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Detection and Prioritization of Developmentally Neurotoxic and/or Neurotoxic Compounds Using Zebrafish

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ABSTRACT

The standard methods for toxicity testing using rodent models cannot keep pace with the increasing number of chemicals in our environment due to time and resource limitations. Hence, there is an unmet need for fast, sensitive, and cost-effective alternate models to reliably predict toxicity. As part of Tox21 Phase III's effort, a 90-compound library was created and made available to researchers to screen for neurotoxicants using novel technology and models. The chemical library was evaluated in zebrafish in a dose-range finding test for embryo-toxicity (ie, mortality or morphological alterations induced by each chemical). In addition, embryos exposed to the lowest effect level and nonobservable effect level were used to measure the internal concentration of the chemicals within the embryos by bioanalysis. Finally, considering the lowest effect level as the highest testing concentration, a functional assay was performed based on locomotor activity alteration in response to light-dark changes. The quality control chemicals included in the library, ie, negative controls and replicated chemicals, indicate that the assays performed were reliable. The use of analytical chemistry pointed out the importance of measuring chemical concentration inside embryos, and in particular, in the case of negative chemicals to avoid false negative classification. Overall, the proposed approach presented a good sensitivity and supports the inclusion of zebrafish assays as a reliable, relevant, and efficient screening tool to identify, prioritize, and evaluate chemical toxicity.

Key words: zebrafish; toxicity screening; malformations; internal concentration; neurotoxicity; prioritization.

There is an increasing number of chemicals in our food, drinking water, air, and soil and some chemicals are becoming worldwide pollutants. Although the risks they pose may be significant, there is little understanding of the health effects of many common chemicals. Since 1950, more than 140 000 new chemicals have been synthesized, of which around 5000 are now ubiquitous in the environment (Gruber, 2018). In 2015, pollution was estimated to have caused almost 9 million deaths worldwide—three times more than from AIDS, tuberculosis, and malaria combined (Gruber, 2018). Long-term exposure to some chemicals can increase the risk of developmental and reproductive disorders, immune-system disruption, endocrine disruption, development of certain cancers, and impaired nervous system function (Yáñez *et al.*, 2002). These facts highlight the importance of performing a comprehensive toxicological profiling of newly manufactured chemicals.

In vitro assays lack the complexity of in vivo systems and cannot easily address metabolism or systemic defects (Roper *et al.*, 2018). On the other hand, although rodents have been used extensively to understand toxicity, screening in rodents is time consuming and expensive (Krewski *et al.*, 2010; National Research Council, 2007; Nishimura *et al.*, 2015). There is an

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unmet need for fast, sensitive, and inexpensive in vivo models that act as safety testing platforms to reliably predict toxicity (Pham *et al.*, 2016). In this line, The National Toxicology Program (NTP), as part of Tox21 Phase III's effort to "Improve on Biological Coverage and Human Relevance," created a 90-compound library of known developmental neurotoxicants (DNTs), and adult neurotoxicants (NTs), as well as compounds of interest to the NTP with unknown DNT or NT activity [eg, flame retardants (FRs), polycyclic aromatic hydrocarbons (PAHs)]. As part of a large collaborative project that was initiated by the NTP to evaluate novel methods to screen for potential NTs, Biobide used morphological and functional endpoints in a zebrafish model as an alternative animal system for the library screening.

The advantages of the zebrafish include their low cost and ease of maintenance and breeding. Zebrafish have external fertilization and can produce hundreds of offspring weekly providing researchers with an abundant supply of embryos to study. Moreover, the zebrafish model is ideal for research purposes due to its small size which allows zebrafish to be plated in standard microplates (6-, 24-, 96-well plates). In addition, they can be handled easily and compound exposure is usually carried out by immersion of fish directly into the media. Their transparency permits visualization of some organs and internal structures using only a simple stereoscope. Importantly, the results of the zebrafish screens show good correlation to mammalian models of toxicology and supports the utility of the zebrafish model in toxicology research (Horzmann and Freeman, 2018). These features allow zebrafish to be a suitable model for screening of toxic profiles of chemicals. Finally, the use of zebrafish larvae is in accordance with the 3R principles because it is considered an alternative model in embryonic stages and minimizes the use of mammals (Bartlett and Silk, 2016; Ducharme *et al.*, 2015).

Behavior can be measured as an approach to determine the functional impact of a chemical exposure in zebrafish itself as an aquatic vertebrate as well as an animal model to predict the possible impact on humans (Bailey *et al.*, 2013; Ek *et al.*, 2016). Zebrafish show spontaneous swimming behavior beginning 3 days post fertilization (dpf) on which allows assessment of locomotor activity. Moreover, embryo tracking systems enable automated analysis of the locomotor activity under different conditions. This approach is useful to identify new drug leads, as well as to detect undesirable effects of chemicals. A common behavioral assay for zebrafish larvae activity analysis consists of tracking larvae movement within a 96-well plate while modifying environmental conditions, such as alternating light and dark phases (Emran *et al.*, 2008; Selderslaghs *et al.*, 2013). Untreated wild-type larvae show basal activity during light periods and when the light is switched off, they react to sudden darkness with an increase in moving distance and velocity. This behavior is believed to be linked to an escape response to a predator that is approaching and is determined not only by neurons but also by the muscular system (Pham *et al.*, 2016).

In this work, we evaluate the reliability and relevance of the use of zebrafish assays to prioritize compounds for further in vivo toxicity testing in rodents as part of Tox21 Phase III's effort. We thoroughly examined morphological alterations in zebrafish as well as locomotor activity alterations in response to light-dark changes induced by these chemicals during developmental exposure. In addition, chemical concentration within the embryo was measured to better understand toxicity related to internal concentrations.

MATERIALS AND METHODS

Subjects. Adult zebrafish from wild-type AB strain and Tg(Cmlc2: copGFP) (transgenic line expressing CopGFP under the myocardium specific promoter cmlc2, Letamendia *et al.*, 2012) were housed and maintained in accordance with standard procedures as described in Alzualde *et al.* (2018). Briefly, fish from both lines were maintained under a photoperiod of 14:10 h light: dark at 28.5°C in water continuously filtered at pH 7–7.8, conductivity 500–800 μS and O_2 saturation at 80%–100%. Adults were fed with both ground dry pellets (Gemma 300, Skretting) and artemia (Catvis) twice a day. Healthy mature zebrafish pairs were used for egg production. The day before the embryos were needed, adults were placed in spawning tanks, 2 females and 2 males per tank separated by a vertical divider. A total of 120 couples per experiment were used for embryo-toxicity assay. The following morning the dividers were removed and 1 h later synchronized eggs from different spawning tanks were pooled and placed in 140 mm petri dishes, 200 embryos per plate. Embryos were collected in E3 embryo media spiked with 0.0001% methylene blue (Acros Organics, >96% purity) and 100 μg/ml ampicillin (Sigma-Aldrich) and kept in the incubator at 28.5°C until they reached the appropriate stage (4 hours post fertilization[hpf] for embryo-toxicity assay and 3 dpf for neurotoxicity assay). Transgenic embryos were used for embryo-toxicity assay to facilitate the detection of the heart function alteration while embryos from wild-type AB strain were used for the neurotoxicity assay. Both zebrafish lines have a similar genetic background, because the transgenics were generated in the AB strain. Our experience with these strains suggests that their response to the chemical exposure is comparable (data not shown).

Zebrafish were maintained in accordance with the European Directive (2010/63/EU) for the protection of animals used for scientific purposes and all experiments were approved by the ethical committee for animal experimentation IIS Biodonostia (San Sebastián, Gipuzkoa, Spain).

Chemicals. Dimethyl sulfoxide (DMSO) (CAS 67-68-5, purity 99.9%) (vehicle control) and the 90 testing compounds were provided blinded to Biobide by the NTP (Table 1). They were sent dissolved in DMSO at 20 mM unless it is stated differently. Stock solutions of each chemical were diluted in DMSO and further diluted to the desired final concentration in E3 media containing 10 mM HEPES (Sigma-Aldrich). The evaluation of chemical toxicity was performed blinded to their identity. Following data analysis and obtaining results, they were decoded and classified in the following NTP categories: drug, FR, industrial, PAHs, pesticide, and negative control. The NTP compound library contains a variety of chemicals, some of which are suspected DNTs and/or NTs. This NTP compound library contains 90 chemicals (86 unique + 4 duplicates). Each chemical was either developmental neuro-, or neuro-toxicant, or a compound with unknown effects on development or neuronal function (Table 1).

