

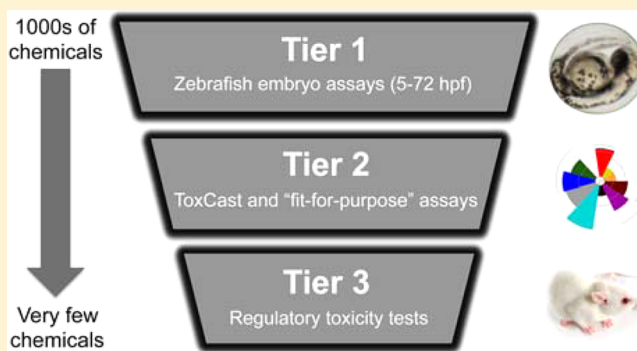
Leveraging Embryonic Zebrafish To Prioritize ToxCast Testing

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S Supporting Information

ABSTRACT: On the basis of two pilot high-content screens of ToxCast Phase I chemicals, we previously demonstrated that exposure of zebrafish embryos to abamectin and butafenacil abolished spontaneous activity and induced severe anemia, respectively. Therefore, the objective of this study was (1) to determine whether high-throughput *in vitro* screening data from the ToxCast program would have prioritized abamectin and butafenacil for further testing and (2) to determine whether a single three-day zebrafish embryo assay is a strong predictor of Toxicological Priority Index (ToxPi) scores derived from ToxCast data. Using publically available ToxCast assay end point data and target information, we calculated assay hit rates, developed hazard classifications, and relied on the ToxPi Graphical User Interface to generate ToxPi charts and scores within a biological process-driven configuration. Overall, our findings suggest that embryonic zebrafish may be valuable for prioritizing ToxCast testing as well as addressing toxicity pathways that may not be represented by the ToxCast assay battery.



INTRODUCTION

As part of a broader multiagency Toxicity Testing in the 21st Century (Tox21) program, the U.S. Environmental Protection Agency (EPA) ToxCast research program was initiated in 2007 to test the hypothesis that a diverse battery of cell-based and cell-free high-throughput screening assays have the potential to predict toxicity *in vivo* and prioritize chemicals for animal-based regulatory toxicity testing.¹ Across Phase I and Phase II of the ToxCast program, more than 1000 unique chemicals have been evaluated within 600–700 high-throughput assays that reflect a wide array of signaling pathways thought to be important for initiating and/or mediating chemical toxicity at the cellular level.² These data are now publicly available via the U.S. EPA's Interactive Chemical Safety for Sustainability (iCSS) Dashboard, allowing scientists, regulators, and the public to mine ToxCast and Tox21 data by assay type and/or chemical of interest. In addition, while currently unavailable via the iCSS Dashboard, the Toxicological Priority Index (ToxPi) Graphical User Interface (GUI) developed by Reif and colleagues is publicly available as a decision-support tool to visualize ToxCast data and aid in chemical prioritization.^{3,4}

Embryonic and larval zebrafish offer one of the most promising alternative and cost-effective models for predicting developmental toxicity and chemical mode of action within vertebrates. Indeed, as part of the ToxCast program, a zebrafish developmental toxicity assay has been used by the U.S. EPA to evaluate the potential human health and ecotoxicological effects of 320 ToxCast Phase I chemicals.⁵ In this assay, chorionated zebrafish embryos are exposed in 96-well plates under static-renewal conditions from 8 to 120 h postfertilization (hpf) and

then visually assessed for mortality and gross malformations at 144 hpf. Similarly, Truong and colleagues recently screened the potential toxicity of 1060 unique ToxCast chemicals to dechorionated zebrafish embryos within 96-well plates under static exposure conditions from 6 to 120 hpf.⁶ Results from these and other studies around the world^{7–10} highlight the potential value of embryonic zebrafish for identifying teratogens within large libraries of chemicals lacking baseline toxicity data. In addition, because of the transparency of zebrafish during embryonic development, a number of high-content, phenotype-driven screening assays using transgenic reporter lines have been developed to assess the potential for targeted chemical impacts on the development of the cardiovascular system,^{11,12} early nervous system,¹³ endocrine pancreas,¹⁴ pronephric kidney,¹⁵ and innate immune system¹⁶ at nonteratogenic concentrations. Unlike assays that rely on apical end points such as survival and gross malformations, these assays are equally important for evaluating targeted effects on organogenesis and classifying chemicals by toxicologically relevant modes of action.

