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FORUM

Adverse Outcome Pathways during Early Fish Development: A Conceptual Framework for Identification of Chemical Screening and Prioritization Strategies

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The fish early life-stage (FELS) test guideline (OECD 210 or OCSPP 850.1400) is the most frequently used bioassay for predicting chronic fish toxicity and supporting aquatic ecological risk assessments around the world. For each chemical, the FELS test requires a minimum of 360 fish and 1 to 3 months from test initiation to termination. Although valuable for predicting fish full life-cycle toxicity, FELS tests are labor and resource intensive and, due to an emphasis on apical endpoints, provide little to no information about chemical mode of action. Therefore, the development and implementation of alternative testing strategies for screening and prioritizing chemicals has the potential to reduce the cost and number of animals required for estimating FELS toxicity and, at the same time, provides insights into mechanisms of toxicity. Using three reference chemicals with well-established yet distinct adverse outcome pathways (AOPs) in early life stages of fish, we proposed FELS-specific AOPs as conceptual frameworks for identifying useful chemical screening and prioritization strategies. The reference chemicals selected as case studies were a cardiotoxic aryl hydrocarbon receptor agonist (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), neurotoxic acetylcholinesterase inhibitor (chlorpyrifos), and narcotic surfactant (linear alkylbenzene sulfonate). Using qualitative descriptions for each chemical during early fish development, we developed generalized AOPs and, based on these examples, proposed a three-tiered testing strategy for screening and prioritizing chemicals for FELS testing. Linked with biologically based concentration-response models, a tiered testing strategy may help reduce the reliance on long-term and costly FELS tests required for assessing the hazard of thousands of chemicals currently in commerce.

Key Words: **adverse outcome pathway; high-throughput screening; zebrafish embryo; fish early life-stage test.**

The use of conventional whole-organism (vertebrate) bioassays for estimating chronic ecological hazards has been limited due to low efficiency, high cost, extensive animal use, and, for aquatic toxicity studies, generation of large volumes of contaminated water. Moreover, most animal guideline tests provide little or no informative mechanistic toxicity data due to the principal focus on apical endpoints such as survival, growth, and reproduction. Due to these limitations, resource-efficient alternatives to conventional toxicity testing, including high-throughput *in vitro* and *in silico* screening assays, have been proposed as key components of a future testing paradigm for mechanism-based regulatory toxicology and ecotoxicology (Bradbury *et al.*, 2004; National Research Council, 2007; Villeneuve and Garcia-Reyero, 2011). However, the predictive power of molecular or cellular perturbations (e.g., modeled or detected *in vitro*) for apical endpoints relevant to ecological risk assessment (i.e., survival, growth, and reproduction) must be sufficiently high to minimize uncertainties and provide meaningful data for regulatory decision making.

To begin identifying useful predictive assays and testing strategies for regulatory ecotoxicology, Ankley *et al.* (2010) recently proposed the use of adverse outcome pathways (AOPs) as a conceptual framework for summarizing existing knowledge about linkages between a direct molecular-level initiating event and an adverse outcome at a level of biological organization relevant to ecological risk assessment. Predictive linkages are implicitly defined when developing AOPs, providing a scientific framework for identification of targeted alternative assays and models that represent key events at multiple levels of biological levels of organization. Linked with biologically based concentration-response models (e.g., toxicokinetic and toxicodynamic

models), these alternative assays, once developed, can facilitate extrapolation to organism- and population-level endpoints and significantly decrease reliance on whole-animal regulatory toxicity tests as the primary source of hazard data for ecological risk assessment and environmental decision making (National Research Council, 2007).

As part of a global effort to identify alternative testing strategies, the ILSI Health and Environmental Sciences Institute sponsored a workshop from 7 to 9 June 2010 in Paris, France, entitled "Development of alternatives to chronic ecotoxicity tests: predicting early life stage and endocrine-mediated toxicity in aquatic vertebrate species." More than 50 scientists representing industry, academia, government, and non-governmental organizations from North America, Europe, and Asia attended the meeting. The workshop focused on identifying critical research gaps and associated short- and long-term research strategies related to the development of alternatives for chronic fish and amphibian toxicity testing. The first half of the workshop explored potential alternatives to a representative, commonly used chronic (long-term) ecotoxicity test—the fish early life-stage (FELS) test. Two session topics were considered within this portion of the workshop: (1) FELS data availability and endpoint evaluation and (2) use of FELS-specific AOPs to identify potential high- and medium-throughput tests that could be incorporated into alternative tiered testing strategies. This paper is focused on key findings and recommendations derived from the latter session topic.

THE FELS TEST

For regulatory ecotoxicity testing, the FELS test guideline (OECD 210 or OCSPP 850.1400) was introduced more than 30 years ago as an alternative to longer-term fish full life-cycle tests (McKim, 1977). Presently, the FELS test is the most frequently used bioassay for predicting chronic fish toxicity and supporting ecological risk assessments for pesticides, biocides, pharmaceuticals, and industrial chemicals. Based on a recent evaluation of publically available and registrant-provided data, approximately 425 chemicals across 51 different fish species have been tested worldwide using the FELS test since 1945 (Norberg-King, U.S. Environmental Protection Agency [EPA], Duluth, MN, personal communication). Currently, the most commonly used freshwater species include rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*), whereas the most commonly used saltwater species is sheepshead minnow (*Cyprinodon variegatus*).