Embryo-toxicity assay. To determine concentrations for the main study, a dose-range finding (DRF) study was first conducted at 5 concentrations ranging from 0.2 to 100 μM to maintain final DMSO concentration at 0.5% as described in Alzualde et al. (2018). Briefly, fertilized Tg(Cmlc2: copGFP) embryos at 4 hpf were placed in 24-well plates with the corresponding chemical concentration. The plates were covered and wrapped with aluminum foil to avoid degradation of light-sensitive compounds. Ten embryos were analyzed per condition after 2 and 4 days of

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Table 1. The List of Chemicals Tested in This Work With the Corresponding Classification and CAS Number

Chemical Name	Classification	CAS	Received Conc. (mM)	Testing Concentration Developmental Toxicity (μM)	Testing Concentration Neurotoxicity (μM)	BA Method
1-Ethyl-3-methylimidazolium diethylphosphate	Industrial	848641-69-0	20.2	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
1-Methyl-4-phenylpyridinium iodide	Drug*	36913-39-0	18.8	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
2-Ethylhexyl diphenyl phosphate (EHDP)	FR	1241-94-7	20.2	1, 2, 3, 6, 10, 15, 20, 40	1, 2, 3, 6, 10	GC/MS
2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB)	FR	183658-27-7	20.0	10, 15, 30, 60, 100	10, 15, 30, 60, 100	GC/MS
2-Methoxyethanol	Industrial*	109-86-4	20.9	10, 15, 30, 60, 100	10, 15, 30, 60, 100	P&T-GC/MS
2,2',4,4',5-Pentabromodiphenyl ether (BDE-99)	FR	60348-60-9	20.0	5, 10, 20, 50, 100	5, 10, 20, 50, 100	GC/MS
2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE-153)	FR	68631-49-2	10.0	5, 10, 20, 50, 100	2, 5, 10, 25, 50	GC/MS
2,2',4,4'-Tetrabromodiphenyl ether	FR	5436-43-1	20.0	5, 10, 20, 30, 50, 75, 100	1, 2, 5, 10, 20	GC/MS
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Industrial	1746-01-6	0.1	0.0001, 0.0003, 0.001, 0.002, 0.005, 0.01, 0.03, 0.1	0.000025, 0.00005, 0.0001, 0.00015, 0.0003	GC/MS
3,3'-Iminodipropionitrile	Industrial*	111-94-4	20.4	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
3,3',5,5'-Tetrabromobisphenol A	FR	79-94-7	20.0	0.5, 1, 1.5, 2, 3, 5, 7.5, 10	0.15, 0.3, 0.6, 1, 1.5	GC/MS
4-H-Cyclopenta(d,e,f)phenanthrene	PAH	203-64-5	20.4	1, 2, 4, 8, 15, 30, 60, 100	1, 2, 3, 6, 10	GC/MS
5-Fluorouracil	Drug*	51-21-8	20.6	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
6-Hydroxydopamine hydrochloride	Drug*	28094-15-7	20.1	5, 10, 20, 50, 100	5, 10, 20, 50, 100	LC/MS
6-Propyl-2-thiouracil	Drug*	51-52-5	20.5	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Acenaphthene	PAH	83-32-9	20.4	5, 10, 15, 20, 30, 50, 75, 100	2, 5, 10, 15, 30	GC/MS
Acenaphthylene	PAH	208-96-8	20.1	5, 10, 15, 20, 30, 50, 75, 100	1, 2, 4, 8, 15	GC/MS
Acetaminophen (4-hydroxyacetanilide)	Negative	103-90-2	20.6	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Acetic acid, manganese (2+) salt	Industrial*	638-38-0	19.6	10, 15, 30, 60, 100	10, 15, 30, 60, 98	ICP-MS
Acetylsalicylic acid	Negative	50-78-2	19.9	10, 15, 30, 60, 100	10, 15, 30, 60, 99.5	LC/MS
Acrylamide	Industrial*	79-06-1	20.1	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Aldicarb	Pesticide*	116-06-3	20.2	0.2, 0.5, 1, 2, 5, 10, 30, 100	0.1, 0.2, 0.3, 0.6, 1	LC/MS
Amoxicillin	Drug	26787-78-0	17.1	10, 15, 30, 60, 100	10, 15, 30, 60, 85.5	LC/MS
Anthracene	PAH	120-12-7	19.9	10, 15, 30, 60, 100	5, 10, 20, 50, 100	GC/MS
Auramine O	Drug	2465-27-2	19.8	1, 1.5, 2, 3, 5, 7.5, 10, 20	0.5, 1, 2, 3, 5	LC/MS
Benz(a)anthracene	PAH	56-55-3	20.1	1, 2, 4, 8, 15, 30, 60, 100	0.25, 0.5, 1, 2, 4	GC/MS
Benzo(a)pyrene	PAH	50-32-8	20.3	5, 10, 15, 20, 30, 50, 75, 100	5, 10, 20, 50, 100	GC/MS
Benzo(b)fluoranthene	PAH	205-99-2	19.6	1, 2, 4, 8, 15, 30, 60, 100	5, 10, 20, 50, 98	GC/MS
Benzo(e)pyrene	PAH	192-97-2	19.9	1, 2, 3, 6, 10, 15, 20, 40	5, 10, 20, 50, 99.5	GC/MS
Benzo(k)fluoranthene	PAH	207-08-9	19.8	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10	0.05, 0.1, 0.2, 0.3, 0.5	GC/MS
Benzo[g, h, i]perylene	PAH	191-24-2	0.8	1, 2, 3, 5, 8	0.5, 1, 1.5, 2.4, 4	GC/MS
Berberine chloride	Drug	633-65-8	20.1	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Bis(2-ethylhexyl) 3,4,5,6-tetrabromophthalate (TBPH)	FR	26040-51-7	20.0	5, 10, 20, 50, 100	5, 10, 20, 50, 100	GC/MS
Bis(tributyltin)oxide	Pesticide*	56-35-9	1.0	0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1	0.005, 0.01, 0.025, 0.05, 0.1	LC/MS

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Table 1. (continued)

Chemical Name	Classification	CAS	Received Conc. (mM)	Testing Concentration Developmental Toxicity (μM)	Testing Concentration Neurotoxicity (μM)	BA Method
Bisphenol A	Industrial*	80-05-7	20.0	5, 10, 15, 20, 30, 50, 75, 100	1, 2, 5, 10, 20	LC/MS
Bisphenol AF	Industrial	1478-61-1	19.9	1, 1.5, 2, 3, 5, 7.5, 10, 20	0.1, 0.2, 0.5, 1, 3	LC/MS
Bisphenol S	Industrial	80-09-1	20.1	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Captan	Pesticide	58-08-2	19.9	5, 10, 15, 20, 30, 50, 75, 100	2.5, 5, 10, 25, 50	GC/MS
Carbamic acid, butyl-, 3-iodo-2-propynyl ester	Pesticide	133-06-2	19.9	0.5, 1, 1.5, 2, 3, 5, 7.5, 10	0.05, 0.1, 0.25, 0.5, 1	GC/MS
Carbaryl	Pesticide*	63-25-2	19.8	1, 2, 4, 8, 15, 30, 60, 100	0.25, 0.5, 1, 2, 4	LC/MS
Chlorpyrifos (Dursban)	Pesticide*	2921-88-2	20.0	1, 2, 5, 7.5, 10, 15, 30, 60, 100	0.5, 1, 2, 3, 5	GC/MS
Chrysene	PAH	218-01-9	9.7	10, 15, 30, 60, 97	2, 5, 10, 25, 48.5	GC/MS
Colchicine	Drug*	64-86-8	19.8	5, 10, 15, 20, 30, 50, 75, 100	5, 10, 25, 50, 99	LC/MS
D-Glucitol	Negative	50-70-4	20.2	10, 15, 30, 60, 100	5, 10, 20, 50, 100	LC/MS
Deltamethrin	Pesticide*	52918-63-5	21.0	0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1	0.0025, 0.005, 0.01, 0.025, 0.05	GC/MS
Di(2-ethylhexyl) phthalate	Industrial*	117-81-7	20.0	10, 15, 30, 60, 100	5, 10, 20, 50, 100	GC/MS
Diazepam	Drug*	439-14-5	20.3	1, 2, 4, 8, 15, 30, 60, 100	1, 2, 4, 8, 15	LC/MS
Dibenz(a,h)anthracene	PAH	53-70-3	10.1	0.2, 0.5, 1, 2, 5, 10, 20, 50	0.1, 0.2, 0.5, 1, 2	GC/MS
Dibenz[a,c]anthracene	PAH	215-58-7	19.9	5, 10, 20, 50, 100	5, 10, 20, 50, 99.5	GC/MS
Dichlorodiphenyltrichloroethane (DDT)	Pesticide*	50-29-3	20.1	1, 2, 4, 8, 15, 30, 60, 100	0.2, 0.5, 1, 2, 4	GC/MS
Dieldrin	Pesticide*	60-57-1	20.0	0.2, 0.5, 1, 2, 5, 10, 20, 50	0.01, 0.03, 0.1, 0.3, 1	GC/MS
Diethylstilbestrol	Drug*	56-53-1	19.9	0.2, 0.5, 1, 2, 3, 6, 10, 20	0.05, 0.1, 0.25, 0.5, 1	LC/MS
Estradiol	Drug	50-28-2	20.0	5, 10, 15, 20, 30, 50, 75, 100	0.5, 1, 2.5, 5, 10	LC/MS
Firemaster 550	FR	860302-33-6	20.0	0.2, 0.5, 1, 2, 5, 10, 30, 100	0.1, 0.25, 0.5, 1, 2	LC/MS
Fluorene	PAH	86-73-7	20.0	5, 10, 15, 20, 30, 50, 75, 100	2, 4, 8, 15, 30	GC/MS
Heptachlor	Pesticide*	76-44-8	20.0	0.5, 1, 2, 4, 8, 15, 30, 60	0.5, 1, 2, 4, 8	GC/MS
Hexachlorophene	Drug*	70-30-4	20.0	0.1, 0.15, 0.2, 0.3, 0.5, 0.75, 1, 2	0.02, 0.05, 0.1, 0.25, 0.5	GC/MS
Hydroxyurea	Drug*	127-07-1	20.2	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Isodecyl diphenyl phosphate	FR	29761-21-5	20.1	5, 10, 15, 20, 30, 50, 75, 100	2.5, 5, 10, 25, 50	LC/MS
L-Ascorbic acid	Negative	50-81-7	20.0	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Lead (II) acetate trihydrate	Industrial*	6080-56-4	20.1	0.2, 0.5, 1, 2, 5, 10, 20, 50	0.2, 0.5, 1, 2.5, 5	ICP-MS
Lindane	Pesticide*	58-89-9	19.9	1, 2, 4, 8, 15, 30, 60, 100	0.25, 0.5, 1, 2, 4	GC/MS
Manganese, tricarbonyl[(1,2,3,4,5-.eta.)-1-methyl-2, 4-cyclopentadien-1-yl]-	Industrial*	12108-13-3	20.0	10, 15, 30, 60, 100	10, 15, 30, 60, 100	GC/MS
Methyl mercuric (II) chloride	Pesticide*	115-09-3	20.0	0.05, 0.1, 0.15, 0.3, 0.6, 1, 2 y 5	0.05, 0.1, 0.25, 0.5, 1	ICP-MS
n-Hexane	Industrial*	110-54-3	20.0	5, 10, 15, 20, 30, 50, 75, 100	5, 10, 25, 50, 100	P&T-GC/MS
Naphthalene	PAH	91-20-3	19.9	5, 10, 15, 20, 30, 50, 75, 100	5, 10, 25, 50, 99.5	GC/MS
Parathion	Pesticide*	56-38-2	19.7	1, 2, 4, 8, 15, 30, 60, 100	1, 2, 4, 8, 15	GC/MS
Permethrin	Pesticide*	52645-53-1	20.1	0.2, 0.5, 1, 2, 5, 10, 30, 100	0.1, 0.2, 0.5, 1, 2	GC/MS
Phenanthrene	PAH	85-01-8	19.6	5, 10, 15, 20, 30, 50, 75, 100	1, 2, 4, 8, 15	GC/MS
Phenobarbital	Drug*	50-06-6	20.4	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Phenobarbital sodium salt	Drug*	57-30-7	20.1	5, 10, 15, 20, 30, 50, 75, 100	5, 10, 25, 50, 100	LC/MS