To this end, using transgenic zebrafish (*fli1:egfp*) embryos that stably express enhanced green fluorescent protein within vascular endothelial cells, we previously leveraged two different 384-well high-content screening assays to screen a small subset of ToxCast Phase I chemicals for potential impacts on

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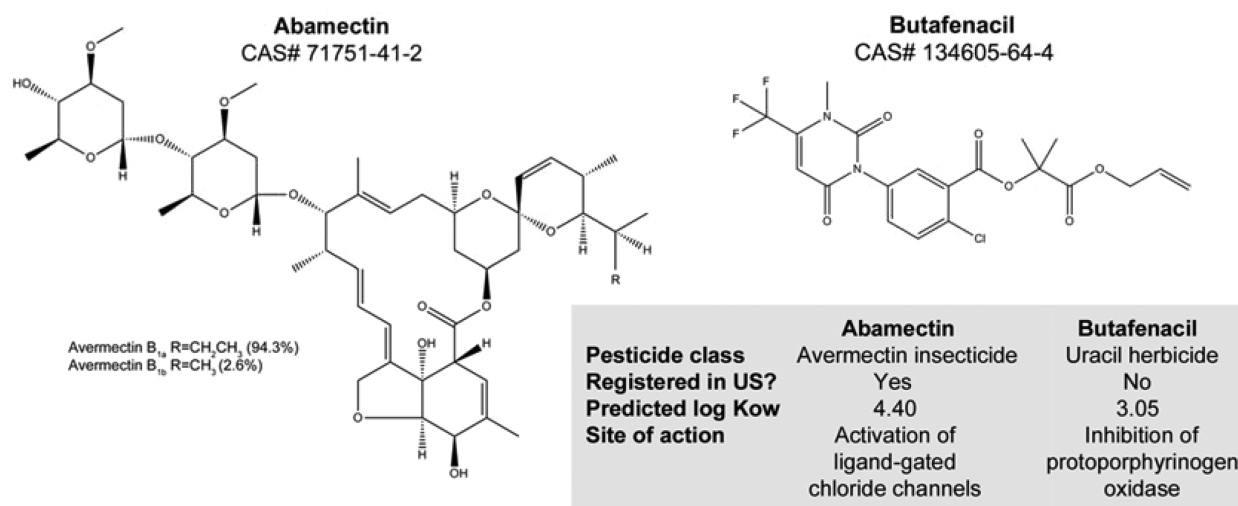


Figure 1. Baseline information for two pesticides (abamectin and butafenacil) previously identified as hits from two pilot screens of a subset of U.S. EPA's ToxCast Phase I chemical library. Abamectin and butafenacil were identified using our zebrafish-based high-content screening assays that evaluate potential impacts of chemical exposure on early nervous system and cardiovascular development, respectively.

cardiovascular and early nervous system development. On the basis of these two pilot screens, we demonstrated that (1) exposure to abamectin (CAS Registry No. 71751-41-2) from 5 to 25 hpf abolished spontaneous activity, an indicator of developmental neurotoxicity,^{17–20} in the absence of effects on survival and gross morphology¹³ and (2) exposure to butafenacil (CAS Registry No. 134605-64-4) from 5 to 72 hpf resulted in severe anemia (because of a complete loss of hemoglobin) in the absence of effects on cardiac function and vascular development²¹ (Figure 1). Abamectin is a broad-spectrum avermectin insecticide registered in the United States and many other countries within Europe and Asia for foliar applications on tree nuts, citrus, tree fruits, potatoes, and vegetables, as well as a seed treatment to control parasitic nematodes on cotton, corn, and soybeans. Butafenacil is a broad-spectrum, postemergent imide herbicide that is registered for agricultural use on cotton, citrus, and cereal grains within Australia, Argentina, Brazil, Japan, Switzerland, and Thailand. Within vertebrates, abamectin activates γ -amino-butyric acid (GABA)-gated chloride channels and induces paralysis,^{19,22} whereas butafenacil inhibits protoporphyrinogen oxidase (PPOX) and abolishes hemoglobin and red blood cell production.²¹ As seen in our findings in early stage zebrafish embryos, abamectin and butafenacil exposure resulted in behavioral and hematologic abnormalities, respectively, in adult rodent toxicity studies required for registration.^{23,24}

As ToxCast data were publicly available for the same Phase I chemicals screened within our high-content screening assays, the objective of this study was (1) to determine whether high-throughput *in vitro* screening data from the ToxCast program would have prioritized abamectin and butafenacil for further testing and (2) to determine whether a single three-day zebrafish embryo assay is a strong predictor of ToxPi scores derived from a large battery of ToxCast assays. Using ToxCast assay end point data and target information, we calculated assay hit rates, developed hazard classifications, and relied on the ToxPi GUI to generate ToxPi charts and scores within a biological process-driven configuration.

