were calculated based on acute fish embryo toxicity. This indicates a weakness of the fish embryo with regard to the acute toxicity (lethality) that has been described for neurotoxic compounds (Knöbel et al. 2012; Klüver et al. 2015; Sobanska et al. 2018; Glaberman et al. 2016). Some of the compounds with a high $ACR_{ZFET}$ (aldicarb, azinphos-methyl, dieldrin, permethrin) were also identified as compounds with a weak sensitivity in fish embryo if compared with acute fish toxicity LC50 (Klüver et al. 2015) and represented AChE inhibitors, γ-aminobutyric acid antagonists, and voltage-gated sodium channel antagonists. Furthermore, when zebrafish embryo acute toxicity and acute fish toxicity data were compared, neurotoxic compounds represented the compounds with the lowest sensitivity in the fish embryo test (Supplemental Data, Figure S8).

## Hydrophobicity of compounds

The internal concentration maxima may not have been reached within the experimental period of acute tests or during the embryonic stages of the fish ELS test. This could result in high ACRs in tests with a prolonged exposure, such as the fish ELS test. Given that the uptake and elimination of a compound are driven to a large extent by its hydrophobicity, the relation of high ACRs to the log $K_{OW}$ may provide information on the role of toxicokinetics for evolving chronic toxicity. Higher ACRs have been observed for log $K_{OW}$s above 4 and associated with prolonged time to reach a steady state in bioconcentration (Ahlers et al. 2006). Higher ACRs may also be expected for polar compounds. For fish embryos it has been shown that equilibrium internal concentrations may not be approached during a 4-d exposure period for hydrophobic and polar compounds (Brox et al. 2014, 2016; Klüver et al. 2015). However, the present study could not reveal any association between ACRs of narcotic compounds and hydrophobicity regardless of whether the ACR was calculated based on acute fish or acute fish embryo toxicity. However, it must be noted that only a limited number of compounds and data were available and that these may be confounded by unknown species-specific differences in toxicokinetics, active transport, or uptake by ingestion (see Neuwoehner and Escher 2011; Luckenbach et al. 2014). A dependency on hydrophobicity may also contribute to a high ACR for nonnarcotic compounds, but this would be difficult to conclude because of the overlying specific modes of action.

## Modes of action and AOPs of compounds with high toxic ratios and ACRs

The data analysis suggests that a specific mode of action increases the likelihood that a compound is provoking a high toxic ratio and/or ACR in the fish ELS test. This could be related partially to the manifestation of certain key events (e.g., respiratory distress) during the course of development or specific interference with growth (e.g., for endocrine-disrupting chemicals or hormones).

Putative hemoglobin oxidation (associated with aniline derivatives), interference with cellular matrix formation (associated with dithiocarbamate fungicides), neurotoxicity (mainly AChE-inhibiting organophosphates and carbamates), and endocrine-disrupting or hormonally active compounds were found in a higher proportion among compounds with a high toxic ratio and/or ACR (Figures 2 and 4) in the fish ELS test. For compounds with an aniline structure (aniline, diuron, propanil, 3,4-dichloroaniline, 4-chloroaniline, 2-imidazolidinethione), the high toxic ratio and ACR may have been related to methemoglobin formation and/or hemolytic anemia (see Supplemental Data, Figure S9, for a putative AOP scheme). These compounds revealed a median toxic ratio and ACR of 67 and 42, respectively. van Leeuwen et al. (1990b) showed that the toxicity of chloroanilines was increased after 7 d of exposure in the fish ELS test in contrast to other presumably narcotic compounds such as chlorobenzenes. Metabolic activation was discussed as a potential reason for the increasing toxicity. Some of the aniline derivates such as diuron or propanil are known to be plant herbicides that interfere with photosynthesis (Koblizek et al. 1998), but this mode of action is not relevant for fish toxicity. However, repeated-dose tests conducted for chronic toxicity in mammals indicated that the observed long-term effects might be associated with methemoglobin formation or hemolytic anemia (Organisation for Economic Co-operation and Development 2011). It was shown that aniline derivates such as propanil are hydrolyzed to 3,4-dichloroaniline by acylamidase and that 3,4-dichloroaniline or further transformation products can lead to a conversion of 50% of the hemoglobin to methemoglobin in mice (Chow and Murphy 1975; McMillan et al. 1991, 1990a, 1990b). Similarly, metabolization via 3,4-dichloroaniline could be involved in diuron toxicity (Wang et al. 1993). The hemolytic oxidation of erythrocytes by *N*-hydroxylamines was considered to be the reason for hemolytic anemia. Hence, the enhanced toxicity in the fish ELS might be primarily related to a respiratory distress resulting in a reduced oxygen binding capacity of erythrocytes. Because in embryonic stages the oxygen supply is suggested to be provided mainly by diffusion, this respiratory distress would affect growth and survival only in later life stages (Rombough 2002; Jacob et al. 2002). Contrary to this hypothesis, however, is the higher range of toxic ratios for this mode of action when fish embryos are compared with the fish ELS test. Furthermore, cumulative survival data are typically not reported for the fish ELS test and/or are not publically available. Therefore, experimental studies targeted to relate respiratory distress to the high ACR and toxic ratio of aniline derivatives in fish would be required to confirm the relevance of this mechanism.

