



Incorporating new approach methodologies in toxicity testing and exposure assessment for tiered risk assessment using the RISK21 approach: Case studies on food contact chemicals



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ABSTRACT

Programs including the ToxCast project have generated large amounts of *in vitro* high-throughput screening (HTS) data, and best approaches for the interpretation and use of HTS data, including for chemical safety assessment, remain to be evaluated. To fill this gap, we conducted case studies of two indirect food additive chemicals where ToxCast data were compared with *in vivo* toxicity data using the RISK21 approach. Two food contact substances, sodium (2-pyridylthio)-N-oxide and dibutyltin dichloride, were selected, and available exposure data, toxicity data, and model predictions were compiled and assessed. Oral equivalent doses for the ToxCast bioactivity data were determined by *in-vitro in-vivo* extrapolation (IVIVE). For sodium (2-pyridylthio)-N-oxide, bioactive concentrations in ToxCast assays corresponded to low- and no-observed adverse effect levels in animal studies. For dibutyltin dichloride, the ToxCast bioactive concentrations were below the dose range that demonstrated toxicity in animals; however, this was confounded by the lack of toxicokinetic data, necessitating the use of conservative toxicokinetic parameter estimates for IVIVE calculations. This study highlights the potential utility of the RISK21 approach for interpretation of the ToxCast HTS data, as well as the challenges involved in integrating *in vitro* HTS data into safety assessments.

1. Introduction

Current established methods of chemical safety testing are based on use of animal bioassays, and are time and resource intensive. Large numbers of chemicals are in use commercially in the United States and globally, and many have little to no toxicological data and await evaluation by these methods. Recently, a strategy was developed to shift toxicity testing towards a more mechanistic, high-throughput basis to enable testing of more chemicals more quickly and cost effectively (National Research Council, 2007). Part of this plan is the development of *in vitro* high-throughput screening (HTS) assays that will evaluate biological pathway perturbations to evaluate chemicals for toxicity. HTS assay development, chemical screening, and data generation have been significant, particularly by the Tox21 effort (Kavlock et al., 2012;

Tice et al., 2013). Interpretation of HTS data and development of best practices for the use of such data in chemical safety assessment remains in progress (Groh and Muncke, 2017). HTS data are already being proposed as a means by which to prioritize chemicals for further testing (USEPA, 2014); however, comparison of HTS data with *in vivo* animal data for predictivity assessments of human toxicity are ongoing (Judson et al., 2010; Liu et al., 2017; Rotroff et al., 2013). Ultimately, the goal is to use *in vitro* data to predict *in vivo* human effects, reducing the reliance on animal testing (NCATS, 2016; NTP, 2016). The EPA's ToxCast program, which is part of the larger Tox21 interagency collaboration, has provided a wealth of HTS data to the toxicology community, generating data on more than 3000 chemicals across 1000 assay endpoints, with the goal of generating screening data that could be used for prioritizing chemicals for further testing (Dix et al., 2007; Richard et al., 2016). So

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Abbreviations

AC ₅₀	Active concentration 50
CFR	Code of Federal Regulations
DBTC	Dibutyltin dichloride
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
HTS	High-throughput screening
IVIVE	<i>in-vitro in-vivo</i> extrapolation
LOAEL	Low observed adverse effect level
NOAEL	No observed adverse effect level

NOEL	No observed effect level
NTP	National Toxicology Program
OED	Oral equivalent dose
PVC	Polyvinyl chloride
rTK	Reverse toxicokinetics
RISK21	Risk Assessment in the 21st Century
SHEDS-HT	High-Throughput Stochastic Human Exposure and Dose Simulation
SPO	sodium (2-pyridylthio)-N-oxide
TK	Toxicokinetics

far, much of the work with the ToxCast data has focused on identifying endocrine disrupting chemicals, with some additional studies done to identify chemicals that potentially target specific pathways (D. Filer et al., 2014; Janesick et al., 2016; Pham et al., 2016; Reif et al., 2010). Little work has been done on a chemical-specific level, where the goal is based on identifying a mode of action or a point of departure for a specific chemical lacking hazard or safety data using this battery of assays (Shah et al., 2016).

