Fish early life-stage toxicity and environmental relevance: What does high-dose toxicity testing tell us?

Abstract

The early-life stage (ELS) toxicity syndrome in fish is well described and has been reported in hundreds of toxicity studies. It is generally characterized by a reduced heart rate, yolk sac and pericardial edemas, and various morphological abnormalities, the most common being spinal curvature. For many of those studies it appears that the ELS toxicity syndrome is the result of non-specific (baseline) toxicity that occurs at aqueous and whole-body concentrations that are just below lethal concentrations. Baseline toxicity is essentially a non-specific response that results from chemicals accumulating in and disturbing function of biological membranes that leads to lethality and sublethal effects at relatively high doses. The commonality of this acute ELS toxicity syndrome among highly diverse organic and inorganic chemicals is remarkable. It is important to identify baseline toxicity because it is considered minimal toxicity that acts in all tissues and cells, and it has the potential to impair all cellular functions. This means if an effect is observed around baseline-toxic concentrations, it is likely that other cellular functions are also affected, i.e., the effect is not specific. The fish ELS toxicity syndrome can also be the result of specific effects involving receptor interactions; therefore, we emphasize the importance of distinguishing between specific and non-specific toxicity responses to provide the most relevant data for environmental risk assessment.

Introduction

There are hundreds, and possibly a few thousand studies that describe toxic effects for fish at high-dose exposure concentrations. Many of these studies are conducted with fish embryos, commonly zebrafish (*Danio rerio*), which are easy to raise and maintain in a laboratory setting. Zebrafish provide a constant supply of eggs and embryos that can be acquired in large numbers and subjected to rapid toxicity tests in shallow, low-volume plate wells, which can exhibit highly variable concentrations (Truong et al. 2011; Knobel et al. 2012).

A common theme for these high-dose studies with early life-stage (ELS) zebrafish (embryos and larvae) concerns developmental effects, such as pericardial and yolk sac edemas, bradycardia, spinal and facial deformities, and mortality. These effects have been considered as a syndrome and in most cases are correlated and occur together at relatively high doses (Ducharme et al. 2013; Jarque et al. 2020). As noted by others, effects on the heart and circulation can result in downstream effects causing edemas and morphological abnormalities due to disruption in peripheral circulation (Incardona et al. 2004). There are many compounds that can cause teratogenic effects in fish; however, the ELS toxicity syndrome discussed here refers to the combination of a reduced heart rate, edemas, and morphological deformities at high exposure doses that likely result from non-specific cell toxicity. In these cases, this syndrome of effects is caused by baseline toxicity, which occurs at constant critical tissue concentrations. Specific effects occur at lower concentrations than baseline toxicity. Classification of specific mechanisms or modes of toxic action in fish have been addressed in several studies (Scholz et al. 2018; Zhu et al. 2018; Wilhelmi et al. 2024) and will not be discussed here.

Baseline toxicity has been described and addressed by several authors (McCarty 1993, Escher et al. 2002; Meador et al. 2011; Klüver et al. 2016; Escher et al. 2020b; Lee et al. 2021). Baseline toxicity is a result of accumulation of organic compounds in tissues to critical levels triggering a non-specific membrane effect that destabilizes cell function. As it is a physical effect in the membranes, it is not dependent on the structure of the chemical but on the molar volume and can be approximated by a constant membrane concentration. Its universality is striking and extremely difficult to refute. It is usually best considered in terms of whole-body (internal) or membrane concentrations, which mitigates the high variability of external exposure concentrations due to organismal toxicokinetics and physicochemical properties (Escher and Hermens 2004; Meador et al. 2008). Toxicity metrics based on water exposure concentrations can vary by orders of magnitude among species and organic compounds, especially as a function of their hydrophobicity, whereas toxicity based on whole-body concentrations is far less variable. Most studies evaluating the baseline toxic response conclude that mortality for a high percentage of species and organic chemicals occurs at a mean concentration of approximately 2.7 $mmol/kg_{whole\text{-}body\ wet\ weight}$ (95%CI = 0.5 – 15 $mmol/kg_{whole\text{-}body\ wet\ weight}$), with sublethal effects occurring at concentrations between 1 and 10 fold lower (McCarty et al. 2013; Scholz et al. 2018; Kluver et al. 2016). This relatively constant and high-dose tissue concentration among fish species and compounds has been directly demonstrated and modeled for hundreds of organic compounds. For many organic compounds with molecular weights commonly in the range of 250 Daltons, baseline mortality in fish occurs at concentrations greater than 125 $mg/kg_{whole\text{-}body\ wet\ weight}$ and sublethal effects above 13 – 25 $mg/kg_{whole\text{-}body\ wet\ weight}$ (assuming a 5-10 fold lower response level). These predictions are variable due to compound characteristics, species, time for the response to develop, duration of exposure, compound molecular weight, health of the organism, organismal lipid content, and other relevant factors. Our goal here is to highlight the misidentification of baseline toxicity and provide guidance on how to tell the difference between baseline toxicity and adverse responses occurring at lower exposure doses due to specific (receptor-mediated) action or reactive toxicity.