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Table 1. (continued)

Chemical Name	Classification	CAS	Received Conc. (mM)	Testing Concentration Developmental Toxicity (μM)	Testing Concentration Neurotoxicity (μM)	BA Method
Phenol, isopropylated, phosphate (3:1)	FR	68937-41-7	20.1	0.1, 0.2, 0.5, 1, 2, 5, 10, 20	0.0025, 0.005, 0.1, 0.25, 0.5	LC/MS
Pyrene	PAH	129-00-0	20.1	1, 2, 4, 8, 15, 30, 60, 100	0.1, 0.2, 0.5, 1, 2	GC/MS
Rotenone	Pesticide*	83-79-4	20.2	0.01, 0.02, 0.05, 0.075, 0.1, 0.15, 0.2, 0.4	0.005, 0.01, 0.025, 0.05, 0.1	LC/MS
Saccharin sodium salt hydrate	Negative	82385-42-0	20.2	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Tebuconazole	Pesticide*	107534-96-3	20.2	5, 10, 15, 20, 30, 50, 75, 100	1, 2.5, 5, 10, 20	LC/MS
tert-Butylphenyl diphenyl phosphate	FR	56803-37-3	20.1	1, 2, 4, 8, 15, 30, 60, 100	0.5, 1, 2, 4, 8	LC/MS
Tetraethylthiuram disulfide	Drug*	97-77-8	20.0	0.01, 0.02, 0.05, 0.1, 0.3, 1, 2, 5	0.01, 0.025, 0.05, 0.1, 0.3	GC/MS
Thalidomide	Drug*	50-35-1	20.0	5, 10, 15, 20, 30, 50, 75, 100	10, 15, 30, 60, 100	LC/MS
Toluene	Industrial*	108-88-3	19.7	10, 15, 30, 60, 100	10, 15, 30, 60, 98.5	P&T-GC/MS
Tricresyl phosphate	FR	1330-78-5	20.0	5, 10, 15, 20, 30, 50, 75, 100	0.5, 1, 2, 5, 10	GC/MS
Triphenyl phosphate	FR	115-86-6	19.9	0.1, 0.2, 0.5, 1, 2, 5, 10, 20	0.1, 0.25, 0.5, 1, 2	LC/MS
Tris(2-chloroethyl) phosphate	FR	115-96-8	20.1	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Valinomycin	Drug	2001-95-8	19.9	0.01, 0.02, 0.05, 0.075, 0.1, 0.15, 0.2, 0.4	0.005, 0.01, 0.25, 0.05, 0.075	LC/MS
Valproic acid sodium salt	Drug*	1069-66-5	20.1	5, 10, 15, 20, 30, 50, 75, 100	5, 10, 25, 50, 75	LC/MS
Tris(chloropropyl) phosphate (TCPP)	FR	13674-84-5	20.0	5, 10, 15, 20, 30, 50, 75, 100	5, 10, 25, 50, 75	LC/MS
Deltamethrin	Pesticide*	52918-63-5	21.0	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2	0.005, 0.01, 0.025, 0.05, 0.1	GC/MS
Methyl mercuric (II) chloride	Pesticide*	115-09-3	20.0	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10	0.01, 0.05, 0.1, 0.2, 0.5	ICP-MS
Saccharin sodium salt hydrate	Negative	82385-42-0	20.2	10, 15, 30, 60, 100	10, 15, 30, 60, 100	LC/MS
Triphenyl phosphate	FR	115-86-6	19.9	0.2, 0.5, 1, 2, 5, 10, 20, 50	0.1, 0.25, 0.5, 1, 2	LC/MS

Concentration at which each chemical was received is also indicated as well as the testing concentrations of embryo-toxicity and neurotoxicity assays. Bioavailability (BA) analysis method is also stated. Chemicals suspected to be DNT/NT are indicated with an asterisk (*) in the Classification column.

Abbreviations: LC/MS, liquid chromatography/mass spectrometry; GC/MS, gas chromatography/mass spectrometry; P&T-GC/MS, purge and trap GC/MS; ICP-MS, inductively coupled plasma mass spectrometry.

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incubation at 28.5°C and the incidence of lethality and the presence of obvious developmental defects were checked under a stereo microscope (Olympus SXZ10) by experienced technicians.

Following the DRF, embryo-toxicity experiments were carried out and embryos were treated with 8 concentrations (or 5 concentrations if no toxicity was detected in DRF study) as described in Alzualde et al. (2018). Embryos were treated in a similar manner as described in the DRF above with the exception that a total of 15 embryos (instead of 10) were tested per experimental condition. Detailed analysis of embryo morphology (including malformations in the head, heart and tail, deformed body shape, and the presence of edemas) and lethality was performed at 2 and 4 dpf. Embryo morphology was visualized under a stereo microscope (Olympus SXZ10) by experienced technicians. Next the lowest effect level (LEL) was determined based on a threshold percentage: if the percentage of malformed embryos was more than 20% it was considered that the compound induced an effect. A no-observed effect level (NOEL) was defined as the highest tested concentration where no significant effects were found.

Neurotoxicity assay. After the embryo-toxicity assay, neurotoxicity experiments (ie, locomotor activity) were performed. In this assay, embryos were exposed to the chemicals at 3 dpf for 48 h until 5 dpf. Differences in exposure period and strains used in embryo-toxicity and neurotoxicity assays are due to the fact that both assays were validated independently (unpublished data). Wild-type AB embryos were obtained and kept at 28.5°C under light: dark cycles until they reached 3 dpf. At this stage, larvae were dispensed in 96 squared-well plates (1 larva per well) and exposed to 5 concentrations per compound based on results from the previous assay. The LEL obtained in embryo-toxicity assay was used as the highest concentration evaluated in behavioral assessments. Sixteen larvae were treated per condition along with vehicle controls (0.5% DMSO). After 48 h of incubation at 28.5°C under light: dark cycles, plates were introduced in the Daniovision automated tracking system powered by Ethovision (Noldus, The Netherlands). Temperature was set at 28.5°C and after 10 min of habituation, tracking, which consisted in 2 rounds of 10 min light and 10 min dark phases, started. Total duration of the tracking was 40 min. After tracking, embryos were visualized under a stereo microscope to detect malformed or dead embryos. Dead embryos were excluded from statistical analysis, as well as malformed embryos if their frequency was less than 20%. If the percentage of malformed embryos was higher, it was related to chemical effect so they were not excluded for the analysis but considered for results interpretation. Several parameters were analyzed including velocity, movement duration, and frequency of activity, but the total distance moved was selected as representative of locomotor activity. The mean total distance moved by larvae in each group was measured in 2-min time bins. Values obtained in each time bin were compared between each treatment and control groups using unpaired Student's *t* test. Previous in-house results indicated that the data are normally distributed (data not shown). A significant threshold was set at 0.005 following Bonferroni's correction. When significant differences were detected in more than 1 point at the same concentration and in both rounds of tracking, the chemical was considered active. When $.005 < p < .05$, the differences were considered as trend. If 2 or more time points in the same tracking phase tended to be different and if this trend was repeated in both tracking rounds, the chemical was also classified as active.

If a chemical induced behavioral alterations, it was classified into these effects: *hypoactivity*, if the treated embryos moved significantly less than controls; *hyperactivity*, if they moved more than controls; or *altered profile* when treated embryos showed inverted/lack response to lighting changes. In Figure 1 an example of each behavioral effect is shown. A chemical could induce several effects depending on the concentrations, even the 3 described effects (see Figure 1C as an example). Embryos could be unhealthy or experiencing toxicity at the highest tested concentration, because this was the LEL in the embryo-toxicity assay. Therefore, embryos tested at the highest concentrations usually showed a reduction in locomotor activity. This effect was emphasized in the dark phase of experiment because this is where activity is naturally higher (Figure 1A). When the only significant locomotor activity differences were found as hypoactivity, embryo-toxicity results were considered for classification. If hypoactivity was found at concentrations where no morphological alterations were present, the chemical was classified also as neurotoxic. On the contrary, if the effects were found at concentrations where morphological changes were present, it was classified as toxic. In the cases where a chemical induces hyperactivity or inversed response to lighting changes, the chemical was classified as neurotoxic.