Risk assessment is the process in which toxicity data, which are used to determine hazard, are combined with exposure data to determine the risk to a population for a particular chemical and use/exposure scenario. RISK21 is a flexible risk assessment framework that provides a transparent process to assess and visualize risk (Pastoor et al., 2014). This iterative process allows the user to evaluate risk (both hazard and exposure information) at each step of the process, refining information and estimates in a tiered manner, until there is enough precision to address the particular decision context (Embry et al., 2014). This can be visualized using the RISK21 webtool, providing a transparent way to present risk at each stage (HESI, 2017).

Chemicals having any use associated with food are of particular concern for chemical safety evaluation, as human exposure to chemicals in food is direct and frequent, and it is estimated that thousands of chemicals are added to foods in the US, either directly or indirectly (Neltner et al., 2011). The ToxCast screening library includes many chemicals having a use associated with food, and many of these chemicals show bioactivity in ToxCast assays (Karmaus et al., 2017, 2016). In the current study, we conduct case studies of two indirect food additives where we compare the animal and ToxCast data for both chemicals, using the RISK21 approach to provide an example and gain insight into a possible use of ToxCast HTS data. The purpose is to give context to HTS data, highlight a potential method for using HTS data in food safety assessment, and identify the challenges involved.

2. Materials and methods

2.1. Selection of chemicals for case studies

Two chemicals were selected for the case studies from the indirect food additives identified in ToxCast (Karmaus et al., 2017, 2016). Initially, the 20 indirect food additives with the highest number of active endpoints in the ToxCast data, after cytotoxicity filtering, were considered. Of these twenty, the two selected chemicals were ultimately picked based on the availability of animal toxicity data and initial exposure estimates (Karmaus et al., 2016). The two chemicals selected were sodium (2-pyridylthio)-N-oxide (also commonly referred to as pyriothione sodium or sodium omadine, abbreviated here as SPO, CASRN 3811-73-2; DTXSID3042390) and dibutyltin dichloride (abbreviated here as DBTC, CASRN 683-18-1; DTXSID8027292). SPO is an antimicrobial used on food contact surfaces and in adhesives used to make food packaging. DBTC is a catalyst and a heat stabilizer in polymers used in plastics (21 CFR§177.1680) (Forsyth, D S et al., 1993; Harper et al., 2005; Kannan et al., 1999; Quevauviller et al., 1991).

DBTC is one of a group of organotin chemicals including dibutyltin diacetate, dibutyltin oxide, dibutyltin dilaurate, and the tributyltins and monobutyltins that are often considered together for toxicity (European Food Safety Authority Panel, 2004; Harper et al., 2005; WHO, 2006). These chemicals are amenable to grouping as the moieties attached to the dibutyltin (dichloride, diacetate, oxide, dilaurate) are relatively weakly bonded to the tin atom and easily interchanged, and thus the chemicals can interconvert (Fisch et al., 1999; Poller, 1978; WHO, 2006). For the purpose of integrating as much *in vivo* animal toxicity and exposure data as possible, we have chosen to include data across this group of organotin chemicals for this analysis.

2.2. Exposure

Registered uses of both chemicals were found in FDA and EPA databases by searching for CAS registry numbers and common names as listed in ChemBook (ChemicalBook, 2016). Databases and resources that were used and/or checked are listed in Supplemental Table 1. DBTC was listed in the Code of Federal Regulations (CFR, 21 CFR§177.1680), and SPO was listed in the CFR (21 CFR§175.105) and in the Inventory of Effective Food Contact Substance (FCS) Notifications database (FCN#1659, FCN #175) from the FDA. Other organotins, including dibutyltin dilaurate, dibutyltin dioxide, and dibutyltin diacetate, are also listed in the CFR as catalysts in various polymers (21 CFR§175.105, 21 CFR§175.300, 21 CFR177.1680, 21 CFR§177.2420). For estimates of exposure, reports from various regulatory agencies were reviewed, including the FDA's CEDI database (U.S.FDA, 2012), the EPA's rapid chemical exposure and dose research (ExpoCast) project (U.S.EPA, 2014), the MIGRESIVES project (European Commission, 2010), and the FACET project (EU, 2012). For SPO, exposure estimates from the CEDI database (U.S.FDA, 2012) and the ExpoCast project (Wambaugh et al., 2014) were used, which are publicly available at the EPA's Computational Toxicology Chemistry Dashboard (U.S.EPA, 2016).