■ DATA SOURCES AND METHODS

Assay end point data for 1860 chemicals were downloaded from the U.S. EPA's iCSS Dashboard version 0.5 (<http://actor.epa.gov/dashboard/>) during January 2015 under the file name "Dashboard_Export_11_14_2014_09_35_59". In addition, assay target information was downloaded from the U.S. EPA's ToxCast Data Web site (<http://www.epa.gov/ncct/toxcast/data.html>) under the file name "ToxCast Assay Annotation Assay_Target_Info_20141021.csv". Assay end point data for 27 ToxCast Phase I chemicals previously screened in our laboratory^{11,13,21} were extracted, and assay target information was added to create a new file used for data mining (File 1 of the Supporting Information). Maximum tolerated concentrations (MTCs) for zebrafish embryo survival for the same 27 ToxCast Phase I chemicals were obtained from the Supporting Information of Yozzo et al.¹¹ and Leet et al.²¹ (File 2 of the Supporting Information). MTCs represent the highest concentration resulting in >70% zebrafish embryo survival following static exposure of one embryo per well within 384-well plates from 5 to 72 hpf.

Half-maximal activity concentrations (AC_{50}) for all 27 ToxCast Phase I chemicals (File 1 of the Supporting Information) were used to calculate assay hit rates, develop hazard classifications, and provide overall summary statistics. As the number of assay end points (range of 786–791) per chemical was not identical across all 27 chemicals, the percent assay hit rate for each chemical was defined as the number of assay end points with an AC_{50} of <1000 μ M, the maximal concentration tested and the basis for an "inactive" activity call, relative to the total number of assay end points per chemical. Likewise, for each chemical, assay end points with an AC_{50} of 1000 μ M were excluded prior to determining the percent of assay end points within each hazard classification (0.001–0.1, 0.1–10, or 10–200 μ M) and developing box plots to visualize the variance in AC_{50} s for "active" activity calls.

ToxPi charts and scores were generated using ToxPi GUI version 1.3 (<http://comptox.unc.edu/toxpi.php>). All assay end point data (including "inactive" activity calls) were organized by biological process (11 total) and reformatted to be compatible for the ToxPi GUI (File 3 of the Supporting Information). After data had been imported, 11 biological process slices of

equal weight (9.1% per slice) were added within the ToxPi GUI and $-\log_{10}(x) + \log_{10}(\max(x))$ scaling was applied to provide greater separation among inactive and active hits. Overall ToxPi scores were based on the sum of individual scores for 11 biological process slices. On the basis of this configuration, a larger slice was associated with increased chemical potency (lower AC_{50}) for assay sets representing a biological process.

RESULTS AND DISCUSSION

After “inactive” activity calls had been removed ($AC_{50} = 1000 \mu\text{M}$), assay hit rates across all 27 chemicals ranged from approximately 1 to 18%, with flufenpyr-ethyl and abamectin having the lowest and highest hit rates, respectively (Figure 2A). Twenty of 27 chemicals (or 74%) resulted in assay hit rates of <10%, and four of the seven remaining chemicals resulted in assay hit rates ranging from 14 to 18%. Butafenacil ranked 20th on the basis of this approach, with an assay hit rate of <5%. Following calculation of hit rates, assay end point data (AC_{50}) were either (1) binned into three different hazard classifications spanning 5 orders of magnitude of concentration (Figure 2B) or (2) summarized using box and whisker plots to visualize data distributions and outliers (Figure 2C). Chemicals within panels B and C of Figure 2 were ranked in the same order as in Figure 2A to determine whether higher assay hit rates were related to increased chemical potency (lower AC_{50} s) within assays with “active” activity calls. However, neither approach revealed a strong relationship even though the majority of “active” activity calls spanned AC_{50} s ranging from 1 to 50 μM (Figure 2C). Therefore, we developed ToxPi charts and scores to account for hit rate and chemical potency within a single metric as well as simultaneously to determine whether certain chemicals differentially impacted assay sets representing unique biological processes.