Internal concentration estimation. Bioanalysis was performed in exposed larvae to estimate the concentration of chemical that entered the larvae as described in Alzualde et al. (2018). All the surviving larvae exposed to the LEL and NOEL concentrations in the embryo-toxicity assay were pooled, washed in E3 media and frozen at −80°C until bioanalysis. The samples were then analyzed using 4 different bioavailability methods depending on their physicochemical properties. The method used for each chemical is indicated in Table 1.

1. For LC/MS (Liquid chromatography/mass spectrometry), a Thermo Fisher Scientific -Dionex Ultimate 3000 ultraperformance liquid chromatography (UPLC) system (Dionex Softron GmbH, Part of Thermo Fisher Scientific Inc., Germany) was used coupled to a mass spectrometer (Exactive, Thermo Fisher Scientific, Germany) for the LC/MS system. Both devices were operated using Trace Finder and Xcalibur software. The UPLC system was equipped with a 2.1 × 100 mm, 2.0 mm (ACE C18-PFP, Hichrom Ltd., England) kept at 4°C. A binary gradient mobile phase was used at a flow rate of 0.5 ml per minute with solvent A (0.1% formic acid in water Type I) and solvent B (acetonitrile). The mass spectrometer was operated in electrospray positive mode (ESI, Thermo Fisher Scientific, Germany), while data acquisition was performed using the Parallel Reactions Monitoring mode. The source settings were as follows: spray voltage 3.500/5.500 V; capillary temperature 28°C; sheath, auxiliary and sweep gas 40, 20 and 1 ad respectively; probe heater temperature 40°C; S-Lens 60 V. The mass resolution was 35.000 and the error mass < 2ppm. The results were quantified using Trace Finder software. Recoveries of all compounds were within 80–120% (data not shown).
2. The GC/MS (gas chromatography/mass spectrometry) system consisted of an AGILENT GC/MS: Agilent 7890 GC coupled to an Agilent quadrupole mass selective detector 5973 MS operated in electron impact (EI) ionization mode. The GC system was equipped with electronic pressure control and an isothermal injector. A total of 2 μl of cleaned extract were injected on a DB-5 column (30 m × 0.25 mm × 0.25 μm) using splitless injection mode. The injection temperature was set

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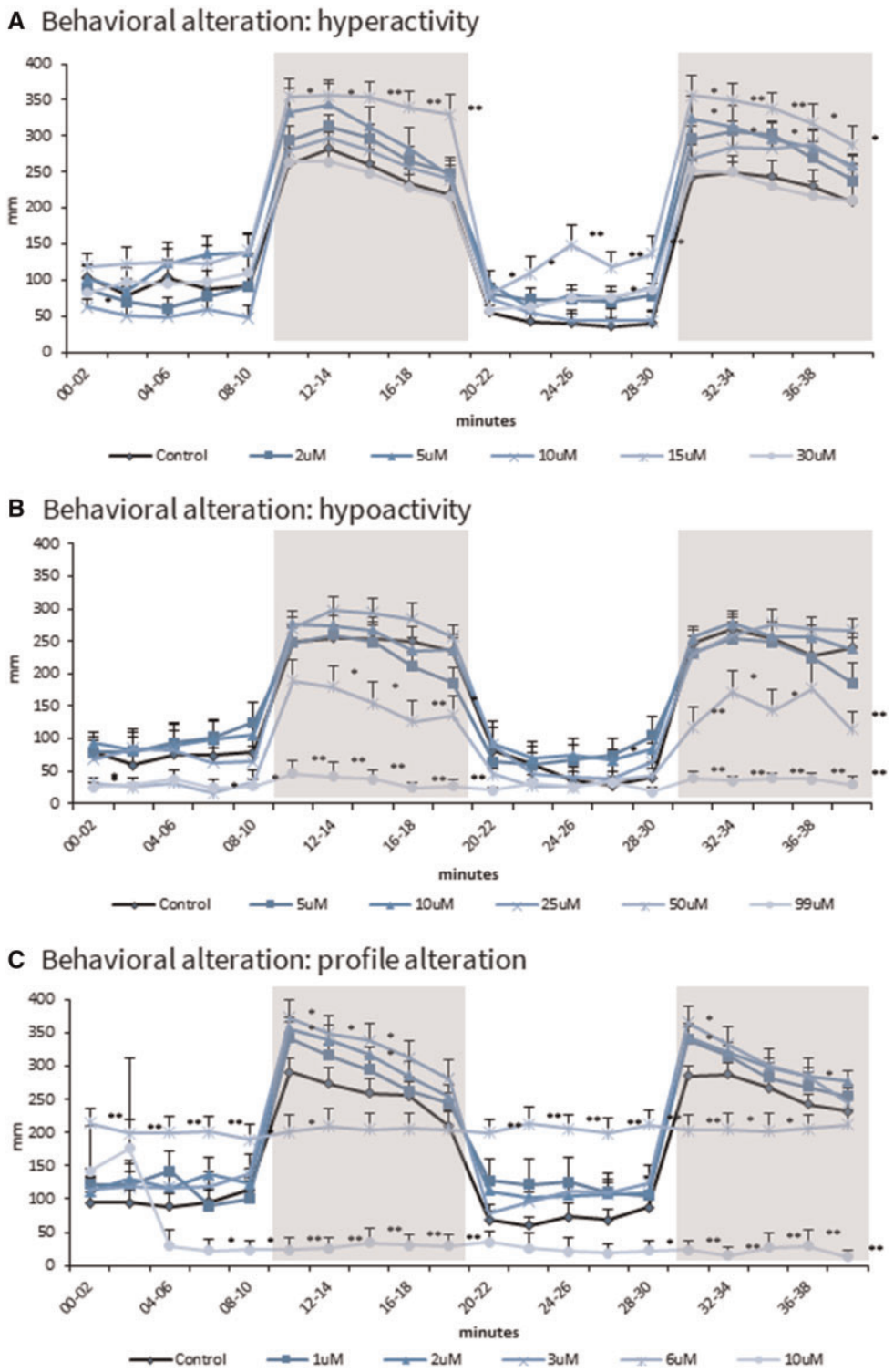

Figure 1. Example of different behavioral alteration effects. Each graph shows the mean of the total distance moved by embryos treated at the 5 tested concentrations together with the vehicle control embryos in 2-minute time bins. The tracking consists of 2 rounds of 10 min under light and 10 min under dark (shadowed in gray) conditions. The top graph (A) is a representative example of chemical-induced hyperactivity following embryo treatment with acenaphthene. At 15 μM acenaphthene induced hyperactivity in both dark phases as well as in the second light phase. At 30 μM, activity levels reduced back down to control embryo levels; which is likely an indicator of acenaphthene toxicity at this dose. The middle graph (B) is a representative example of chemical-induced hypoactivity following embryo treatment with colchicine. Decreased activity was observed within the dark phases at 50 μM and 99 μM. The bottom graph (C) is a representative example of chemical-induced behavioral alteration following embryo treatment with EHDP. At concentrations lower than 3 μM, EHDP caused hyperactivity in dark phases while at 6 μM embryos did not respond to light/dark phases and finally at 10 μM, hypoactivity was seen in all phases. $^{**}p < .005$, $^{*}0.005 < p < .05$.

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at 280°C. The GC temperature program was 60°C, hold 2 min, ramp 25°C/min to 180°C, hold 10 min, ramp 5°C/min to 300°C, hold 5 min. Helium was used as carrier gas with a flow rate of 1.2 ml/min. The mass spectrometer was employed in selected ion monitoring (SIM) mode. The ion source, quadrupole, and interface temperatures were set at 250, 150, and 300°C. The results were quantified using MassHunter Workstation software, Quantitative Analysis Ver B.06.00. Recoveries of all compounds were within 80%–120%.

3. P&T-GC/MS (purge and trap GC/MS) analysis was done with the following systems: P&T: Teledyne Tekmar (Model: Velocity XPT Purge and Trap Sample Concentrator) coupled with AGILENT GC/MS: Agilent 6890 GC coupled to an Agilent quadrupole mass selective detector 5973 MS operated in EI ionization mode. The GC system was equipped with electronic pressure control and a CIS4 injector. A total of 5 ml are injected into the P & T and after the online extraction process the compounds arrive through the transfer line (150°C) to the column that is in the gas chromatograph, the column is a DB-5MS column (30 m × 0.25 mm × 0.5 μm). The P & T work conditions are: purge time 11 min, purge flow 40 ml/min, and desorb temp. 250°C a type "K" trap was used. The temperature program (column) was 40°C, hold 9.5 min, ramp 25°C/min to 120°C, hold 6 min. Helium was used as carrier gas with a constant pressure of 9.15 psi. The mass spectrometer was employed in SIM mode. The ion source, quadrupole, and interface temperatures were set at 250, 150, and 300°C. The results were quantified using CHEMSTATION ver. B04 03 2.
4. The ICP-MS (inductively coupled plasma mass spectrometry) system consisted of an AGILENT 7800 ICP-MS system with an Octopole Reaction System (ORS) and fitted with a standard sample introduction system (MicroMist glass concentric nebulizer, a quartz Peltier-cooled spray chamber, and quartz torch with 2.5 mm internal diameter injector) was used for all measurements. For interference suppression, the ORS was operated in helium collision mode (He mode) only, which is effective at removing a wide range of plasma and matrix-based polyatomic species using kinetic energy discrimination (KED). Example: manganese (Mn). Elements that do not suffer from polyatomic interferences can be analyzed with He mode as well. But to achieve better limits of detection, they were analyzed with no gas in the cell (no gas mode). Elements as lead (Pb) and mercury (Hg). The results were quantified using MassHunter Workstation software ver. 4.3. Recoveries of all compounds were within 80%–120%.