DBTC had an exposure estimate in ExpoCast, but was lacking a CEDI database entry or other exposure estimate. Accordingly, to increase confidence in the exposure estimates for DBTC, exposure modeling was conducted using the approach developed by Biryol et al. (2017) for chemicals in polymer food packaging (note this model was not applicable to SPO since it is not used as a polymer additive). This HTS-level modeling approach estimates exposure for chemicals used in food packaging. It combines physicochemical properties of chemicals (log of the octanol water partition coefficient, molecular weight, and solubility) with known uses and levels of chemicals in food packaging polymers to parameterize a model of migration from a food's packaging material into various food types under different storage conditions (Biryol et al., 2017). DBTC physicochemical properties were obtained from 2006 WHO CICAD #73 and Chembook (ChemicalBook, 2016; WHO, 2006). These migration estimates are then combined with food consumption data from NHANES food diaries within EPA's High-Throughput Stochastic Human Exposure and Dose Simulation (SHEDS-HT) (Isaacs et al., 2014) model to estimate exposure to foods containing

the chemicals used in packaging (USDA, 2015). DBTC is listed for use as a catalyzer in polyurethanes (21 CFR§177.1680), but is also listed in the literature as a heat stabilizer in PVC and has been detected in plastics in addition to polyurethanes such as PVC (Forsyth, D S et al., 1993; Pappaspyrou et al., 2007; Rosenberg, 2013). Accordingly, a SHEDS-HT exposure simulation was run with DBTC as a catalyst and another with DBTC as a heat stabilizer in PVC. Amounts of DBTC allowed for use in polymers are 0.5–1.5% as a heat stabilizer in PVC, and 0.001–0.5% as a catalyst in polymers, and these values were used to parameterize the exposure model simulations for the two uses (WHO, 2006). Because several dibutyltins are registered for use in different plastics, it was conservatively assumed that DBTC was used in all types of plastics as a catalyst in lieu of additional data. First, migration of DBTC from polymer packaging into food was estimated using the physicochemical properties of DBTC and the initial concentration in the polymer for various food categories, which were broken into aqueous, fatty, alcoholic, and acidic groups stored at various temperatures (U.S.FDA, 2007). This gave predictions of DBTC concentrations in foods. Secondly, exposure to food containing the migrated chemical was estimated using US food consumption data from NHANES food diaries. This results in a range of exposures to DBTC from the diet for a population of people. This is a conservative model, meant for chemical screening or prioritization, and makes several assumptions designed to favor over-estimation of exposure to the chemical, including the assumptions that dibutyltin is used in all of the polymers in which it allowed (full market penetration), the assumption that the polymer packing is a monolayer in direct contact with the food, and the assumption that the concentration of dibutyltin in foods is the same across the foods in a specific food category for the purposes of modeling individual consumption.

2.3. *In vivo* toxicity data

In vivo animal data for both chemicals were gathered from various sources. Searches were done for summary documents from regulatory agencies across the globe (Supplemental Table 1), and in databases such as ToxLine and PubMed. Data were collated and summarized. Reported NOEL, NOAEL and LOAEL values from animal bioassays were collected from reports from agencies and organizations involved in chemical safety, and used in this study as doses shown to cause activity in whole animal models (European Food Safety Authority Panel, 2004; Harper et al., 2005; OECD, 2008; U.S.EPA, 1996; WHO, 2006). Modes of action for both chemicals were identified if possible.

2.4. *In vitro* ToxCast data analysis

Assay-specific ToxCast data for both chemicals were downloaded from the ToxCast dashboard version 2 (U.S.EPA, 2015). Background and control assays, determined as assays that had “background measurement” or “null” as the intended target family, were removed. For SPO, 550 assays were obtained from the ToxCast Dashboard, and 447 remained for analysis after this removal. For DBTC, 325 assays were obtained from the ToxCast Dashboard and 228 remained for analysis after this removal. The remaining assays run were evaluated for activity using the hit calling criteria defined by the ToxCast Pipeline (tcp1) (D. L. Filer et al., 2014; Filer et al., 2017). If the assay was labeled a hit, it was labeled as an active assay for our purposes; all other tested assays were termed negative assays. A hit in ToxCast indicates that the response caused by the chemical in the assay exceeded the background noise of the assay and that a concentration-dependent trend (indicated by a best-fit model of Hill or gain-loss) could be fit to the data. For each active assay, a measure of potency in the form of an AC₅₀ value (representing the concentration (in μM) at which half of the maximal activity/efficacy is achieved with the chemical in the assay) was reported as well as a value for efficacy represented as the maximal magnitude of response for the compound in the modeled concentration-response