We hypothesized that interference with cellular matrix formation represents a further mode of action leading to a high median toxic ratio ($9.3 \times 10^4$) and $ACR_{AFT}$ (89) in the fish ELS test (see Supplemental Data, Figure S9, for a putative AOP scheme). This mode of action was associated with dithiocarbamate fungicides. The precise fungicidal mode of action of dithiocarbamates is not known, and they are described to act on

multiple sites (Maltby et al. 2009). For mammals, a neuropathic effect via the production of $CS_2$ was controversially discussed (US Environmental Protection Agency 2001a). For some, but not all, dithiocarbamates an inhibition of AChE inhibition was also reported (US Environmental Protection Agency 2001b). There are various other modes of action that have been discussed for dithiocarbamates (US Environmental Protection Agency 2001a; United Nations 2002). However, strong evidence that the enhanced toxicity in the fish ELS test is related to developmental toxicity and inhibition of extracellular matrix formation was provided by studies using zebrafish. Haendel et al. (2004) and Tilton et al. (2006) observed that exposure of fish embryos to various dithiocarbamates elicited distinct notochord distortions at exposure concentrations starting from 0.08 (sodium metam) and 0.02 μM (thiram). Growth inhibitions in the fish ELS (LOEC 0.013–0.15 μM) test for thiram, ziram, maneb, and sodium dimethyldithiocarbamate (NaDMDC) were observed in a range of concentrations close to those causing notochord distortions in zebrafish embryos (Tilton et al. 2006). This notochord distortion appears to be caused by inhibition of the enzyme lysyl oxidase, and repression of the enzyme using antisense morpholinos provoked the same phenotype (van Boxtel et al. 2010). The rescue of the wild-type phenotype using triazine as an anesthetic indicates that notochord malformation requires muscle contractions to be established (Tilton et al. 2006). It can be assumed that the notochord distortions may affect swimming behavior and feeding, leading to the observed reduction in survival and growth observed in the fish ELS test.

The toxicity of neurotoxic compounds in fish and other vertebrates has been associated with respiratory distress (De Candole et al. 1953; Bradbury et al. 2008; Russom et al. 2014). Particularly for AChE inhibitors, the AOP has been well described (Russom et al. 2014). It is known that exposure of fish to neurotoxic compounds can lead to reduced blood oxygen levels caused by interference with the cholinergic system and neuromuscular junctions. Diverse mechanisms or a combination of these, such as hemorrhages in the vertebral column, vasoconstriction of gill sphincter muscles, decreased heart rate, and reduced movement, have been discussed as lowering the oxygen level, which can ultimately lead to the death of the animal (Bradbury et al. 2008). The dependency on oxygen supply for neurotoxicity is further supported by comparison of acute toxicity between fish embryos and later life stages. Mortality in fish embryos exhibits a weak sensitivity for neurotoxic compounds (Knöbel et al. 2012; Klüver et al. 2015). It is likely that the weak sensitivity of embryos to neurotoxic compounds is associated not with a lack of the compound's target (e.g., binding to AChE inhibition as the molecular initiating event) but with a lack of subsequent key events such as the respiratory distress syndrome—as described for the formation of hemoglobin by aniline derivates. Hence, the lower median $ACR_{AFT}$ of 5.8 and the higher median $ACR_{ZFET}$ of 22 obtained for compounds with a neuromuscular mode of action are plausible because of the lack of the respiratory failure syndrome in embryonic stages. The relatively low median toxic ratio of 22 may be the result of a number of neurotoxic compounds with low ACRs. Many of these compounds exhibit high log $K_{OW}$, and for these compounds the low ECs for baseline toxicity may result in relatively low toxic ratios. Toxic ratios well below 1 observed for a number of hydrophobic compounds may also indicate potential artifacts because of experimental limitations such as water solubility. Another potential reason that requires further investigation and may apply to other groups of compounds could be the interference with biotransformation. Many organophosphates require metabolic activation, and some are also rapidly transformed. This could lead to internal bioavailable concentrations that differ between different life stages and/or exposure durations (e.g., as discussed for malathion [de Bruijn and Hermens 1993]).