Analysis

The commonality of studies reporting this ELS toxicity syndrome of effects for a wide diversity of organic compounds leads to a simple hypothesis that these effects are the result of a non-specific response by the organism exposed to doses high enough to result in critical tissue concentrations. In most cases, this response can be attributed to baseline toxicity, which is also known as "narcosis". The underlying syndrome of effects is disturbance of cellular membrane structure and functioning (van Wezel 1995), leading to cytotoxicity, which manifests in whole-organism toxicity tests as well as *in vitro* cellular assays (Escher et al. 2020b). This observation is a good example of Occam's razor, that prescribes a simple explanation over one that is more complex based on speculation about toxicological function. Given the commonality of this response, the working hypothesis is that most of these high-dose studies are reporting on a non-specific response that occurs at doses slightly below those causing mortality. In many cases the results are provocative showing severe effects and a variety of physiological, morphological, and gene expression alterations, which is not surprising for organisms that are near death. Differential expression of cardiac or developmental genes are not proof of a specific effect causing the ELS syndrome because such alterations are expected given the near lethal, high-dose exposure. A common theme among many of these studies is oxidative stress, which is the hallmark of cytotoxicity (Chakraborty et al. 2023; Simmons et al. 2009). With *in vitro* toxicology, this observation has been named "cytotoxicity burst" (Fay et al. 2018; Escher et al. 2020b).

It is no coincidence that many of the compounds causing the ELS toxicity syndrome fall along the regression line defining aqueous exposure, bioaccumulation, and toxicity as a function of hydrophobic partitioning (Konemann 1981; Kluver et al. 2016; Scholz et al. 2018; Meador 2021). This partitioning can be expressed by the octanol-water partition constant K_{ow} for neutral chemicals (Figure 1), which forms a quantitative structure-activity relationship (QSAR). For ionizable compounds, the K_{ow} can be simply replaced with the lipid membrane-water distribution ratio (D_{lipw}) without substantially changing the equations (Kluver et al. 2019; Escher et al. 2020a). The key point here is that compounds along this regression line bioaccumulate to critical levels triggering the baseline toxicity response. For many compounds this occurs at relatively high aqueous concentrations (“high dose”); however, for hydrophobic compounds this can occur at lower external aqueous concentrations.

While the vast majority of studies reporting biological effects consistent with the fish ELS toxicity syndrome are due to baseline toxicity, this syndrome can also be observed at lower exposure doses and is not the result of baseline toxicity. The concentration for organic compounds causing toxic responses by receptor-specific interaction is generally well below values predicted with the baseline toxicity QSAR. Many of these are due to specific action on mitochondrial processes that produce very similar phenotypic responses to those non-specific responses (Meador, 2021). As hypothesized by Meador (2021), the high-dose baseline toxicity response also manifests in mitochondrial membrane disruption leading to altered calcium handling, which affects the developing heart and downstream effects such as edema and morphological abnormalities. It has also been shown that the specific effect of uncoupling of oxidative phosphorylation, which is caused by a protonophoric shuttle mechanisms can transition into baseline toxicity depending on the membrane concentration of toxicant needed to trigger the observed response (Escher and Schwarzenbach 2002). Examples of chemicals causing the ELS toxicity syndrome by specific action include cyanide, tralopyril, tributyltin, and retinoic acid (Meador 2021) (Figure 1).

Additionally, the ELS toxicity syndrome has been demonstrated for many chemicals at baseline toxicity concentrations and for different toxicity responses at far lower exposure concentrations. A good example of this can be seen in Meador (2021) for ethinylestradiol (EE2), which causes the ELS toxicity syndrome at baseline concentrations and specific receptor-mediated effects at concentrations approximately 5 to 6 orders of magnitude lower. Similar examples can be seen in Meador (2021) for atrazine, carbamazepine, bisphenol S, and several other chemicals.