curve. For each chemical, active assays were organized by AC₅₀ value from low to high. Summary statistics were compiled for each chemical to find the mean and various percentiles across the AC₅₀ values for assays in which the chemical was active. The cytotoxicity center was calculated as the median AC₅₀ value among active cytotoxicity assays. The cytotoxicity limit (the concentration three global cytotoxicity median absolute deviations below the cytotoxicity center for a given compound) is reported on the ToxCast dashboard and was used to filter the results for potential confounding effects of cytotoxicity or cell stress (Judson et al., 2016). Active assays with an AC₅₀ value less than the cytotoxicity limit were counted separately as active assays after cytotoxicity filtering; cytotoxicity assays below the cytotoxicity limit were excluded from this count.

2.5. Estimation of steady state concentrations (C_{ss}) and oral equivalent doses (OEDs)

First, steady state concentrations (C_{ss}) for the two chemicals were estimated in order to compare the ToxCast AC₅₀ values with *in vivo*-derived NOAEL, NOEL, and LOAEL values. These C_{ss} values were used in conjunction with the ToxCast data, utilizing a process known as reverse toxicokinetics (rTK), to estimate oral equivalent doses (OEDs) (Wetmore et al., 2012). OEDs are defined as the external dose required to achieve plasma concentrations of the chemical equal to the *in vitro*-derived ToxCast AC₅₀ values (in μM). For SPO, *in vivo* toxicokinetic (TK) data from the literature was used to calculate C_{ss}; and for DBTC, an *in-vitro-in-vivo* extrapolation (IVIVE) approach was used (Mitoma et al., 1983).

In the case of SPO, *in vivo* toxicokinetic parameters such as the elimination rate, half-life, and volume of distribution were available for three animal species (rat, rabbit, and rhesus monkey) and were used to estimate the clearance (where the clearance equals the volume of distribution multiplied by the elimination constant) and C_{ss} (where C_{ss} is the rate of administration divided by the clearance) assuming a 1 mg/kg-bw/day chemical administration (Mitoma et al., 1983).

To calculate the OEDs, the following equation was employed.

$$\text{OED}(\text{mg/kg/d}) = \text{AC}_{50}(\mu\text{M}) \times \left(\frac{1\text{mg/kg/d}}{C_{\text{ss}}(\mu\text{M})} \right)$$

This equation assumes a daily oral exposure of 1 mg/kg-bw/day. OEDs were calculated for each ToxCast assay endpoint, for each chemical respectively.

With the absence of reliable *in vivo* toxicokinetic data for DBTC, we modified an approach used by Wetmore et al. to estimate TK parameters using parameters measured *in vitro*, and came up with several scenarios for DBTC to estimate the C_{ss} (Wetmore et al., 2015). This model estimates C_{ss} by combining estimates of nonmetabolic renal clearance (estimated as a function of glomerular filtration of the unbound fraction of the chemical in the kidney) with estimates of hepatic clearance based on clearance of the chemical by hepatocytes in culture:

$$C_{\text{ss}} = \frac{ko}{\frac{(Q_H \times F_{\text{ub}} \times Cl_{\text{intH}})}{(Q_H + F_{\text{ub}} \times Cl_{\text{intH}})} + (GFR \times F_{\text{ub}})}$$

Where ko = chemical exposure rate, Q_H = mean hepatic blood flow approximated as 90 L/h (Davies and Morris, 1993), F_{ub} = unbound fraction of parent chemical in the blood, Cl_{intH} = hepatic intrinsic metabolic clearance; and GFR = glomerular filtration rate. Cl_{intH} estimate is scaled to represent whole organ clearance (in liters/h) from a Cl_{u, in vitro} measurement of chemical clearance by hepatocytes in culture in (ml/(min × million cells)) based on the following:

$$Cl_{\text{intH}} = (Cl_{u, \text{in vitro}}) \times (\text{HPGL}) \times (V_1) \times \left(\frac{1}{10^6 \mu\text{L}} \right) \times \left(\frac{60 \text{ min}}{1 \text{ h}} \right)$$