Using static-renewal or flow-through conditions, ≥ 60 newly fertilized eggs per treatment group are equally divided between a minimum of two replicate chambers and continuously exposed to a negative and/or vehicle control and at least five concentrations of a test chemical suspended within vehicle. Therefore, this design requires a minimum of 360 fish for each FELS test; however, up to 720 fish per chemical is common depending on replication, brood stocks, range-finding studies, and/or preliminary investigations. Fish are exposed until at least all vehicle-exposed fish are swimming and free feeding. The typical test includes embryo, eleutheroembryo, larval, and juvenile stages and is continued until 32 days (fathead and sheepshead minnows) to 91 days (rainbow trout) post-hatch. Hatching, mortality, gross morphology, and behavioral abnormalities are assessed and documented throughout the entire test (usually daily), whereas body length and weight (dry and wet) are assessed at test termination. Based on these endpoints, hypothesis testing is used to calculate the lowest-observable effect concentration and no-observable effect concentration (NOEC) for all endpoints at one or more time-points. Alternatively, for each endpoint, regression modeling may be used to model the concentration that adversely affects 10% of the population relative to controls (EC_{10}). The chronic NOEC and/or EC_{10} based on the most sensitive endpoints are then used as hazard thresholds for regulatory risk assessments.

AOPs DURING EARLY FISH DEVELOPMENT

In the future, a battery of alternative *in silico*, *in vitro*, and *in vivo* bioassays will likely decrease the number of, and may ultimately replace, labor-intensive and costly animal experiments performed for chemical hazard assessments. A critical step in the development of tiered testing approaches, however, is the identification of common chemically mediated mechanisms of toxicity that could be used to predict potential adverse effects during fish development. Therefore, to provide a conceptual framework for development of alternative testing strategies, FELS-specific AOPs must first be identified and summarized using best-available data from the scientific literature. Using three reference chemicals with well-established yet distinct modes of action in early life stages of fish, we summarized three example FELS-specific AOPs to begin conceptualizing a tiered testing approach for chemical screening and prioritization. The reference chemicals we selected as case studies were a cardiotoxic aryl hydrocarbon receptor (AHR) agonist (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]), neurotoxic acetylcholinesterase (AChE) inhibitor (*O*,*O*-diethyl *O*-[3,5,6-trichloro-2-pyridyl] phosphorothionate or chlorpyrifos [CPF]), and narcotic surfactant (linear alkylbenzene sulfonate [LAS]). For each chemical, we briefly summarized the mode of action in early life stages of fish. Using these qualitative descriptions, we proposed an AOP for each chemical (Fig. 1) and, based on these three examples, proposed an overall three-tiered testing scheme for screening and prioritizing chemicals for FELS testing (Fig. 2).

Case Study #1: TCDD-Induced Cardiotoxicity

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a widely studied nongenotoxic halogenated aromatic hydrocarbon that is a by-product of incomplete municipal/industrial waste combustion. TCDD is known to induce adverse effects in humans