On the basis of output from the ToxPi GUI, 27 chemicals were ranked from highest (top left) to lowest (bottom right) ToxPi score within Figure 3. ToxPi scores across all 27 chemicals ranged from 0.44 to 9.06, with carfentrazone-ethyl and chlorothalonil having the lowest and highest ToxPi scores, respectively (Figure 3). On the basis of this approach, abamectin and butafenacil ranked fourth and 23rd on the basis of ToxPi scores of 5.537 and 1.198, respectively (Figure 3). Relative to assay hit rate alone (Figure 2A), the rank order changed for the top seven chemicals and tebufenpyrad (ToxPi score of 3.106) was replaced with dazomet (ToxPi score of 3.731). Nevertheless, consistent with Figure 2A, chlorothalonil (ToxPi score of 9.06) and thiram (ToxPi score of 6.419) remained within the top three chemicals with the highest ToxPi scores.

Within the configuration used within the ToxPi GUI, the hit rate across multiple assay sets, rather than the chemical potency within a smaller number of assay sets, was the primary driver for calculation of overall ToxPi scores. For example, clodinafop-propargyl strongly affected assay end points representing “regulation of catalytic activity” (Figure 3) but did not strongly affect assay end points representing the 10 remaining biological processes. As a result, clodinafop-propargyl was ranked 21st on the basis of the overall ToxPi score. Therefore, as a higher ToxPi score was driven by impacts on multiple biological processes, we hypothesized that MTCs for zebrafish embryo survival, an *in vivo* end point that integrates converging pathways of toxicity, would be predictive of ToxPi scores generated from ToxCast assay data. Indeed, there was a

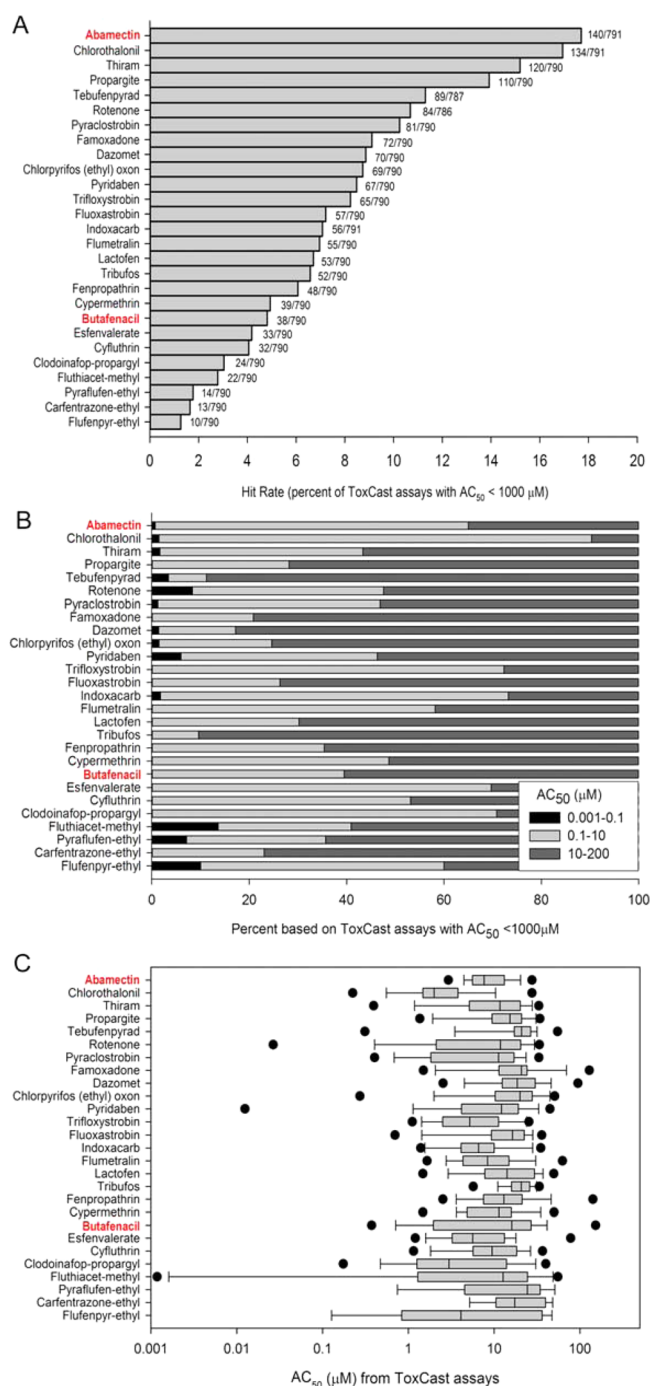


Figure 2. (A) Hit rate, (B) hazard classification, and (C) summary statistics based on AC_{50} s for 27 ToxCast Phase I chemicals screened within the ToxCast research program. Within panel A, chemicals are ranked from highest (top) to lowest (bottom) hit rate; chemicals within panels B and C are ranked in the same order as those in panel A. Abamectin and butafenacil are shown in red to highlight where these two chemicals ranked relative to the 25 remaining chemicals.

significant negative correlation ($p < 0.001$) between ToxPi scores and MTCs based on a linear regression in log MTC space, where lower log MTCs were strongly associated with higher ToxPi scores (Figure 4).