Where HPGL is the hepatocytes per gram liver (110 million cells/g liver (Barter et al., 2007)); and V₁ is the liver volume (1596 g (Johnson

et al., 2005);). As there were no experimental measurements of plasma protein binding or intrinsic hepatic clearance for DBTC (and metals are outside the domain of applicability for *in silico* prediction tools), DBTC C_{ss} was estimated using different binding and clearance scenarios as a surrogate. Plasma protein binding can theoretically range from 0% (none bound) to 100% (all of the chemical bound), and DBTC binding to plasma proteins was set to three different values (95.5% bound, 90% bound, 50% bound). Similarly, one of four possible liver clearance rates were also employed (e.g., no, low, medium or high clearance), which were chosen following review of the range of values reported for the over 400 chemicals that have been analyzed in *in vitro* hepatic clearance assays to date (Wetmore, 2015; Wetmore et al., 2012). These values were used to calculate C_{ss} as previously described (Wetmore et al., 2012). The OEDs were then calculated as described for SPO, but across a range of scenarios (Fig. 4C).

2.6. Generation of graphs and RISK21 plots

RISK21 plots were developed using the RISK21 webtool (HESI, 2017). Input data were generated as described in the section above. Other graphs were generated using SigmaPlot software (Systat, Chicago, IL).

3. Results

3.1. Estimates of exposure

A variety of sources and tools were mined for exposure estimates. Registered food uses of both chemicals were identified by CAS registry number and common names (ChemicalBook, 2016). SPO (CAS# 3811-73-2) is listed for use as an antimicrobial agent for use on food contact materials and as an antimicrobial in adhesives used to make packaging (21 CFR§175.105, FCN#1659, FCN #175) (U.S.EPA, 2011). DBTC (CAS# 683-18-1) is used as a catalyst in polymers used to make plastic packaging. DBTC is registered for use in polyurethanes (21 CFR§177.1680), but other dibutyltins which have the ability to interconvert (dibutyltin oxide, dibutyltin dilaurate, dibutyltin diacetate) are registered for use in other polymers (21 CFR§175.105, 21 CFR§175.300, 21 CFR177.1680, 21 CFR§177.2420). DBTC is also reported to be used as a heat stabilizer in polymers such as PVC, though this use was not found in the CFR (Forsyth, D S et al., 1993; Pappaspyrou et al., 2007; Rosenberg, 2013).

SPO has an exposure estimate in the CEDI (Cumulative Estimated Daily Intake) database of 0.00016 mg/kg-bw/d (U.S.FDA, 2012). DBTC did not have an entry in the CEDI database, or in the other databases used in this study.

Both chemicals had a screening-level exposure estimate available from the EPA's ExpoCast project. The ExpoCast approach computationally estimates exposure using a high-throughput heuristic model to get a broad estimate of exposure to use to prioritize chemicals for further study, particularly intended to be used to estimate exposure for chemicals when no other data are available (Wambaugh et al., 2014). It uses the production volume of a chemical and four simple use

“heuristics” to estimate exposure via a regression against exposures inferred from biomonitoring data. In the case of SPO, the ExpoCast prediction corresponding to the upper 95% credible interval around the geometric mean, was below the CEDI value. Therefore, the median ExpoCast value for the whole population was taken as the low estimate of exposure for our analysis, and the CEDI value as the high estimate of exposure (Table 1).

Since only the ExpoCast exposure estimate was available for DBTC, an additional estimate of exposure to DBTC from migration from plastic packaging into foods was estimated as described in the Methods. Several dibutyltin chemicals, which have the ability to interconvert, are currently in use in plastic packaging with the relative usage amounts unknown (OECD, 2008). Therefore, we conservatively assumed dibutyltin use as both a heat stabilizer and a catalyst in a variety of polymers. This resulted in a range of exposures to dibutyltin in foods across the population, due to differences in food type consumption and body weights (Fig. 1). The estimates of exposure derived from this method were higher than the ExpoCast predictions, which is expected as the food contact exposure model makes a number of conservative assumptions including full market penetration of dibutyltin in the polymers for which it is permitted. For comparison with other data, the median values for the total population estimate for both the catalyst and stabilizer uses were added together and used as the high estimate of exposure (Table 1).

3.2. *In vivo* data (Traditional Toxicology Data)

Both chemicals were selected because of the availability of traditional *in vivo* toxicology data reviewed by various agencies and organizations involved with chemical safety (European Food Safety Authority Panel, 2004; Harper et al., 2005; OECD, 2008; U.S.EPA, 1996; WHO, 2006). The available data are summarized in Table 2.