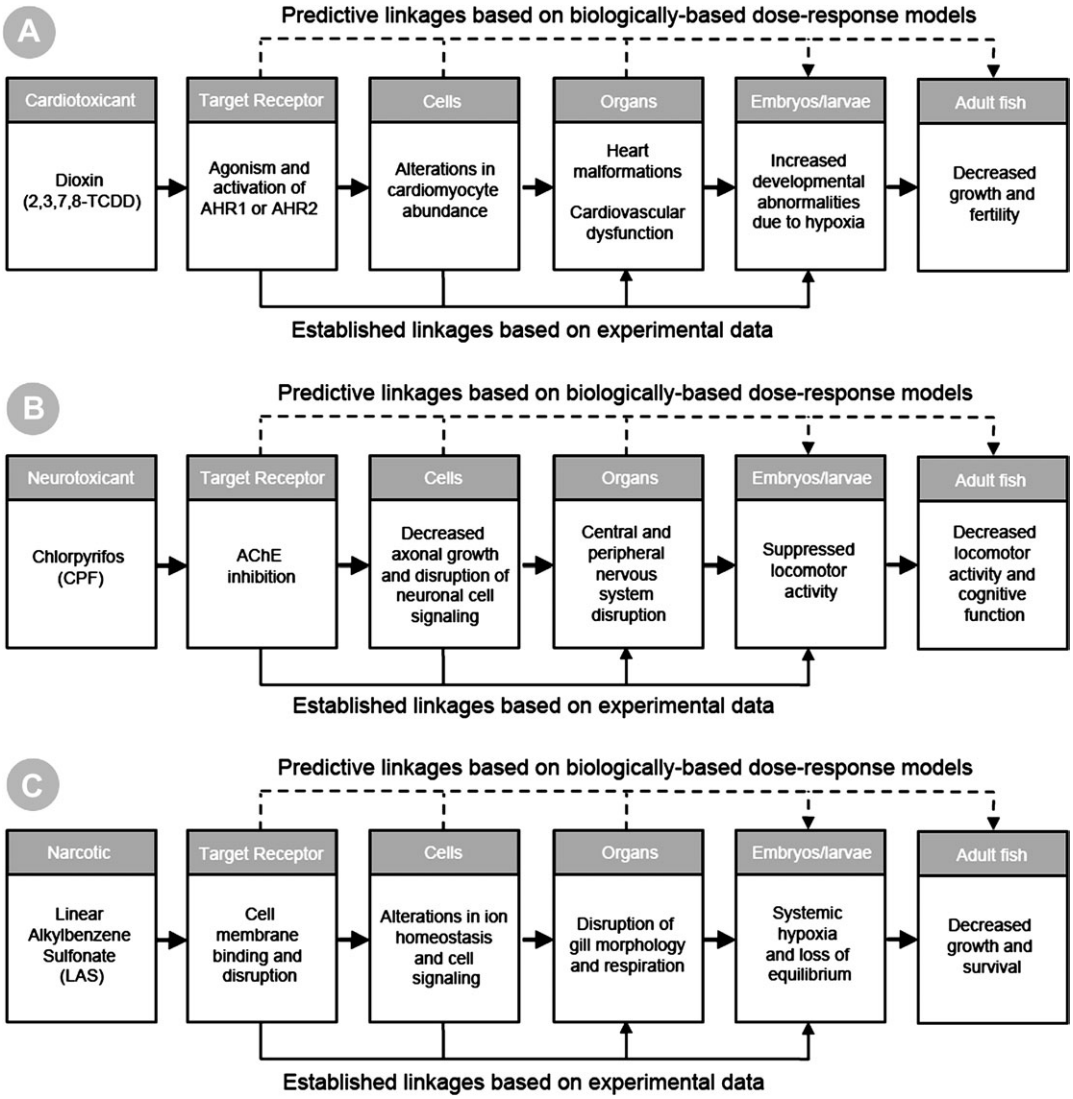

FIG. 1. Proposed AOPs for (A) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)–induced, (B) chlorpyrifos (CPF)–induced, and (C) LAS-induced toxicity in early life stages of fish development.

and wildlife, including cancer, reproductive/developmental effects, immunotoxicity, nephrotoxicity, and cardiovascular disease (DeVito and Birnbaum, 1994), and is highly lipophilic (log $K_{ow} = 6.58–7.58$), exhibits very little solubility in water (19 ng/l at 25°C) (ATSDR, 1998), and bioconcentrates within vertebrates, including fish (log BCF = 3.4–5.1 depending on the species) (ATSDR, 1998). Due to biological persistence and high sensitivity of early life stages, TCDD exhibits persistent effects on teleost health and reproduction (King Heiden *et al.*, 2009) and has been implicated in declining recruitment and population sizes of feral fish inhabiting TCDD-contaminated water bodies (e.g., lake trout populations within Lake Ontario) (Cook *et al.*, 2003).

Upon entry into cellular cytosol, TCDD is highly specific for the AHR, a ligand-activated transcription factor identified in a variety of vertebrates, including species of mammals, birds, amphibians, bony fishes, cartilaginous fishes, and jawless fishes (Hahn, 2002). Classic AHR-responsive genes (otherwise known as the "*Ah* gene battery") in mammals and fish include cytochrome P450 1A (CYP1A), NAD(P)H:quinone oxidoreductase (NQO1), aldehyde dehydrogenase 3 (ALD3A1), UDP glucuronosyltransferase (UDPGT), and glutathione transferase (GST), all phases I and II enzymes that comprise a group of genes coordinately transcribed and translated in response to AHR ligand exposure (Nebert *et al.*, 2000). In addition to dioxin-like chemicals, the AHR pathway and target genes are responsive to a wide variety of structurally diverse chemicals (Denison and Heath-Pagliuso, 1998). Other chemicals that potently activate the AHR likely initiate a TCDD-like AOP.

Peterson *et al.* published the first comprehensive study of TCDD-induced early life stage toxicity in zebrafish (Henry *et al.*, 1997). In this study, 5 h post-fertilization (hpf) zebrafish embryos (40 embryos per treatment) were exposed for 1 h to