RESULTS

A total of 86 (+4 duplicated) blinded chemicals were tested first in the DRF test at concentrations covering a wide range (0.2–100 μM). Based on results from this study, concentrations were set for the subsequent embryo-toxicity assay performed to determine the LEL of each chemical and obtain samples for bioavailability analysis. Finally, the chemicals were assayed for behavioral alterations to investigate their potential to target the nervous system. The highest concentration evaluated in behavioral assessments was set around the LEL obtained in embryotoxicity assay.

Overall, 59 chemicals were detected as active for zebrafish embryos. Based on the LEL, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was the most potent chemical, noted by induction of morphological alterations at picomolar levels. Chemicals which altered the normal development of zebrafish at nanomolar levels were valinomycin, bis(tributyltin)oxide and deltamethrin. Deltamethrin also induced hyperactivity apart from morphological alterations. On the other hand, amongst the chemicals whose toxicity was not detected, there were the 5 included as negative controls as well as other chemicals not detected inside the embryos by bioavailability.

Chemicals Suspected to Be Developmental Neurotoxicants or Neurotoxicants

Among the tested chemicals, there were 38 (+ 2 duplicates) which were suspected DNTs and/or NTs based on the literature by different modes of action. In this assessment, 26 (+ 2 duplicates) out of 38 DNT/NT chemicals (68.4%) were detected as toxic or neurotoxic for zebrafish larvae (Table 2). Fifteen chemicals (57.7%) induced behavioral alterations at concentrations where no morphological alterations were detected; the other 11 chemicals induced toxicity in zebrafish embryos (ie, alterations were observed at lower or the same concentration as the behavioral alterations).

Chemicals suspected to be DNTs or NTs are divided into different categories: drugs, industrial, and pesticides (Figure 2).

Drugs. There were 14 chemicals suspected to be DNT/NT which were classified as drugs. Some of these chemicals are well-known NTs in humans as well as in the zebrafish. For instance, MPP+ and 6-OHDA are drugs widely used to induce a parkinsonism-like phenotype in animal models including zebrafish (Cronin and Grealy, 2017; Li *et al.*, 2018). As anticipated, these chemicals were classified as neurotoxic in this study (Table 2, Supplementary Figure 1). Another example is valproic acid, a well-known anticonvulsant drug whose effect on zebrafish has already been described (ie, Bailey *et al.*, 2016; Grone *et al.*, 2017; Martinez *et al.*, 2018,). In our study, valproic acid was classified as neurotoxic.

Phenobarbital and phenobarbital sodium salt were tested in this study up to 100 μM. The former induced a reduction in locomotor activity in the absence of morphological alterations, so it was classified as neurotoxic. The latter did not cause any alteration in zebrafish, so it was classified as nontoxic. These different results could be explained by the differences in internal concentration. At the same treatment concentration (100 μM), phenobarbital was detected at 22.52 μM and phenobarbital sodium salt at 10.54 μM.

Colchicine and diazepam were classified in the behavior assay as hypoactivity-inducing drugs at concentrations where no morphological alterations were found. On the other hand, diethylstilbestrol and hexachlorophene induced morphological alterations (mainly microcephaly) and death.

Expected toxicity induced by 5-fluorouracil, 6-propyl-2-thiouracyl, hydroxyurea, and tetraethylthiuram disulfide was not detected, most likely because of their low penetrance into the embryos, as these chemicals were not detected inside the embryos by our analytical methods. Thalidomide did not induce morphological alterations nor did it affect locomotor activity, however, it also showed a limited penetration: When embryos were treated at 100 μM, the concentration found in the embryo was 8.69 μM. In addition, it has been recently shown that the teratogenic effect of thalidomide cannot be detected in zebrafish embryos, because the mechanism responsible of its effect (Sall4 degradation) is not conserved (Donovan *et al.*, 2018).

Industrials. There were 10 chemicals suspected to be DNT/NT classified as industrials. None of them was classified as

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Table 2. Results Obtained in Embryo-Toxicity Assay (NOEL and LEL) and Neurotoxicity Assay (LEL) and the Classification of the Effect Found for Each Chemical

Classification		Chemical Name	Embryo Toxicity		Neurotoxicity	Characteristical Effect	Classification
			NOAEL (μM)	LOAEL (μM)	LOAEL (μM)		
Negative controls		Acetaminophen (4-hydroxyacetanilide)	>100/0.843	—	—	—	ND
		Acetylsalicylic acid	>100/24.88	—	—	—	ND
		D-Glucitol	>100/ND	—	—	—	ND
		L-Ascorbic acid	>100/230.4	—	—	—	ND
		Saccharin sodium salt hydrate	>100/ND	—	—	—	ND
		Saccharin sodium salt hydrate	>100/2.78	—	—	—	ND
DNT/NT	Drugs	1-Methyl-4-phenylpyridinium iodide	>100/16.16	—	30	Hyperactivity	Neurotoxic
		5-Fluorouracil	>100/ND	—	—	—	ND
		6-Hydroxydopamine hydrochloride	>5/ND	—	100	Hypoactivity	Neurotoxic
		6-Propyl-2-thiouracil	>100/ND	—	—	—	ND
		Colchicine	75/—	100/1.05	50	Hypoactivity	Neurotoxic
		Diazepam	4/18.05	8/47.73	15	Hypoactivity	Toxic
		Diethylstilbestrol	0.5/18.55	1/35.34	—	—	Toxic
		Hexachlorophene	0.2/ND	0.3/ND	0.5	Mortality	Toxic
		Hydroxyurea	>100/ND	—	—	—	ND
		Phenobarbital	>100/22.52	—	100	Hypoactivity	Neurotoxic
		Phenobarbital sodium salt	>100/10.54	—	—	—	ND
		Tetraethylthiuram disulfide	0.1/ND	0.3/ND	0.3	Hypoactivity	Toxic
		Thalidomide	>100/8.69	—	—	—	ND
		Valproic acid sodium salt	50/130.0	75/154.7	50	Hyperactivity	Neurotoxic
	Industrials	2-Methoxyethanol	>100/—	—	—	—	ND
		3,3'-Iminodipropionitrile	>100/17.46	—	—	—	ND
		Acetic acid, manganese (2+) salt	>60/126.9	—	—	—	ND
		Acrylamide	>100/13.18	—	—	—	ND
		Bisphenol A	10/640.4	15/12.7	—	—	Toxic
		Di(2-ethylhexyl) phthalate	>15/14.76	—	—	—	ND[a]
		Lead (II) acetate trihydrate	1/15.84	2/32.33	5	Hypoactivity	Toxic
		Manganese, tricarbonyl[(1,2,3,4,5-.eta.)-1-methyl-2, 4-cyclopentadien-1-yl]-	>100/2.53	—	10	Mortality	Toxic
		n-Hexane	>100/-	—	—	—	ND
		Toluene	>100/-	—	—	—	ND
	Pesticides	Aldicarb	0.5/ND	1/ND	—	—	Toxic
		Bis(tributyltin)oxide	0.02/ND	0.05/ND	0.1	Hypoactivity	Toxic
		Carbaryl	2/ND	4/ND	4	Hypoactivity	Toxic
		Chlorpyrifos (Dursban)	2/882.1	5/22.2	1	Profile alteration	Neurotoxic
		Deltamethrin	0.02/ND	0.05/ND	0.025	Hyperactivity	Neurotoxic
		Deltamethrin	0.05/ND	0.1/ND	0.005	Hyperactivity	Neurotoxic
		Dichlorodiphenyltrichloroethane (DDT)	1/443.5	2/10.2	0.5	Profile alteration	Neurotoxic
		Dieldrin	0.2/288.4	0.5/567.8	0.05	Profile alteration	Neurotoxic
		Heptachlor	4/337.2	8/20.8	0.5	Profile alteration	Neurotoxic
		Lindane	2/282.9	4/504.7	0.5	Profile alteration	Neurotoxic
		Methyl mercuric (II) chloride	0.15/50.56	0.3/114.4	1	Mortality	Toxic
		Methyl mercuric (II) chloride	0.1/40.27	0.2/75.98	—	—	Toxic
		Parathion	4/696.7	8/15.1	8	Profile alteration	Neurotoxic
		Permethrin	1/5.02	2/14.26	0.5	Hyperactivity	Neurotoxic
		Rotenone	0.075/ND	0.1/ND	—	—	Toxic
		Tebuconazole	15/408.0	20/556.4	10	Hypoactivity	Neurotoxic
Unknown toxicity	Drugs	Amoxicillin	>100/ND	—	15	Hyperactivity	Neurotoxic
		Berberine chloride	>100/12.01	—	60	Hyperactivity	Neurotoxic
		Valinomycin	0.02/ND	0.05/ND	—	—	Toxic
	Industrials	1-Ethyl-3-methylimidazolium diethylphosphate	>100/71.01	—	—	—	ND

Continued

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Table 2. (continued)