SPO is registered with the EPA as a pesticide due to its antimicrobial properties and as such, the data on SPO comes through the EPA (U.S.EPA, 2011, 1996). The EPA established reference dose for SPO is 0.005 mg/kg-bw/d based on a NOEL of 0.5 mg/kg-bw/d (U.S.EPA, 1996). The most sensitive endpoint observed with SPO was neurotoxicity, comprising hind limb paralysis and skeletal muscle atrophy (U.S.EPA, 1996). SPO is an ionophore, but beyond this a mode of action has not been determined (Kim et al., 1999; Knox et al., 2004; Kondoh et al., 2002; Lind et al., 2009).

Several groups have reviewed DBTC, either alone or as a one of a number of organotin chemicals (European Food Safety Authority Panel, 2004; Harper et al., 2005; OECD, 2006; WHO, 2006). DBTC is an immunotoxicant, with changes in blood cell counts and hematocrit as the most often noted effects (OECD, 2006). Higher doses have been shown to cause reproductive toxicity as well as damage to the liver and pancreas. A definitive mode of action is unknown, but DBTC has been shown to induce apoptosis (Matsushita et al., 2012). DBTC also interacts with thiol-containing compounds, and may disrupt cell membranes and cytoskeletons (Ali et al., 1990).

DBTC and other dibutyltin chemicals are made and used on their own, but are also metabolites of tributyltin, and can break down into

Table 1
Human exposure estimates.

Compound	Exposure: Lower Bound (mg/kg-bw/d)	Exposure: Upper Bound (mg/kg-bw/d)
Sodium (2-pyridylthio)-N-oxide	2.39E-08 (ExpoCast) ^a	0.00016 (CEDI) ^b
Dibutyltin Dichloride	1.07E-07 (ExpoCast) ^a	0.015 (SHEDS-HT) ^c

^a ExpoCast predictions are from a high-throughput model based on production volume and listed uses providing broad estimate of exposure (Wambaugh et al., 2014).

^b Cumulative Estimated Daily Intake (CEDI) is a database maintained by the US FDA on exposure to food-use chemicals.

^c SHEDS-HT estimates dietary exposure to chemicals in food contact materials by modeling migration of the chemical from the food contact substance polymer into different foods, and then modeling exposure to the food containing the chemical using NHANES food intakes, and is a very conservative model, meant for prioritization (Biryol et al., 2017).