On the basis of our findings discussed above, this study has led to three major conclusions. First, although ToxPi scores are useful metrics that integrate ToxCast assay hit rates and chemical potency, the assay hit rate was the primary driver for

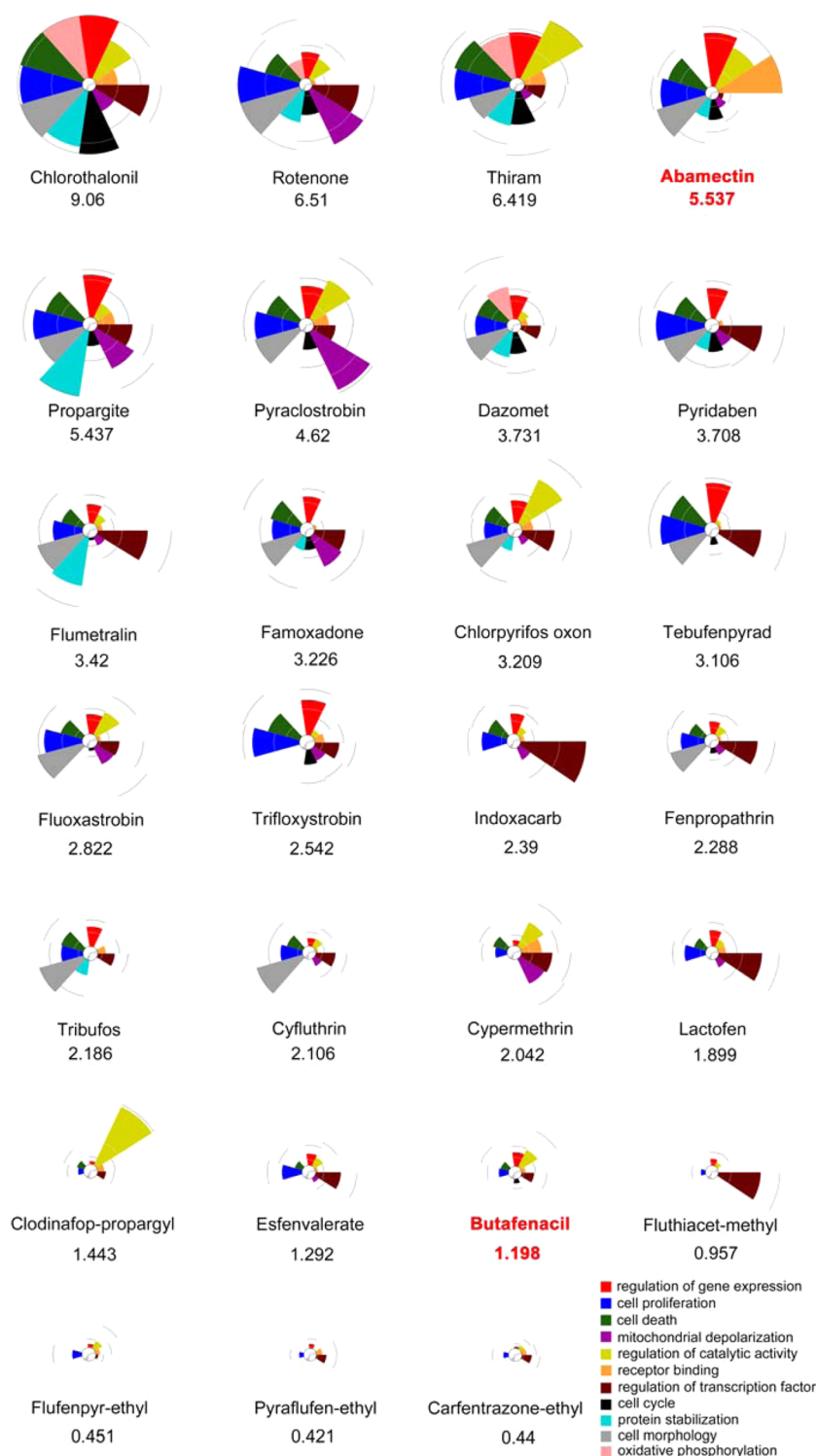


Figure 3. ToxPi charts and scores for 27 ToxCast Phase I chemicals based on biological processes assigned to assay end points. ToxPi charts are color-coded by biological process (11 total) and organized from the highest (top left) to lowest (bottom right) ToxPi score. Abamectin and butafenacil are shown in red to highlight where these two chemicals ranked relative to the 25 remaining chemicals.