Classification	Chemical Name	Embryo Toxicity		Neurotoxicity	Characteristical Effect	Classification
		NOAEL (μM)	LOAEL (μM)	LOAEL (μM)		
	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	0.0001/*ND*	0.0003/*ND*	—	—	Toxic
	Auramine O	2/*60.76*	3/*87.85*	—	—	Toxic
	Bisphenol AF	1.5/*47.39*	2/*62.91*	—	—	Toxic
	Bisphenol S	>100/*22.09*	—	—	—	ND
	Estradiol	5/*446.3*	10/*472.5*	—	—	Toxic
Pesticides	Captan	30/*ND*	75/*ND*	—	—	Toxic
	Carbamic acid, butyl-, 3-iodo-2-propynyl ester	0.5/*ND*	1/*ND*	—	—	Toxic
Flame retardants	2-Ethylhexyl diphenyl phosphate (EHDP)	6/*363.6*	10/*852.3*	3	Profile alteration	neurotoxic
	2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB)	>15/*ND*	—	—	—	ND[a]
	2,2',4,4'-Tetrabromodiphenyl ether	15/*559.0*	20/*780.3*	2	Profile alteration	Neurotoxic
	2,2',4,4',5-Pentabromodiphenyl ether (BDE-99)	>10/*88.66*	—	—	—	ND[a]
	2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE-153)	>5/*36.12*	—	—	—	ND[a]
	3,3',5,5'-Tetrabromobisphenol A	1/*198.2*	1.5/*201.4*	1.5	Mortality	Toxic
	Bis(2-ethylhexyl) 3,4,5,6-tetrabromophthalate (TBPH)	>5/*ND*	—	—	—	ND[a]
	Firemaster 550	0.5/*76.49* 0.5/*152.6* 0.5/*34.41*	1/*175.2* 1/*275.8* 1/*107.8*	2	Hypoactivity	Toxic
	Isodecyl diphenyl phosphate	30/*110.8*	50/*229.8*	50	Hypoactivity	Toxic
	Phenol, isopropylated, phosphate (3: 1)	0.2/*41.98* 0.2/*36.38* 0.2/*7.76*	0.5/*105.2* 0.5/*83.83* 0.5/*19.14*	—	—	Toxic
	tert-Butylphenyl diphenyl phosphate	2/*12.0*	4/*12.5*	4	Hypoactivity	Toxic
	Tricresyl phosphate	5/*233.6*	10/*622.5*	10	Hypoactivity	Toxic
	Triphenyl phosphate	1/*72.95*	2/*143.4*	1	Hypoactivity	Neurotoxic
	Triphenyl phosphate	0.5/*60.38*	1/*125.5*	2	Hypoactivity	Toxic
	tris(Chloropropyl) phosphate (TCPP)	50/*40.23*	75/*87.15*	75	Hypoactivity	Toxic
	tris(2-chloroethyl) phosphate	>100/*8.67*	—	—	—	ND
PAH	4-H-Cyclopenta(d,e,f) phenanthrene	4/*13.2*	8/*24.3*	—	—	Toxic
	Acenaphthene	20/*25.52*	30/*60.78*	15	Hyperactivity	Neurotoxic
	Acenaphthylene	10/*84.89*	15/*887.8*	—	—	Toxic
	Anthracene	>10/*16.4*	—	99.5	Hypoactivity	Neurotoxic
	Benz(a)anthracene	2/*33.37*	4/*51.20*	—	—	Toxic
	Benzo(a)pyrene	>5/*2.03*	—	5	Hyperactivity	Neurotoxic
	Benzo(e)pyrene	>10/*44.04*	—	—	—	ND[a]
	Benzo[g,h,i]perylene	>5/*2.59*	—	—	—	ND[a]
	Benzo(b)fluoranthene	30/*7.84*	60/*29.24*	—	—	Toxic
	Benzo(k)fluoranthene	0.05/*1.95*	0.1/*2.67*	0.5	Hyperactivity	Neurotoxic
	Chrysene	>10/*53.28*	—	25	Hypoactivity	Neurotoxic
	Dibenz[a,c]anthracene	>5/*ND*	—	—	—	ND[a]
	Dibenz(a,h)anthracene	0.2/*ND*	0.3/*ND*	—	—	Toxic
	Fluorene	15/*18.6*	20/*14.8*	4	Hyperactivity	Neurotoxic
	Naphthalene	75/*3.04*	100/*5.16*	—	—	Toxic
	Phenanthrene	5/*13.0*	10/*22.0*	—	—	Toxic
	Pyrene	1/*901.9*	2/*16.3*	2	Hypoactivity	Toxic

Internal concentration found inside the embryos is stated at NOEL and LEL of embryo toxicity (in italics after /).
Abbreviation: ND, toxicity not detected.
[a]Limited testing concentrations due to solubility issues.

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neurotoxic as they did not induce behavioral alterations at concentrations where morphological alterations were not detected. Bisphenol A, a high production volume chemical, was recently included in the list of substances of very high concern (ECHA/PR/18/01) due to its properties as an endocrine disruptor. In this study, bisphenol A showed a great capacity of bioaccumulation in zebrafish. Embryos treated with 15 μM of bisphenol A for 96 h had an internal concentration of 1247 μM and presented developmental malformations (mainly microcephaly and heart edema), but no clear behavioral alteration. Lead (II) acetate trihydrate was also classified as toxic. Although behavior was altered in embryos exposed to this chemical, the effect was found at concentrations where morphological alterations were induced.

Manganese, tricarboryl [(1,2,3,4,5-.eta.)-1-methyl-2, 4-cyclopentadien-1-yl] induced mortality in behavioral assay in a dose-dependent manner although it was not detected in embryotoxicity assay. This difference in mortality detection could be explained by the differences in exposure time, embryo stage, as well as the size of the wells where embryos were placed for treatment.

3,3'-Iminodipropionitrile, bis(2-ethylhexyl)phthalate, and acrylamide did not alter morphology or behavior of zebrafish embryos at tested concentrations, even though internal concentration measurements confirmed their uptake (internal estimated concentration was at least 10% of the nominal concentration).

Other industrial chemicals such as methoxyethanol, *n*-hexane, and toluene were also negative. However, they were not detected in the embryo. This fact could be due to limited penetration or volatility of the chemicals (especially in the case of *n*-hexane and toluene).

Pesticides. The 14 pesticides suspected to be DNT/NT were detected in the present study either as neurotoxic (10 out of 14, 71.4%) or toxic (4 out of 14, 28.6%). Chlorpyrifos, an organophosphate pesticide that is known to inhibit acetylcholinesterase activity and considered moderately hazardous to humans by the World Health Organization (WHO, 2010) was classified as neurotoxic. The banned chemical, DDT, and the insecticide developed as its alternative, dieldrin, were also classified as neurotoxic. All these 3 pesticides were found to alter the locomotor activity profile by inducing an increase in activity during light phases and decrease during dark phases; other pesticides with a similar behavioral effect were heptachlor and parathion (Figure 3). Permethrin and deltamethrin induced hyperactivity in the dark phases of tracking so they were also classified as neurotoxic. Tebuconazole and lindane were also neurotoxic as they induced hypoactivity at concentrations where no morphological alterations were found. Bis(tributyltin)oxide, carbaryl, aldicarb, methyl mercury (II) chloride, and rotenone were toxic for zebrafish embryos as they altered the behavior at the LEL of embryotoxicity.

Chemicals With Unknown Effects

Apart from the described groups of chemicals (chemicals suspected to be DNT/NT), there were 43 (+ 1 duplicate) more chemicals tested with unknown DNT/NT effect. Among them, there were 2 main groups analyzed, FRs (15 test items + 1 duplicate) and PAHs (17 test items). The remaining 11 chemicals consisted of 3 drugs, 2 pesticides, and 6 industrials with unknown effect.

Flame retardants. Ten out of the 15 FRs were toxic for zebrafish embryos as all of them induced morphological alterations

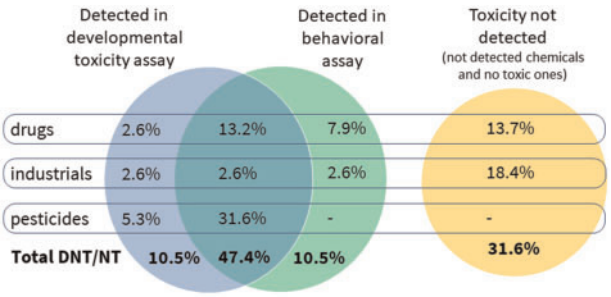

Figure 2. Representation of the percentage of chemicals suspected to be DNT/NT detected as toxic in embryo-toxicity and/or behavioral assay (far left). Overlapping percentages are those detected as toxic in both assays. On the right, percentages of chemicals whose toxicity was not detected. The chemicals are divided into drugs, industrials, and pesticides, following NTP classification.

(Table 2). Moreover, 2-ethylexyl diphenyl phosphate (EHDP), 2,2′,4,4′-tetrabromodiphenyl ether, tricresyl phosphate, and triphenyl phosphate were classified as neurotoxic chemicals.

The ones that appeared to be safe for zebrafish embryos at tested concentrations were BDE-99, BDE-153, and TBPH. TBB was also negative, however it could not be detected inside embryos which suggests this result could be a false negative. On the other hand, it is noteworthy to highlight the difference found between nominal and internal concentrations. FRs showed much higher internal concentration than the nominal one due to the accumulation of the FRs in zebrafish embryos in the majority of cases (Table 2).