vehicle (0.1% acetone) or increasing concentrations of [^{3}H]TCDD (35–2100 ng/l = 0.1–6.5nM) and then placed in clean water until 10 days post-fertilization (dpf). From exposure initiation to complete hatch (~72 to 96 hpf), there were no significant differences in embryo survival or time-to-hatch within all TCDD treatments compared with vehicle controls. Immediately prior to and following hatch, zebrafish embryos and larvae exhibited a significant concentration-dependent decrease in heart rate, blood flow, and swim bladder inflation, as well as a significant time- and concentration-dependent increase in pericardial edema, yolk sac edema, craniofacial malformations, and, as a result, mortality. Interestingly, TCDD-induced heart malformations and cardiotoxicity preceded systemic toxicity. This indicates that structural malformations, altered looping, and decreased cardiomyocyte abundance (due to increased cell death and/or decreased cell proliferation) within the developing zebrafish heart leads to retrograde blood flow, ventricular standstill, and, subsequently, impairments to peripheral circulation and proper organ functioning (Antkiewicz *et al*., 2005). AHR is required for TCDD-induced toxicity in zebrafish embryos, as morpholino (MO)-based knockdown of AHR translation eliminated cardiotoxicity observed by 72 hpf in non-MO–exposed control embryos exposed to TCDD (Prasch *et al*., 2003; Teraoka *et al*., 2003).

Case Study #2: CPFO-Mediated Inhibition of Neurite Outgrowth

Chlorpyrifos (CPF) is a lipophilic (log K_{ow} = 4.96) organophosphorus insecticide used for broad-spectrum insect control on ornamentals, turfgrass, and certain vegetable and field crops. Bioconcentration factors in fish range from approximately 0.2 to 28,000 depending on the species and exposure duration (U.S. EPA ECOTOX http://cfpub.epa.gov/ecotox/), a variation that is likely explained by species-specific differences in chemical metabolism. Following cellular uptake, CPF is metabolized to CPF-oxon (CPFO), a potent metabolite that, at acutely toxic concentrations, inhibits AChE via phosphorylation of a catalytic serine residue, resulting in decreased acetylcholine (ACh) hydrolysis, and increased ACh-induced activation of post-synaptic ACh receptors (Eaton *et al*., 2008). For acute exposure events, overstimulation of the cholinergic synapses often leads to paralysis and death. Following sublethal exposures during early development, cholinergic- and non-cholinergic–related toxicity results in decreased axonogenesis, suppressed locomotor activity, and altered brain development (Das and Barone, 1999; Li and Casida, 1998; Slotkin, 1999), leading to persistent neurobehavioral effects into adulthood (Dam *et al*., 2000; Icenogle *et al*., 2004; Johnson *et al*., 2009; Levin *et al*., 2002).

Mechanisms of CPF-induced neurotoxicity during early fish development have only recently been characterized using zebrafish as an animal model. In the absence of gross malformations, CPF (and CPFO) exposure during zebrafish embryogenesis decreased baseline locomotor activity and touch-induced swimming bursts of young larvae (Levin *et al*., 2004; Yang *et al*., 2011). Interestingly, similar to rodents, neurodevelopmental effects on locomotor activity and choice accuracy (using a zebrafish maze) appear to persist into adulthood after transfer to clean water at 5 dpf (Levin *et al*., 2003). Although CPFO does not directly affect axial muscles or abundance of secondary motoneurons, non-teratogenic concentrations of CPFO do inhibit axonal growth of primary and secondary motoneurons as well as Rohon-Beard and dorsal root ganglia sensory neurons (Jacobson *et al*., 2010; Yang *et al*., 2011). Therefore, CPFO-induced functional effects on zebrafish behavior are directly linked to structural effects on the developing neural circuitry, phenotypes that are likely due to AChE inhibition within developing embryos (Behra *et al*., 2002; Jacobson *et al*., 2010; Yang *et al*., 2011) and/or alterations in tubulin dynamics due to direct phosphorylation of microtubules (Jiang *et al*., 2010).

Case Study #3: LAS-Induced Gill Toxicity and Narcosis

LAS is a complex mixture of anionic surfactants that are added to many household laundry and dish detergents. Commercially available alkyl chain lengths vary from 10 to 13 carbons, and sulfophenyl groups can be located at any nonterminal carbon. LAS is moderately hydrophobic (log K_{ow} = 3.32 for C11.6 LAS), negatively charged at environmental pH (due to presence of a sulfophenyl group), and exhibits low bioaccumulation potential (Human and Environmental Risk Assessment, 2009). In addition, LAS is readily biodegradable in the environment and undergoes rapid metabolism and elimination within fish (Tolls *et al*., 2000). Following biological uptake, LAS binds to cellular membranes, resulting in disruption of membrane integrity and fluidity; alterations in ion homeostasis; aberrant cell signaling; and, at high concentrations, cell death (necrosis) (Bernhard and Dyer, 2005; Jifa *et al*., 2006; Romani *et al*., 2003). Within fish, toxicity increases with increasing LAS alkyl chain length, as longer hydrophobic alkyl chains or phenyl isomers lead to increased hydrophobicity and increased binding and disruption of cellular membranes (Rosen *et al*., 2001). Therefore, unlike TCDD and CPF, LAS toxicity is nonspecific and induces a narcotic mode of action.