generation of higher ToxPi scores within the ToxPi GUI. Second, because of the absence of ToxCast assays that identify PPOX inhibitors and/or chemicals affecting red blood cell production, butafenacil may not have been prioritized by the ToxCast program for future testing even though this chemical was identified as a potent inducer of anemia in zebrafish embryos. Lastly, on the basis of a single three-day assay using

one 384-well microplate, 72 hpf zebrafish embryo survival was a strong predictor of ToxPi scores derived from a large, complex battery of nearly 800 ToxCast assay end points.

Taken together, these conclusions suggest that ToxCast assays and the ToxPi GUI appear to prioritize acutely toxic chemicals that impact multiple biological pathways. Moreover, these conclusions suggest that, similar to conclusions by

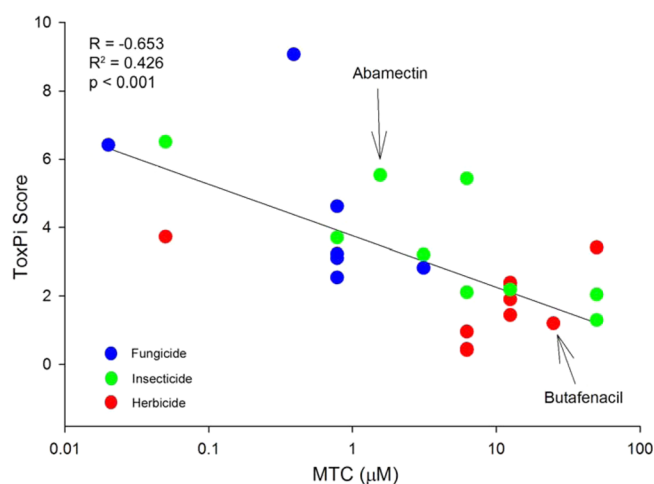


Figure 4. Correlation of ToxPi scores and maximal tolerated concentrations (MTCs) for zebrafish embryo survival for 27 ToxCast Phase I chemicals. MTCs represent the highest concentration resulting in >70% zebrafish embryo survival following a static exposure within 384-well plates from 5 to 72 hpf.

Truong and colleagues,⁶ a single three-day microplate assay using zebrafish embryo survival and development as apical end points may be used as an initial testing tier to help identify and prioritize chemicals for testing within the ToxCast program as well as “fit-for-purpose”, mode-of-action-driven assays using zebrafish embryos and larvae as well as other non-mammalian alternative models such as the nematode *Caenorhabditis elegans*.²⁵ In addition to their use streamlining the time and cost investments associated with ToxCast testing, zebrafish embryos that are ≤72 hpf are considered nonprotected life stages^{26–31} and, like cell-based and cell-free assays, are similarly defined as alternative testing models. Moreover, although organogenesis is incomplete at 72 hpf, longer-term screening assays using 96 to 144 hpf zebrafish, such as the Fish Embryo Toxicity test guideline (OECD Test 236) adopted in 2013, are low-throughput and more labor-intensive, requiring treatment solution renewals (and, therefore, more technical material) and excessive embryo/larval handling. Therefore, future research is needed to explore and leverage the utility and application of high-throughput screening assays with early stage zebrafish embryos for prioritization of chemicals for ToxCast testing.