Polycyclic aromatic hydrocarbons. Regarding PAHs, 14 out of 17 were found to be toxic for zebrafish embryos. Acenaphthene, benzo(k)-fluoranthene, fluorene, benzo(a)pyrene were classified as neurotoxic as they induced behavior alteration. Interestingly, these 4 PAHs induced a similar effect on behavior, ie, hyperactivity during dark phases. Anthracene and chrysene also altered zebrafish behavior at concentrations where no morphological alterations were found. Therefore, they were also classified as neurotoxic. PAHs that did not induce any toxic effect included benzo(e)pyrene, benzo[g,h,i]perylene, and dibenz[a,c]anthracene, however the latter was not detected in embryos which might indicate a false negative results. The other 8 PAHs were toxic for zebrafish embryos (Table 2).

Others. Among the group of compounds with unknown DNT/NTs, 2 were neurotoxic (berberine chloride and amoxicilin), other 7 induced embryo-toxicity and 2 were not toxic (1-ethyl-3-methylimidazolium diethylphosphate and bisphenol S) (Table 2).

Quality Control: Negative Controls and Replicates

The fact that all the replicates were similarly classified indicates the reproducibility of the assay. The 2 samples of *Deltamethrin* were classified as neurotoxic in both cases based on an increase in locomotor activity during dark phases at an intermediate testing concentration. At higher concentrations, this effect was lost, possibly due to systemic toxicity. The 2 samples of *methyl mercury (II) chloride* induced morphological malformations, mainly microcephaly and heart edema (Figure 4) so this test item was classified as embryo toxic in both replicates. There was not a clear alteration of locomotor activity observed at tested concentrations. In the case of *triphenyl phosphate*, the only difference between replicates was the concentration at which

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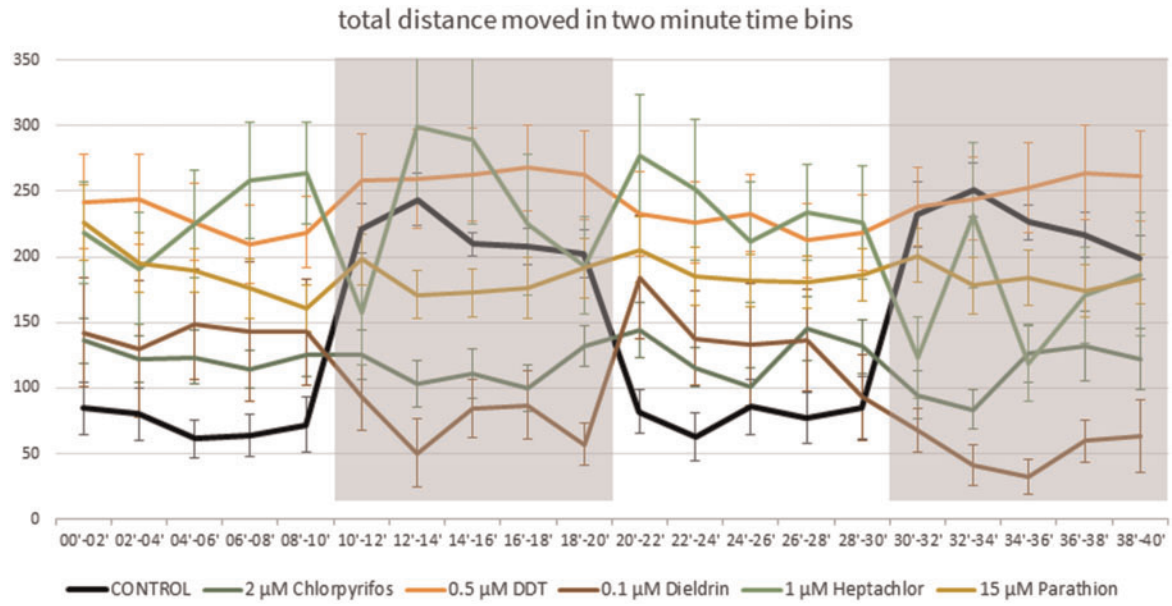

Figure 3. Behavioral alteration induced by pesticides: Line graphs are representing the mean and standard deviation of the total distance moved in each 2 minute time bin of the tracking, which consist of 2 rounds of 10 minute light and 10 minute dark phases (shadowed). Embryos treated with vehicle (Control = black line) are compared to those treated with chlorpyrifos, DDT, dieldrin, heptachlor, and parathion at each behavioral lowest effective level. Embryo treatment with pesticides induced the loss of response to light/dark phases within the experiment and were considered to cause behavioral alterations.

malformations and behavioral alterations were initially detected. The first replicate did not induce any significant alterations until 2 µM (60% displayed heart edema) and the other replicate induced morphological alterations from 1 µM (26.7% of the embryos displayed heart edema). In the behavioral alteration assay the first one induced significant alterations at 1 µM and the other at 2 µM. Thus, this chemical would be classified as neurotoxic considering the first replicate and toxic considering the second one. The slight differences in LEL obtained in embryo-toxicity and behavioral assay resulted in different classification of the chemical. However, the results are very similar. The 2 samples of *saccharin sodium salt* did not induce any effect on zebrafish embryos so they were classified as nontoxic.

In addition, there were 5 negative controls (+1 duplicate) which did not cause any developmental toxicity nor behavioral changes (Table 2). Therefore, they were classified as nontoxic at tested concentrations (up to 100 µM). By bioanalysis, L-ascorbic acid and acetylsalicylic acid were detected in the embryos at 230.4 µM and 24.9 µM, respectively at the highest tested concentration (100 µM). On the contrary, acetaminophen, D-glucitol, and saccharin sodium salt were detected in the embryo at a very low concentration or were not detected.

DISCUSSION

The NTP initiated a collaborative project to discuss how different test methods could be integrated into a "battery" of medium- and high-throughput cell-based models, and alternative animal systems to prioritize compounds for further in vivo testing in rodents and/or to complement current regulatory DNT guideline studies (Behl *et al.*, 2016, 2019). This effort brought together investigators from academia, industry, and the government who evaluated the library presented here in their respective laboratories using assays that informed on some aspect of DNT.

Zebrafish is described as a flexible model that fits between in vitro models and mammalian rodent models of toxicity (Horzmann and Freeman, 2018). The aim of this work was to evaluate the reliability and relevance of zebrafish assays to prioritize compounds for further in vivo toxicity testing. We checked the potential of the tested chemicals to induce morphological and/or locomotor activity alterations. Importantly, embryo chemical concentration was also measured as an approach to real chemical exposure.

Following the described procedure, 59 out of 86 chemicals were found to be active (ie, toxic or neurotoxic) in zebrafish embryos in the current study. A different, independent study by Hagstrom *et al.* (2019) screened the same library in zebrafish embryos and noted all the compounds as active with one exception (ie, hydroxyurea). They carried out an extra assay that consisted of an embryo photomotor response that was not performed in the present study. However, the additional end-point did not contribute significantly towards the detection of actives (Hagstrom *et al.* (2019). Although we treated embryos with intact chorion (in embryo-toxicity assay) or embryos out of chorion (in neurotoxicity assay), embryos were enzymatically dechorionated before treatment in Hagstrom *et al.* protocol. We found that the internal concentration in 24 hpf embryos treated for 20 h was higher than in embryos whose chorion was manually disrupted (data not shown). These results indicate that chemical dechorionation can sensitize embryos. Another reason for this difference could be the interpretation of the results obtained in the neurotoxicity assay. Our criteria to determine which effects are biologically significant could be stricter than others, because the effects have to be repeated at the 2 dark or 2 light phases to consider them truly neurotoxic. In another publication from the same group where 14 FRs were tested, (Noyes *et al.*, 2015) they detected 13 active chemicals while we detected 9. For instance, they consider one active compound (isodecyl diphenyl phosphate) that induced hypoactivity only in one of the light phases at the lowest tested concentration. Based on our criterium, we would not consider it active. In this regard, there is a publication focused on providing a new strategy for processing and analyzing data obtained from zebrafish

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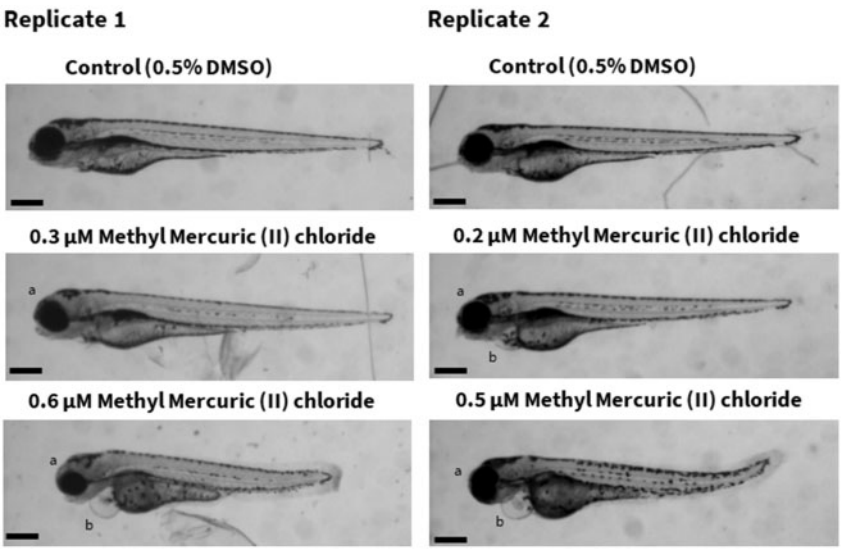

Figure 4. Performance of assay reproducibility. Several chemicals, including methyl mercury (II) chloride, were tested twice in a blinded experimental design. Images highlight that similar phenotypic changes (a = microcephaly, b = heart edema) were observed in a dose-dependent manner for replicate 1 and replicate 2.

developmental toxicity and neurotoxicity assessments to avoid inconsistency in data analysis strategies to classify chemical activity outcomes (Hsieh *et al.*, 2019). They have used our dataset to construct this new strategy and the concordance in classification of active compounds between their approach and ours was 100% in case of embryo-toxicity and 74% in the case of neurotoxicity assay. We detected five actives that they did not: 1-methyl-4-phenylpyridinium iodide, acenaphthene, anthracene, benzo(k)fluoranthene, and valproic acid. At least 2 of them already had robust data supporting their neuroactivity in zebrafish (Dukes *et al.*, 2016; Farrell *et al.*, 2011; Lee *et al.*, 2018; Li *et al.*, 2018; Zhang and Zhao, 2018). On the contrary, Hsieh *et al.* (2019) detected 4 additional active chemicals, all of them detected only in one of the rounds of the tracking.