Endocrine-disrupting chemicals are considered to be of high concern, primarily because of their ability to interfere with reproduction and subsequent population development (Scholz and Mayer 2008; Ankley et al. 2010; Scholz et al. 2013a). Reproductive endpoints are not covered in the fish ELS test. However, our data analysis indicated that growth and survival in the fish ELS test (median toxic ratio = 478) were affected for compounds known or discussed as interfering with the endocrine system (17-methyltestosterone, ethinylestradiol, genistein, nonylphenol, tributyltin oxide; see Supplemental Data, Tables S1 and S6 for further details and references) and could be observed at concentrations well below those causing acute toxicity. Some of these compounds (genistein, tributytin oxide [Akiyama et al. 1987; World Health Organization 1999]) are known to exhibit other modes of action as well and, hence, the observed higher toxic ratios may or may not exclusively relate to the endocrine mode of action. It is not precisely known how endocrine-disrupting compounds interfere with growth and survival in the fish ELS test. As shown for ethinylestradiol, the interference with regulation of the growth hormone/insulin-like growth factor in fish could link estrogenic effects to growth, leading to high toxic ratios and ACRs (Shved et al. 2008). A high median ACR (53) was only observed for the comparison of fish embryo test LC50s versus fish ELS toxicity. This may, however, be caused by a lack of availability of LC50s for adult/juvenile fish for compounds such as methyltestosterone and ethinylestradiol.

## Conclusions and perspectives for the development of alternatives to the fish ELS test

The meta-analysis demonstrated that certain modes of action were associated with an enhanced toxic ratio and/or ACR in the fish ELS test. On the basis of the data set analyzed, neurotoxicity, lysyl oxidase inhibition, endocrine disruption, and methemoglobin formation/hemolytic anemia were identified as the (putative) major modes of action leading to high toxic ratios and ACRs in the fish ELS test. This observation provides a basis to use and develop targeted bioassays linked to the AOP concept (Ankley et al. 2010). These could be short-term assays using in vitro cellular or other alternative test systems such as fish embryos and would allow identification of the molecular initiation or a key event leading to the adverse effects (i.e., an enhanced fish ELS toxicity). Furthermore, mode of action–specific QSARs beyond baseline toxicity may be used to predict the fish ELS toxicity. The analysis of additional data sources for fish ELS ECs such as

European Chemicals Agency or European Food Safety Authority dossiers and proprietary databases of environmental agencies may provide additional modes of action that may not have been covered in the present data set.

Given that the fish ELS test includes the embryonic period, exposure could be restricted to the embryonic period, if endpoints related to relevant modes of action and predictive of chronic toxicity could be identified and already measured in embryonic stages. For some of the major modes of action leading to enhanced ACRs or toxic ratios described in the present study, appropriate fish embryo assays have been developed and described: 1) the alterations in embryonic behavior (movements) have been shown to identify neurotoxicity (Klüver et al. 2015); 2) endocrine-disrupting effects can already be detected in fish embryos using, for example, aromatase as a sensitive target gene in a transgenic zebrafish model (Brion et al. 2012)—likewise, thyroid hormone–disrupting compounds (Fetter et al. 2015; Thienpont et al. 2011) and androgens (Sébillot et al. 2014) can be identified using fish embryos; and 3) the lysyl oxidase–inhibiting dithiocarbamates provoked visible notochord distortion (Tilton et al. 2006; van Boxtel et al. 2010), indicating that the observation of malformations may serve as an indicator of potential enhanced fish ELS toxicity.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4090.

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*Data Availability*—Raw data are available as Supplemental Data files (S1 and S2).

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