■ ASSOCIATED CONTENT

Supporting Information

Assay end point data and target information for 27 ToxCast Phase I chemicals previously screened in our laboratory (File 1), chemical classifications and MTCs for zebrafish embryo survival for the same 27 ToxCast Phase I chemicals (File 2), and input file for the ToxPi GUI (File 3). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00123.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Dix, D. J.; Houck, K. A.; Martin, M. T.; Richard, A. M.; Setzer, R. W.; Kavlock, R. J. The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol. Sci.* **2007**, *95* (1), 5–12.
- (2) Kavlock, R.; Chandler, K.; Houck, K.; Hunter, S.; Judson, R.; Kleinstreuer, N.; Knudsen, T.; Martin, M.; Padilla, S.; Reif, D.; Richard, A.; Rotroff, D.; Sipes, N.; Dix, D. Update on EPA's ToxCast program: Providing high throughput decision support tools for chemical risk management. *Chem. Res. Toxicol.* **2012**, *25* (7), 1287–1302.
- (3) Reif, D. M.; Martin, M. T.; Tan, S. W.; Houck, K. A.; Judson, R. S.; Richard, A. M.; Knudsen, T. B.; Dix, D. J.; Kavlock, R. J. Endocrine profiling and prioritization of environmental chemicals using ToxCast data. *Environ. Health Perspect.* **2010**, *118* (12), 1714–1720.
- (4) Reif, D. M.; Sypa, M.; Lock, E. F.; Wright, F. A.; Wilson, A.; Cathey, T.; Judson, R. R.; Rusyn, I. ToxPi GUI: An interactive visualization tool for transparent integration of data from diverse sources of evidence. *Bioinformatics* **2013**, *29* (3), 402–403.
- (5) Padilla, S.; Corum, D.; Padnos, B.; Hunter, D. L.; Beam, A.; Houck, K. A.; Sipes, N.; Kleinstreuer, N.; Knudsen, T.; Dix, D. J.; Reif, D. M. Zebrafish developmental screening of the ToxCast Phase I chemical library. *Reprod. Toxicol.* **2012**, *33* (2), 174–187.
- (6) Truong, L.; Reif, D. M.; St Mary, L.; Geier, M. C.; Truong, H. D.; Tanguay, R. L. Multidimensional in vivo hazard assessment using zebrafish. *Toxicol. Sci.* **2014**, *137* (1), 212–233.
- (7) Ducharme, N. A.; Peterson, L. E.; Benfenati, E.; Reif, D.; McCollum, C. W.; Gustafsson, J. A.; Bondesson, M. Meta-analysis of toxicity and teratogenicity of 133 chemicals from zebrafish developmental toxicity studies. *Reprod. Toxicol.* **2013**, *41*, 98–108.
- (8) Pardo-Martin, C.; Allalou, A.; Medina, J.; Eimon, P. M.; Wahlby, C.; Fatih Yanik, M. High-throughput hyperdimensional vertebrate phenotyping. *Nat. Commun.* **2013**, *4*, 1467.
- (9) Yamashita, A.; Inada, H.; Chihara, K.; Yamada, T.; Deguchi, J.; Funabashi, H. Improvement of the evaluation method for teratogenicity using zebrafish embryos. *J. Toxicol. Sci.* **2014**, *39* (3), 453–464.
- (10) Ali, S.; Aalders, J.; Richardson, M. K. Teratological effects of a panel of sixty water-soluble toxicants on zebrafish development. *Zebrafish* **2014**, *11* (2), 129–141.
- (11) Yozzo, K. L.; Isales, G. M.; Raftery, T. D.; Volz, D. C. High-content screening assay for identification of chemicals impacting cardiovascular function in zebrafish embryos. *Environ. Sci. Technol.* **2013**, *47* (19), 11302–11310.
- (12) Tran, T. C.; Sneed, B.; Haider, J.; Blavo, D.; White, A.; Aiyegoro, T.; Baranowski, T. C.; Rubinstein, A. L.; Doan, T. N.; Dingledine, R.; Sandberg, E. M. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res.* **2007**, *67* (23), 11386–11392.
- (13) Raftery, T. D.; Isales, G. M.; Yozzo, K. L.; Volz, D. C. High-content screening assay for identification of chemicals impacting spontaneous activity in zebrafish embryos. *Environ. Sci. Technol.* **2014**, *48* (1), 804–810.
- (14) Tsuji, N.; Ninov, N.; Delawary, M.; Osman, S.; Roh, A. S.; Gut, P.; Stainier, D. Y. Whole organism high content screening identifies stimulators of pancreatic β -cell proliferation. *PLoS One* **2014**, *9* (8), e104112.
- (15) Westhoff, J. H.; Giselsbrecht, S.; Schmidts, M.; Schindler, S.; Beales, P. L.; Tonshoff, B.; Liebel, U.; Gehrig, J. Development of an automated imaging pipeline for the analysis of the zebrafish larval kidney. *PLoS One* **2013**, *8* (12), e82137.
- (16) d'Alencon, C. A.; Pena, O. A.; Wittmann, C.; Gallardo, V. E.; Jones, R. A.; Loosli, F.; Liebel, U.; Grabher, C.; Allende, M. L. A high-throughput chemically induced inflammation assay in zebrafish. *BMC Biol.* **2010**, *8*, 151.