Of course, membrane disruption is not the only mechanism that is consistent with the ELS toxicity syndrome. In general, if these symptoms occur at lower membrane or whole-body concentrations than observed for baseline toxicity, they must be specific. Other low-dose effects directly affecting the heart or related physiological pathways may result in the syndrome of responses similar to those caused by baseline toxicity. One example is activation of the aryl hydrocarbon receptor (AhR) (Shankar and Villeneuve 2023) that can result in the ELS toxicity syndrome. A good example of these is dioxin and dioxin-like compounds such as PCBs and 3-methylcloanthrene (Seok et al. 2008). Also, compounds that act specifically on cardiac heart rate and rhythms can result in the ELS syndrome at low exposure concentrations (D'Amico et al. 2012). The problem is that there are far too many reports of high-dose exposures that are likely the result of baseline toxicity, which incorrectly conclude that toxicity is caused by a specific, receptor-mediated response, such as AhR activation.

Even though baseline toxicity is commonly applied to organic compounds, the syndrome of effects for ELS fish can also be seen for metals, microplastics, and nanoparticles. This response for non-organic compounds is also a product of non-specific cytotoxicity. For example, Chen et al. (2024) reported bradycardia, edemas, and morphological abnormalities in zebrafish resulting from exposure to polystyrene particles. They attributed these responses to oxidative stress and increased levels of reactive oxygen species (ROS). Several metals are also known to cause the ELS toxicity syndrome with observations of the same suite of developmental abnormalities and bradycardia. As noted in a recent review by Rana (2020), elements such as arsenic, cadmium, lead, mercury, copper, chromium, and nickel, in addition to nanoparticles, are known to cause endoplasmic reticulum stress, which is suspected in causing the ELS toxicity syndrome (Meador, 2021). The toxicity syndrome has also been reported for zebrafish embryos exposed to algal extracts (González-Penagos et al. 2024). This commonality of effects for organic compounds, elements, and micro/nano particles is astonishing and it deserves far more research on the mechanisms involved.

Given the commonality of high-dose toxic responses, the default assumption should be baseline toxicity. It is researchers' responsibility to determine if their high-dose results are something other than a non-specific, cytotoxic response. For most toxicity results, and the fish ELS toxicity syndrome, it is important to perform an initial check to determine if toxicity is a baseline toxic response or a truly specific effect.

A chemical is likely to be a baseline toxicant if:

1. Whole-body concentrations fall within range of those defining baseline toxicity as highlighted above (mortality > 0.5 mM and sublethal effects > 0.05 mM (lower 95% confidence interval)). These concentrations can be measured or modeled with aqueous exposure concentrations and partition coefficients.

2. Aqueous toxicity metrics for organic compounds fall on or nearby the QSAR regression line defined in Figure 1 and the toxic ratio (TR_{LC50}, eq. 1) is <10.

$$TR_{LC50} = \frac{\text{LC50 baseline}}{\text{LC50 observed}} \qquad (1)$$

3. An aqueous sublethal response concentration is within an order of magnitude of the mortality response. This can be determined with an acute to chronic ratio that is < 10 (Scholz et al. 2018). In most cases, the LOECs for sublethal ELS toxicity syndrome responses are close to the LC50 (Figure 1).

4. Because the ELS toxicity syndrome can occur by specific toxicity, the aqueous sublethal response should be compared to the baseline LC50 (i.e., if the ratio of $LC50_{baseline}$ to $EC_{observed}$ (ELS toxicity syndrome) is <10, then it is baseline toxicity).

5. Reactive oxygen species (ROS) and apoptosis markers are elevated, in addition to many of the gene markers associated with sarcoplasmic/endoplasmic reticulum (ER) membrane stress. If the response occurs close to predicted baseline toxicity, it is likely a result of the cytotoxicity burst and not a specific effect.

A chemical is likely to be specific-acting if it does not fulfill 1-4, and if:

6. There is a direct demonstration of receptor interaction at environmentally relevant concentrations, such as AhR activation. Additional support for receptor interaction could come from competitive or non-competitive receptor interaction results. Further support for potential receptor interaction may be found in the ECOdrug database (Verbruggen et al. 2018), Drugbank (Wishart et al. 2018) or the T3DB (Wishart et al. 2015)

7. An adverse outcome pathway (AOP) or target-specific sequence of events has been defined for the observed response at the reported non-baseline level exposure concentrations. An important component of an AOP is the identification of a receptor as the molecular initiating event.

8. Only one of ROS and ER stress markers are elevated below predicted baseline toxicity concentrations, which indicates a specific stress response. If both are elevated close to predicted baseline toxicity concentrations, the ROS and ER stress are likely a consequence of the cytotoxicity burst (Fay et al. 2018; Escher et al. 2020b) and not a specific effect.