In early life stages of fish, gills are the primary target organs for LAS toxicity. Toxic concentrations of LAS disrupt epithelium of primary and secondary lamellae (but not chloride cells), resulting in pathologic alterations (hypertrophy and edema) that, if persistent, lead to decreased respiration, systemic hypoxia, loss of equilibrium, and death (Versteeg and Rawlings, Central Product Safety, Procter & Gamble, personal communication). Based on partial life cycle and FELS tests using smallmouth bass, northern pike, fathead minnow, and white sucker (McKim *et al*., 1975), fish embryos and larvae are the most susceptible life stages to LAS-induced toxicity, with survival representing the most sensitive endpoint across a wide range of teleosts (Mallett *et al*., 1997; Versteeg

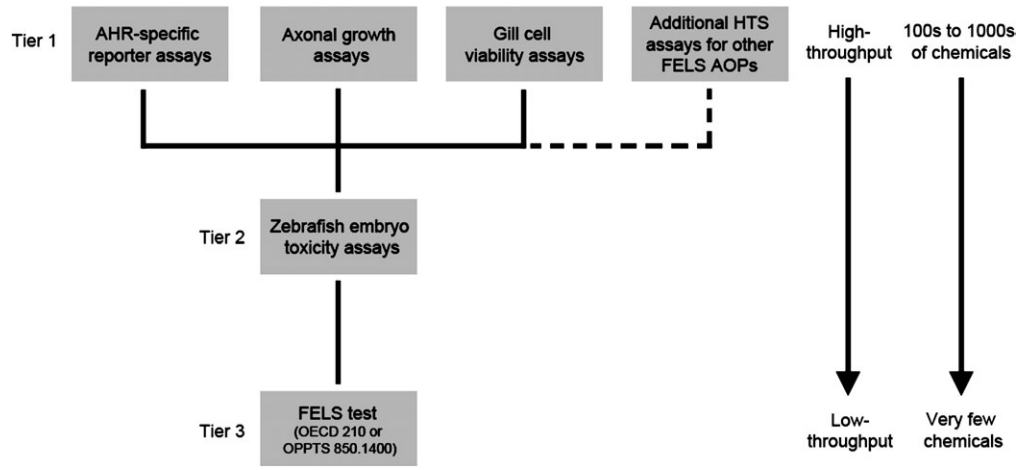

FIG. 2. Proposed three-tiered testing strategy for screening and prioritizing chemicals for FELS toxicity testing.

and Rawlings, 2003); however, LAS-induced gill toxicity is reversible, and fish that survive beyond the first 30–60 days of exposure are reproductively viable (Pickering and Thatcher, 1970). Therefore, effects on larval mortality and population growth could be predicted based on adverse effects on gills during early stages of fish development.

TIERED TESTING STRATEGY TO SCREEN AND PRIORITIZE CHEMICALS FOR FELS TOXICITY TESTING

Although a small subset of regulated chemicals (principally pesticides) continues to be tested using the FELS test, the need to screen and prioritize hundreds to thousands of currently registered chemicals regulated under, e.g., Registration, Evaluation, Authorization and Restriction of Chemical (REACH) or Toxic Substances Control Act (TSCA) requires development of a rapid, cost-efficient, and mechanism-based tiered testing strategy. Therefore, to begin addressing this challenge, we propose development of a three-tiered testing strategy, with the first tier representing high-throughput AOP-based *in vitro* (cell-based) assays; the second tier representing medium-to-high-throughput *in vivo* (zebrafish embryo) assays; and the highest tier representing the current FELS guideline test (Fig. 2).

The primary goal of this tiered strategy would be to identify rapidly a subset of potentially hazardous chemicals for FELS testing using the current regulatory guideline (OECD 210 or OCSPP 850.1400). Although this approach is tailored to chemical screening and prioritization programs, we recognize that the application and progression of this tiered testing scheme may be dependent on the specific needs and risk assessment objectives of regulatory program(s) around the world. In addition, although the proposed strategy is illustrated using three chemical-specific AOPs, this tiered approach must be expanded to a broad range of toxicologically relevant AOPs in order to provide a reliable and effective testing strategy that accounts for concentration-dependent multiple mechanisms of toxicity within and across chemical classes. Based on comparisons to positive and negative reference chemicals for each assay, hazard thresholds will need to be developed within and across each testing tier to determine whether additional higher-tier testing is warranted. Moreover, for tier 1 and 2 assays, the application of biologically based concentration-response modeling (e.g., toxicokinetic and toxicodynamic models) will be critical to accurately extrapolate findings from cells (tier 1) and embryos (tier 2) to later life stages of fish development (tier 3).