(17) Selderslaghs, I. W.; Hooyberghs, J.; Blust, R.; Witters, H. E. Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryos and larvae. *Neurotoxicol. Teratol.* **2013**, *37*, 44–56.

(18) Kokel, D.; Bryan, J.; Laggner, C.; White, R.; Cheung, C. Y.; Mateus, R.; Healey, D.; Kim, S.; Werdich, A. A.; Haggarty, S. J.; Macrae, C. A.; Shoichet, B.; Peterson, R. T. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat. Chem. Biol.* **2010**, *6* (3), 231–237.

(19) Raftery, T. D.; Volz, D. C. Abamectin induces rapid and reversible hypoactivity within early zebrafish embryos. *Neurotoxicol. Teratol.* **2015**, *49*, 10–18.

(20) Noyes, P. D.; Haggard, D. E.; Gonnerman, G. D.; Tanguay, R. L. Advanced morphological-behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. *Toxicol. Sci.* **2015**, *145* (1), 177–195.

(21) Leet, J. K.; Lindberg, C. D.; Bassett, L. A.; Isales, G. M.; Yozzo, K. L.; Raftery, T. D.; Volz, D. C. High-content screening in zebrafish embryos identifies butafenacil as a potent inducer of anemia. *PLoS One* **2014**, *9* (8), e104190.

(22) Bloomquist, J. R. Ion channels as targets for insecticides. *Annu. Rev. Entomol.* **1996**, *41*, 163–190.

(23) Abamectin Registration Review Docket: ID#: EPA-HQ-OPP-2013-0360-0003. Supporting and Related Material: Abamectin. Human Health Assessment Scoping Document in Support of Registration Review. H.E.D. Risk Assessment Branch III, Office of Pesticide Programs, U.S. Environmental Protection Agency: Washington, DC, 2013.

(24) Evaluation of the new active butafenacil in the products Logran B-Power Herbicide & Touchdown B-Power Herbicide. National Registration Authority for Agricultural and Veterinary Chemicals: Canberra, Australia, 2002.

(25) Boyd, W. A.; McBride, S. J.; Rice, J. R.; Snyder, D. W.; Freedman, J. H. A high-throughput method for assessing chemical toxicity using a *Caenorhabditis elegans* reproduction assay. *Toxicol. Appl. Pharmacol.* **2010**, *245* (2), 153–159.

(26) Volz, D. C.; Belanger, S.; Embry, M.; Padilla, S.; Sanderson, H.; Schirmer, K.; Scholz, S.; Villeneuve, D. Adverse outcome pathways during early fish development: A conceptual framework for identification of chemical screening and prioritization strategies. *Toxicol. Sci.* **2011**, *123* (2), 349–358.

(27) Embry, M. R.; Belanger, S. E.; Braunbeck, T. A.; Galay-Burgos, M.; Halder, M.; Hinton, D. E.; Leonard, M. A.; Lillicrap, A.; Norberg-King, T.; Whale, G. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. *Aquat. Toxicol.* **2010**, *97* (2), 79–87.

(28) Halder, M.; Leonard, M.; Iguchi, T.; Oris, J. T.; Ryder, K.; Belanger, S. E.; Braunbeck, T. A.; Embry, M. R.; Whale, G.; Norberg-King, T.; Lillicrap, A. Regulatory aspects on the use of fish embryos in environmental toxicology. *Integr. Environ. Assess. Manage.* **2010**, *6* (3), 484–491.

(29) Braunbeck, T.; Kais, B.; Lammer, E.; Otte, J.; Schneider, K.; Stengel, D.; Strecker, R. The fish embryo test (FET): origin, applications, and future. *Environ. Sci. Pollut. Res.* **2014**, DOI: 10.1007/s11356-014-3814-7.

(30) Belanger, S. E.; Rawlings, J. M.; Carr, G. J. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. *Environ. Toxicol. Chem.* **2013**, *32* (8), 1768–1783.

(31) Strahle, U.; Scholz, S.; Geisler, R.; Greiner, P.; Hollert, H.; Rastegar, S.; Schumacher, A.; Selderslaghs, I.; Weiss, C.; Witters, H.; Braunbeck, T. Zebrafish embryos as an alternative to animal experiments: A commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reprod. Toxicol.* **2012**, *33* (2), 128–132.