In most cases, high-dose responses occur at concentrations that are not environmentally relevant. For organic compounds, aqueous levels in the mg/L or high µg/L range in the environment are extremely rare. The baseline response is such a severe and universal response for so many compounds, any species-specific response occurring at baseline levels for these high environmental concentrations would have deleterious ramifications for all species. The observation of severe responses at such high doses does not necessarily translate to an adverse response at lower doses, especially those that are non-specific, cytotoxic insults. However, baseline toxicants are concentration additive in mixtures. Therefore, baseline toxicity might play a role for complex environmental mixtures even if all components of the mixture are not individually toxic at their concentrations in the mixture.

An important application for the default assumption of baseline toxicity concerns the determination of toxicity metrics for complex mixtures. In some cases, the adverse response is based on a small percentage of compounds comprising the total concentration. One example of this is the myriad studies on oil toxicity claiming that PAHs (specifically tricyclic PAHs) are the main toxic component causing the fish ELS syndrome, when the default assumption should be baseline toxicity. PAHs are a minor component of oil and are nowhere near as toxic in isolation as is concluded when exposing fish to the total water-soluble fraction without invoking unsubstantiated synergism. In those studies, the potential toxicity of thousands of compounds is ignored in favor of PAHs that can be quantified with well-developed protocols and analytical standards, as opposed to myriad known, predicted, and suspected compounds in the WSF that cannot be easily quantified (Meador and Nahrgang 2019). As the funnel hypothesis states, the more components a mixture has, the more likely toxicity of the mixture is due to additive effects resulting from the baseline toxic component (Warne and Hawker 1995). A recent study by Harsha et al. (2024) demonstrated the ELS toxicity syndrome for a crude oil WSF without detectable PAHs. This study supports the hypothesis that PAHs are not the primary cause of the fish ELS toxicity syndrome due to crude oil exposure and that baseline toxicity is the more reasonable conclusion. No one component or class of compounds in crude oil has been shown to cause the ELS toxicity syndrome in isolation at the concentrations at which they occur in the mixture. Importantly, there are exceptions such as that from Tian et al. (2021) who identified the primary casual toxicant (6PPD-quinone) in stormwater, which explained toxicity of the mixture (stormwater) and left no doubt that the response was due to one single specific-acting compound that matched the toxic response for the mixture and causative agent. Such cases are spectacular, but rare and in the big scheme of things the unspectacular process of concentration addition of underlying baseline toxicity can overall do more harm than a few synergistically acting toxic mixture components.

In aquatic toxicity studies, high-dose exposure results are useful for determining mortality or near lethal toxicity but not for identifying low-dose specific toxicity, which is more relevant for environmental risk assessment. There is little reason to assume that biochemical targets activated by high-dose exposure will be relevant when examined in terms of environmentally relevant exposure levels. Also, there is no guarantee that effects from short-term, high-dose exposures will be biologically significant for long-term, low dose exposures. Effects due to receptor interaction at high doses may be very weak, or non-existent, at lower doses and apical effects such as impaired growth, reproduction, behavior, and others will likely be absent. This is also critical for baseline toxicity because membrane disruption and biological effects ~~resulting from high-dose exposure~~ will not occur at lower doses. It must be noted that baseline toxicity for very hydrophobic chemicals occurs at low external aqueous concentrations because the bioaccumulation is high for hydrophobic chemicals, but the baseline toxicity occurs always the highest concentrations for any given compound, any specific effect occurs at lower concentrations. Additionally, in some cases environmental concentrations may be very high due to intentional or unintentional releases of contaminants, therefore baseline toxicity for these situations will be informative.

Adequate protection of aquatic populations will be accomplished with an accurate evaluation for all chemicals in the environment capable of inducing receptor-based sublethal effects that over the long term may impact population viability. The goal should be to identify the lowest observed effect concentrations for specific effects, not the highest dose that affects all bodily functions regardless of the mechanism of effect. In simple terms, baseline toxicity is not a useful or relevant response for environmental protection under most situations.

Conclusions

It is important to realize that it is common for organic compounds to cause non-specific baseline toxicity at high doses and specific, receptor-mediated toxicity at low doses leading to different adverse outcomes (Meador 2021). In many cases, chemicals causing abnormal morphological effects at high exposure concentrations are mistakenly classified as teratogens when in fact the response is nothing more than baseline toxicity. As toxicologists, we should focus our research efforts on those specific-acting compounds that affect endocrine systems, behavior, immune dysfunction, morphological abnormalities and other important responses occurring at low and environmentally realistic concentrations with the potential to affect organisms and their probability of completing a successful life cycle.