Tier 1: High-Throughput In Vitro *Screening*

Tier 1.1–AHR-specific reporter assays. Based on the mechanism of TCDD-induced cardiotoxicity in zebrafish, *in vitro* luciferase reporter assays using recombinant zebrafish AHR2 are likely valuable for predicting AHR-mediated developmental abnormalities and mortality in early life stages of fish. Given known intra- and interspecies variation in AHR isoform functionalities within teleosts (Hahn, 2002), additional high-throughput and multiplexed fish AHR-specific luciferase reporter assays may be needed to develop models that can account for genetic variation of feral teleosts to AHR activation by dioxin-like chemicals. Full-length AHR isoforms (e.g., AHR1, AHR2, etc.) should be isolated from one or more model FELS test teleosts (e.g., fathead minnow, medaka, zebrafish, and rainbow trout) and cloned into commercially available expression vectors. Expression constructs for functional zebrafish and rainbow trout AHRs are currently available (Abnet *et al.*, 1999), whereas functional expression constructs for fathead minnow, medaka, and sheepshead minnow AHRs will need to be developed. For high-throughput assays, these constructs would be individually transfected into an AHR-negative mammalian or fish host cell line, and

AHR-driven luciferase reporter activity, based on a fish aryl hydrocarbon response element–driven reporter vector—would be quantified following a 24- to 48-h exposure to positive controls (e.g., TCDD, benzo[a]pyrene, etc.), negative controls, or test chemicals in concentration-response format. The potency of test chemicals (based on EC_{50}s) at non-cytotoxic concentrations would be compared with reference AHR agonists and negative controls to determine the potential for AHR activation and toxicity within FELS. Luciferase reporter induction thresholds that trigger tier 2 testing will need to be defined based on the magnitude of AHR activation known to induce developmental malformations in early life stages of fish.

Tier 1.2–Axonal growth assays. The toxicity of neurotoxic compounds such as AChE inhibitors is generally associated or paralleled by inhibition of neurite outgrowth. Therefore, high-throughput cell line–based systems capable of identifying whether neurotoxic chemicals exhibit the potential to inhibit axonal growth of fish-specific sensory neurons and/or motoneurons should be developed. Although numerous mammalian lines are available, the use of fish-specific neuronal cell lines will likely minimize uncertainties with extrapolating from mammals to fish; however, to date, the comparative sensitivity of mammalian versus fish neuronal cells to chemical exposure has not been directly tested. To our knowledge, the only immortal fish-specific neuronal line available is the rainbow trout brain cell line developed by Niels Bols at the University of Waterloo (Ontario, Canada). For high-throughput assays, axonal growth would be quantified using automated high-content imaging following a 24- to 48-h exposure to positive controls (e.g., CPFO, synthetic pyrethroids, etc.), negative controls, and test chemicals in concentration-response format. In addition, enzymatic or biochemical assays could be used to assess whether decreased axonal growth is associated with target receptor inhibition (e.g., AChE), activation (e.g., nicotinic ACh receptors), or modulation (e.g., Na^+ channels). The potency of test chemicals (based on EC_{50}s) at non-cytotoxic concentrations would be compared with reference neurotoxicants and negative controls to determine the potential for neurotoxicity within FELS. Thresholds for axonal growth inhibition that trigger tier 2 testing will need to be defined based on the magnitude of neurite growth inhibition known to result in behavioral effects in early life stages of fish.

Tier 1.3–Gill cell viability assays. High-throughput cell line–based systems should be developed to identify whether narcotic chemicals exhibit the potential to disrupt gill cell epithelium and viability. Immortal epithelial cells derived from a primary culture of rainbow trout gills (RTgill-W1) are currently available from ATCC (cat. no. CRL-2523) (Bols *et al.*, 1994), precluding the need to develop a new fish-specific gill cell line. For high-throughput assays, gill cell membrane integrity and viability should be quantified using standard live/dead viability assays (Dayeh *et al.*, 2004; Schirmer *et al.*, 1997, 1998) following a 24- to 48-h exposure to positive controls (e.g., LAS, alkylphenols, etc.), negative controls, or test chemicals in concentration-response format. The potency of test chemicals (based on EC_{50}s) would be compared with reference narcotic chemicals and negative controls to determine the potential for gill epithelium disruption within early life stages of fish. Gill cell viability thresholds that trigger tier 2 testing will need to be defined based on the magnitude of gill epithelium disruption known to affect survival of early life stages of fish.

Tier 1.n–Additional assays and FELS AOPs. Tier 1 assays should be developed to address the potential for chemical metabolism, as these data will be critical for better understanding chemical half-life and persistence within early life stages of fish. To account for potential chemical metabolism, fish-specific hepatocyte lines should be used to assess the rate of metabolism relative to positive and negative reference chemicals. Hepatocytes derived from one or more model FELS test teleosts would be ideal, and model teleost(s) used for metabolism assays should be identical to species used for other tier 1 assays. In addition, although freshly isolated hepatocyte suspensions would be ideal, the use of immortal cell lines would greatly facilitate the development and application of high-throughput assays, as well as significantly reduce (or eliminate) the need for additional use of adult fish. Immortal hepatocytes derived from normal rainbow trout liver (RTL-W1) are currently available within Niels Bols' laboratory (Lee *et al.*, 1993) and, similar to primary cultures of rainbow trout hepatocytes, are sensitive to polycyclic aromatic hydrocarbon–mediated induction of cytochrome P450 1A (Behrens *et al.*, 2001).