Besides zebrafish, Hagstrom *et al.* (2019) also described a dataset obtained using freshwater planaria as a new alternative animal model for toxicity screening (Hagstrom *et al.* 2019; Zhang *et al.*, 2019). The results of this dataset are more similar to ours. Among the 38 chemicals suspected to be DNT/NT, the pesticide group is the one where more chemicals were detected, followed by drugs and finally by the industrials. They detected 13 out of 14 pesticides as active, 6 out of 14 drugs, and 4 out of 10 industrials; while we detected toxicity in all the pesticides, 9 of out of 14 drugs and 3 out of 10 industrials.

Chemicals Suspected to Be Developmental Neurotoxicants or Neurotoxicants

We were able to detect all the pesticides with anticipated neurotoxicity as active in zebrafish larvae. Moreover, 71.4% of them altered embryos' behavior at concentrations where no morphological alterations were detected, confirming that nervous system might be one of the principal targets for these chemicals. Seven pesticides tested in this work were previously analyzed for malformations by Padilla *et al.* (2012) as part of ToxCast Phase I studies. All 7 pesticides produced malformations in agreement with our findings, although the LEL or NOEL was not available for a more detailed dose comparison. Another recent study by Özdemir *et al.* (2018) described that chlorpyrifos and deltamethrin, among other pesticides, disrupt normal neural activity which correlates with disruption of both *c-fos* and *bdnf* genes and protein expression in the zebrafish brain. We also noted that several drugs induced alterations in zebrafish behavior. All the drugs except ones that showed a low percentage of uptake (internal dose $\leq$10% of the nominal dose) were classified as toxic or neurotoxic. Among the detected ones, 62.5% altered embryos' behavior at concentrations in the absence of morphological alterations.

In comparison, industrial chemicals that were suspected to be DNT/NT did not induce any behavioral alterations in zebrafish embryos at tested concentrations; only 3 out of 10 induced toxicity in zebrafish embryos. Most of the compounds that were nontoxic were detected inside the embryos at more than 10% of nominal concentrations so the absence or low uptake could not explain the absence of effect. We are unsure of why these zebrafish assays, as well as planaria models (Zhang *et al.*, 2018) might be insensitive to industrial chemical exposure; further studies are warranted.

Chemicals With Unknown Effects

Amongst these chemicals, there were 4 drugs, 2 pesticides, 5 industrial, 15 FRs, and 17 PAHs. The 2 pesticides caused embryo toxicity, however, they did not induce any behavioral alteration in zebrafish larvae. Amongst the 4 drugs tested, 2 induced embryo toxicity without any behavioral changes. The other 2 drugs (berberine chloride and amoxicillin) were classified as neurotoxic as both induced a slight hyperactivity in zebrafish larvae. Limited evidence suggests some neurological adverse effects related to amoxicillin treatment in humans (Mattappalil and Mergenhagen, 2014; Raposo and Bento, 2016), and in juvenile rats (Atli *et al.*, 2016). Moreover, berberine chloride is supposed to be neuroprotective against toxicity induced by mercury (Moneim, 2015). This fact may indicate that this chemical could target the nervous system. On the other hand, none of the industrial chemicals with unknown toxicity were found to be neurotoxic as they did not alter the behavior of zebrafish larvae. However, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, bisphenol AF, and auramine O were found to be toxic in zebrafish embryo.

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Most of the FR and PAH were toxic to zebrafish embryos. A total of 66.7% of FR and 82.3% of PAH induced some alteration in zebrafish. In addition, 4 out of 10 active FR (40%) and 5 out of 14 active PAH (36%) also induced a behavioral effect at concentrations where overt toxicity was not detected so they were classified as neurotoxic. Many of these FRs have been studied before and noted to have effects on developmental toxicity and neurotoxicity in other in vitro and alternate animal systems (Behl *et al.*, 2015, 2016; Jarema *et al.*, 2015; Glazer *et al.*, 2018; Shi *et al.*, 2018). In our recently published work with these chemicals (Alzualde *et al.*, 2018), 9 of the 15 FRs were assessed for systems toxicity. Results showed concordance between the findings across both studies. The significant amount of toxicity and neurotoxicity noted reinforces the NTP's concern to investigate the potential neurotoxicity of this chemical class.

Bioavailability of Chemicals to Zebrafish Embryos

A total of 12 out of 38 (31.6%) DNT/NT suspected chemicals did not induce any toxicity in zebrafish larvae at tested concentrations. However, 8 out of the 12 nontoxic were not detected in the zebrafish embryos or were detected at $\leq$10% of nominal concentration. Therefore, it is possible that in many cases the lowest effective concentrations were not reached inside the embryos. For instance, this might have been the case of acrylamide. Acrylamide was detected in the embryo at 13.18 μM when the nominal concentration was 100 μM. It has been described elsewhere to induce neurotoxicity in zebrafish, however, at higher treatment concentrations (Prats *et al.*, 2017). For other compounds such as phenobarbital, it is possible that differences in activity is due to the salt form tested as previously discussed in the results section. Although increasing testing concentrations could improve the detection of possible effects of chemicals with low uptake, maximum concentration that could have been reached in this study was 100 μM as they were received at 20 mM in DMSO; the maximum DMSO concentration was set at 0.5%. In some cases, solubility issues limited the testing concentrations. For instance, 4 out of the 5 FRs (TBB, BDE-99, BDE-153, and TBPH) and the 3 PAHs whose toxicity was not detected precipitated, so a higher concentration could not have been tested.

In this study, chemicals were detected inside the embryos at various percentages of the nominal concentrations. Some chemicals were not detected in the embryos while others highly accumulated (ie, pyrene was found almost 1000 times more concentrated in the embryos than the concentration at which they were treated). Several physicochemical properties of chemicals have been described to influence the uptake such as size, molecular weight, or lipophilicity, as well as the presence of active transport into and within embryos (de Koning *et al.*, 2015) and the dissolved organic matter in the media (Li *et al.*, 2018). Chemicals exceeding 3 kDa or 3000 g/mol show a restricted uptake due to the chorion presence in zebrafish embryos until 48–72 hpf (de Koning *et al.*, 2015). This restriction probably did not affect this study as none of the testing chemicals exceeds this molecular weight, and because the bioavailability assay was conducted at 4 dpf, 1–2 days after hatching of embryos that were in constant contact with the chemicals. One of the most important factors for internal exposure is the log *P* (lipophilicity) of a chemical. Internal exposure for chemicals with a higher log *P* value is higher than for chemicals with a lower log *P* value (de Koning *et al.*, 2015; Berghmans *et al.*, 2008). However, a low log *P* value does not mean there is no uptake, the uptake increases with the applied dose, so in the case of lipophobic chemicals they should be tested at higher doses (de Koning *et al.*, 2015). The fact that chemicals show such a different internal concentration percentage, highlights the importance of performing a bioanalytical procedure to confirm the true internal exposure of zebrafish to testing chemicals.

In summary, disregarding the chemicals with limited uptake (internal concentration $\leq$10%) or precipitation that were not toxic in zebrafish embryos, 89.6% (26 out of 29) were classified as embryo toxic or neurotoxic. Moreover, 48.3% (14 out of 29) induced neurotoxicity selectively. On the other hand, 94.3% (33 out of 35) of the chemicals with unknown effect were toxic to zebrafish embryos, with 25.7% (9 out of 35) being selectively neurotoxic. Although specific neurotoxic effects could not always be well-distinguished from general toxicity, this information allows for prioritization of compounds for further in-depth testing. The coupling of the zebrafish embryo-toxicity and behavior alteration assays support the inclusion of zebrafish in screening batteries for DNT and NT. Although not investigated in this study, the availability of genetic and molecular tools allows further exploration into toxicity mechanisms in this animal model that provide a powerful way to identify pathways perturbed by environmental exposure (Planchart *et al.*, 2016; Groh and Suter, 2015).

CONCLUSION

The approach presented here has been proven to be suitable to detect a set of chemicals with known or suspected developmental neurotoxicity or neurotoxicity. Zebrafish is postulated as a good translational model to screen for the impact of chemicals in humans and also a suitable aquatic vertebrate model to detect environmental toxicity.

By Including zebrafish in the process of toxicological screening, profiling, and in the analysis of newly synthetized chemicals we could speed up the process in a cost-effective manner. Behavioral profiling in zebrafish larvae could be an essential part of this process to detect chemicals that could interfere with nervous system development and consequently could be involved in earlier or later neurological disorders. Further studies are warranted to compare the relevance and predictivity of findings in zebrafish with exposures in humans and aquatic wildlife.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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