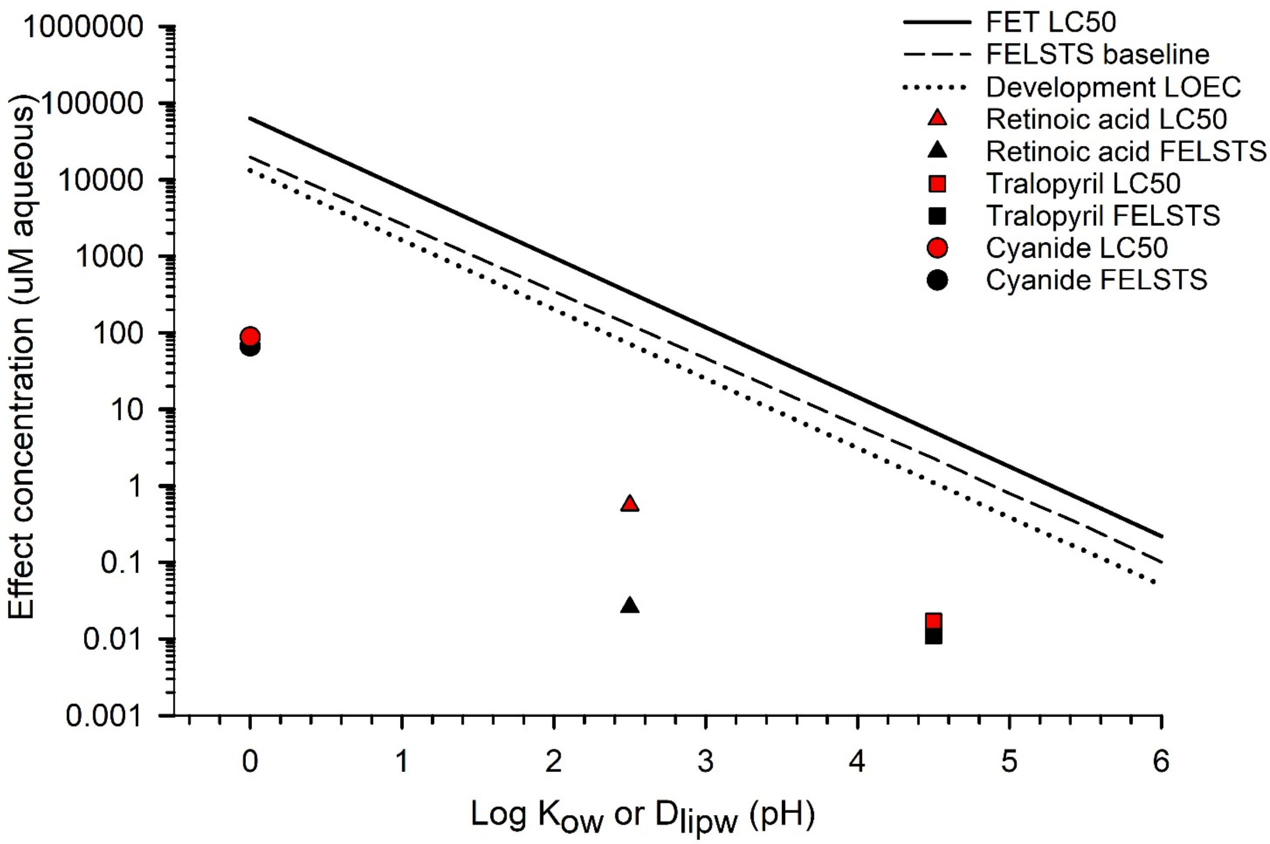

Figure 1: Aqueous lethal and sublethal effect concentrations (µM) against hydrophobicity expressed by the octanol-water partition coefficient log K_{ow} or the lipid membrane-water distribution ratio log D_{lipw}. FET refers to the baseline toxicity QSAR for the 96 h LC50 fish embryo mortality (FET) using data from Kluver et al. (2016) (n=99, r^2=0.75). FELSTS baseline refers to the baseline toxicity QSAR for the fish early life-stage toxicity syndrome related to development, edema, and reduced heart rate endpoints after exposure of 4 – 34 days (Scholz et al. 2018 and Meador 2021) (n=36, r^2=0.82). Development LOEC refers to the QSAR for developmental abnormalities at days 28 - 34 for ELS fish (n=14, r^2=0.83) (Scholz et al. 2018) to show slightly higher toxicity for longer term results. All QSARs for TR_{LC50}<10. Examples for the FELSTS for specific action (TR_{LC50}>10) are shown for retinoic acid (△), cyanide (•), and tralopyril (□). LC50 (red) and sublethal endpoints (black). $TR_{LC50} = \frac{\text{LC50 baseline}}{\text{LC50 observed}}$

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