Importantly, implementation of only three assays would not account for other toxicologically relevant AOPs during FELS development, potentially undermining the confidence in tier 1 as a priority-setting tool. Therefore, a tier 1 battery should include high-throughput screening assays relevant to other known FELS AOPs, and research should be directed toward systematically characterizing other AOPs related to FELS toxicity. For example, using the same host cell lines, tier 1.1 reporter assays may be altered to address the potential activation of a broad range of nuclear receptors by developing expression and reporter constructs specific to other nuclear receptor–mediated pathways (e.g., estrogen, androgen, and peroxisome proliferator–activated receptors). In theory, all toxicologically relevant FELS AOPs should be represented with an appropriate assay in order to account for concentration-dependent multiple mechanisms of toxicity within and across chemical classes. Using a battery of tier 1 assays, a "positive" finding from one or more of the tier 1 AOP-specific assays would trigger tier 2 testing, whereas "negative" findings from all tier 1 assays would preclude the need to conduct additional testing within fish embryos and larvae. A "positive" finding may occur when the test chemical EC_{50} is within the range of EC_{50}s for positive controls or when the ratio of baseline

(nonspecific) toxicity to specific biological effects is similar to positive controls. A "negative" finding may occur when the test chemical EC_{50} is orders of magnitude higher than EC_{50}s for positive controls or when the test chemical only induces baseline (nonspecific) toxicity at high concentrations. Although comprehensive coverage does not preclude the potential for false negatives, a manageable and appropriately sized battery of high-throughput tier 1 assays should be sufficient to identify chemicals exhibiting potential FELS toxicity. As a potential testing requirement for chemical registrations in the future, this approach, while not perfect, would represent an improvement over the current strategy that relies on minimal to no hazard testing for most chemicals in commerce.

Tier 2: Medium-to-High-Throughput In Vivo *Screening*

Definitions of protected and nonprotected stages of fish for regulatory testing and scientific experimentation vary widely around the world (Halder *et al*., 2010). According to current legislation within the European Union, fish embryos (pre-hatch stages) and eleuthereoembryos, the time-period between immediately post-hatch and stages of independent feeding, are nonprotected life stages and are considered as alternative testing models (Belanger *et al*., 2010; Embry *et al*., 2010; Halder *et al*., 2010; Scholz *et al*., 2008). Other regulatory jurisdictions do not clearly distinguish protected and non-protected stages of fish but do provide indications based on acceptance of certain assays or through the encouragement of new assays and approaches (e.g., in Japan and the United States).

In contrast to cell-based assays, fish embryos provide the complexity and interaction of an intact organism, enabling the evaluation of chemically induced effects on multiple target organs. Given the accessibility, global use, and research tools available for zebrafish, a tier 2 zebrafish embryo toxicity assay should be implemented to predict the potential toxicity of chemicals on early life stages of model FELS test teleosts. As part of the chemical screening and prioritization research (ToxCast) program within the U.S. EPA, a zebrafish developmental toxicity assay has been used by EPA to evaluate the potential human health effects of 320 ToxCast Phase-I chemicals (Padilla *et al*., 2009). In this assay, zebrafish are continuously exposed in 96-well plates from late gastrula stage (approximately 8 hpf) to 5 dpf and then visually assessed for mortality and gross malformations at 6 dpf. In collaboration with EPA, this assay should, in addition to gross assessments of mortality and morphology, be modified to include high-content quantitative imaging of cardiovascular development, neurogenesis, and gill morphology using whole-mount immunofluorescence-based approaches. In addition, for functional assessments of cardiotoxicity and neurotoxicity, larval heart rates and behavior (locomotor activity and photomotor response) could be rapidly quantified within developing zebrafish embryos (Burns *et al*., 2005; Kokel *et al*., 2010; MacPhail *et al*., 2009; Milan *et al*., 2003; Rihel *et al*., 2010). These live imaging assays have the potential to classify accurately cardiotoxic and neurotoxic AOPs and could be very useful for development of predictive models of cardiotoxicity and neurotoxicity.

Overall, a 3- to 5-day zebrafish embryo toxicity assay would be consistent with existing definitions of embryo and eleuthereoembryo life stages (Belanger *et al*., 2010), as well as considerations under the European Animal Protection Directive where protected stages of fish occur at the transition to exogenous feeding (Halder *et al*., 2010). Based on this tier 2 assay, target organ toxicity thresholds that trigger tier 3 FELS testing will need to be defined based on concentration-by-time response comparisons to positive controls with known early life stage toxicity.

Tier 3: Low-Throughput FELS Test (OECD 210 or OCSPP 850.1400)

The final testing tier would represent the current FELS test guideline. Applied in a chemical screening and hazard characterization context, this tier would only be implemented if a chemical tested positive based on the previous two testing tiers. Following tier 2 testing, a "positive" finding may occur when the test chemical's EC_{50} for organ-specific effects is within the range of EC_{50}s for positive controls. Based on this definition, these chemicals would likely have a higher probability of inducing persistent chronic effects and, consequently, would be viewed as higher priority targets for tier 3 testing.

SHORT- AND LONG-TERM RESEARCH GOALS

In the short term (3–5 years), the following research objectives are recommended to implement a tiered testing strategy as outlined above. First, toxicologically relevant AOPs should be identified, described, and annotated to provide a conceptual and scientific foundation for identification and development of tier 1 assays. Recently, an open-source and open-knowledge program named Effectopedia (available at http://www.softpedia.com/get/Science-CAD/Effectopedia.shtml) was released in late 2010 by the International QSAR Foundation to provide a Wiki-based tool for describing, cataloging, and updating AOPs in an encyclopedic manner. Using Wiki-based software such as Effectopedia, the global scientific community can collectively identify and systematically annotate currently known and newly discovered FELS-specific AOPs relevant to chemical toxicity. Following annotation, tier 1 assays and corresponding reference chemicals may then be identified based on a catalog of FELS AOPs.

Following identification of potential tier 1 assays, positive and negative reference chemicals for currently known FELS AOPs should be identified for tiers 1 and 2 assays using criteria similar to those previously described (Schirmer *et al*., 2008). At minimum, positive reference chemicals should (1) exhibit

a range of potencies (low to moderate to high) for target receptors or endpoints represented within tier 1 assays, (2) exhibit sufficient hydrophobicity (log K_{ow} > 2) to ensure adequate chemical movement across the chorion during embryogenesis for tier 2 assays, and (3) have documented target organ toxicity within early life stages of fish based on existing FELS toxicity data derived from the FELST OECD 210 or OPPTS 850.1400 guideline. Once these chemicals have been identified, tier 1 assays should be developed and tested using the general strategy described above. Using reference chemicals for neurotoxic AOPs, the comparative sensitivity of rainbow trout brain cells versus commonly used mammalian neuronal cell lines should be assessed to determine whether mammalian neurons could be used as surrogate models for fish neurons. Following completion of tier 1 testing with reference chemicals, the EPA's ToxCast zebrafish developmental toxicity assay should be formally adapted and implemented as a tier 2 assay using tier 1 reference chemicals. This assay should rely on a robust strain of wild-type zebrafish exhibiting low background mortality rates during embryogenesis and, at minimum, include assessments of heart rate, behavior (locomotor activity and photomotor response), cardiovascular development, neurogenesis, and gill morphology. Finally, based on comparisons of tiers 1 and 2 results, false-negative and -positive error rates should be quantified and, if necessary, non-predictive tier 1 assays should be removed and new tier 1 tests should be developed to improve the ability to predict effects observed within tier 2 assays.

In the long term (5–10 years), the early life stage toxicity of tiers 1 and 2 reference chemicals should be tested (if data are not already available) using a modified OECD 210 or OPPTS 850.1400 guideline that includes endpoints reflecting AOPs represented within the tier 1 assays. These results should then be compared with results from tiers 1 and 2 assays to quantify the ability of high- and medium-throughput assays to predict FELS toxicity. Following successful completion of pilot tiers 1 and 2 tests, a large-scale inter-laboratory validation of these assays must be developed and completed prior with adoption by regulatory authorities. Multitier assay validation would include the use of a wide range of chemical classes that cover multiple FELS AOPs. Finally, based on comparisons of tiers 2 and 3 results, false-negative and -positive error rates for predicting FELS toxicity should be quantified, and, if necessary, the tier 2 assay should be modified to improve the ability to predict effects observed within tier 3 assays. These modifications may include the use of (1) additional endpoints at the histologic or biomarker level, (2) transgenic strains or reverse genetic approaches (e.g., morpholino knockdown), and/or (3) pharmacological co-exposures (e.g., co-exposure with competitive inhibitor) to help uncover target pathways. Finally, as new FELS AOPs are discovered and characterized, a Wiki-based tool such as Effectopedia should be continuously updated in order to augment the existing catalog of known FELS AOPs. Following consensus within the scientific community, new tier 1 assays and reference chemicals that represent newly discovered FELS AOPs may then be recommended for development and validation as described above.

CONCLUSIONS

In conclusion, using FELS-specific AOPs as conceptual frameworks, the development and implementation of a tiered testing approach will likely enable rapid screening and identification of chemicals with the potential to cause early life stage toxicity, allowing for effects-based identification of high-priority targets for additional tiers of testing that utilize integrated and complex biological models. This strategy would preclude the need to (1) rely on low-throughput FELS tests for each chemical hazard assessment or (2) conduct chemical hazard assessments in the absence of biological effects data. As outlined above, we propose development of a three-tiered testing strategy that includes high-throughput cell-based assays, medium-to-high-throughput zebrafish embryo toxicity assays, and low-throughput guideline FELS tests. Although our recommendations are based on three example AOPs, the proposed approach must account for other toxicologically relevant AOPs by developing a battery of FELS AOP-based tier 1 assays. In general, the use of AOPs for assay identification holds promise for facilitating the implementation of alternatives to animal testing for environmental toxicology and risk assessment. Although the proposed methodology is tailored for chemical screening and prioritization, in the future, the use of alternative tests (e.g., tiers 1 and 2) alone may provide the basis for regulatory decision making and environmental risk assessment.

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