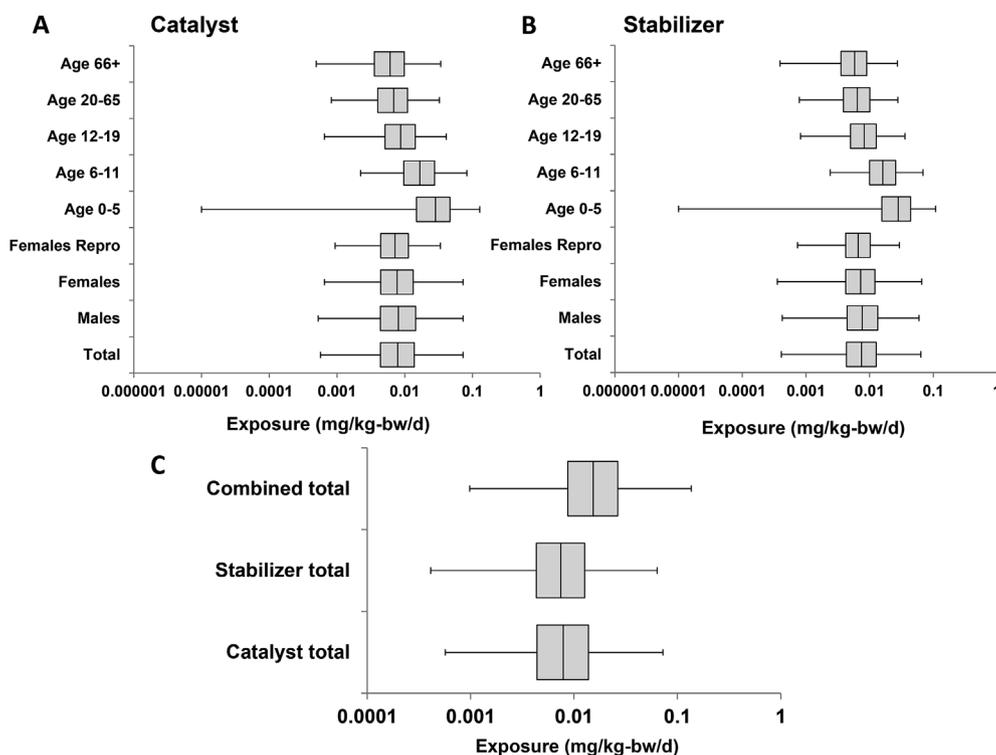


Fig. 1. DBTC Estimate of Exposure. Estimates of exposure for DBTC were generated by modeling the migration of dibutyltin from plastic food packing into foods based on physicochemical parameters and initial amounts of the chemical in the packaging. Human exposure to foods containing dibutyltin was estimated using food diaries from NHANES. The 1st, 25th, 50th, 75th, and 99th percentiles of exposure were calculated for the total population and for various subgroups. Estimates were computed and plotted separately when considering different uses for DBTC as A) a catalyst or B) a heat stabilizer. C) Exposure values for the total population were plotted for use of DBTC as a catalyst, heat stabilizer, or combined use.

monobutyltin (Kimmel et al., 1977; Ueno et al., 1994). In addition, the various dibutyltin chemicals can interconvert under certain conditions, such as acidic conditions seen in the stomach (OECD, 2008). Therefore, some groups consider these chemicals together in a group (European Food Safety Authority Panel, 2004). Tributyltin is the most well studied of the group, and all three have been shown to act similarly in the body. Several groups have come up with tolerable daily allowances for DBTC and other organotin compounds (Table 2). The European Food Safety Authority (EFSA) holds the lowest limit that is applied across the group of organotin chemicals after deeming them to possess a similar mode of action due to their similarity in structure and in effects observed in short-term assays (European Food Safety Authority Panel, 2004). EFSA set the limit based on the most sensitive endpoint effect level from a longer term tributyltin study (European Food Safety Authority Panel, 2004).

The exposure data were combined with the *in vivo* data for both chemicals to generate a plot using the RISK21 risk visualization matrix (Fig. 2). The matrix plot is an integration of traditional *in vivo* animal study toxicity data (Table 2) and exposure estimates (Table 1). For each chemical, the maximum and minimum exposure estimates were plotted on the X-axis, and the reference dose or tolerable daily intake was

plotted on the Y-axis as a point estimate (using the NOEL for SPO and the NOAEL from the 2 year chronic rat study for DBTC in Table 2) plus the uncertainty factor of 100 to account for interspecies and intraspecies variation.

3.3. ToxCast data

ToxCast data were processed to remove background and control assays, resulting in SPO having been evaluated across 447 assays, of which 131 were reported as active. The cytotoxicity center and cytotoxicity limit for filtering, determined as described in the methods, were 6.23 μM and 2.06 μM respectively. After comparing the AC_{50} values from active assays to the cytotoxicity limit, there were 18 active assays that were not considered cytotoxicity-confounded based on our approach (Fig. 3A and B). These 18 assays are listed in Table 3 and reflect many target groups, with no specific biological targets based on intended target as listed in ToxCast appearing as distinctly sensitive to SPO.

For DBTC, 228 assays were run after removal of control assays, and 85 of these were reported as active. Similar to SPO, the assays determined to be active span a range of target groups, and no one group

Table 2
Traditional toxicological data.

Compound	NOAEL/LOAEL	Study type	Endpoint	Limit	Source
Sodium (2-pyridylthio)-N-oxide	0.5mg/kgbw/d (NOEL)	2yr chronic rat (oral)	Neurotoxicity (hind limb paralysis)	0.005mg/kg-bw/d	USEPA, 1996
Dibutyltin Dichloride	2mg/kgbw/d (NOAEL)	90d rat (oral)	Reduced body weight and food intake, hematological effects	None determined	OECD SIDS, 2006
Dibutyltin Dichloride	5mg/kgbw/d (LOAEL)	6wk rat (oral)	Immunotoxicity	0.005mg/kg-bw/d	ATSDR, 2005
Dibutyltin Dichloride	2.5mg/kgbw/d (LOAEL)	6wk rat (oral)	Immunotoxicity	0.003mg/kg-bw/d	WHO, 2006
Organotin Compounds	0.025mg/kgbw/d (NOAEL) ^a	2yr chronic rat ^a	Immunotoxicity	0.00025mg/kg-bw/d ^b	EFSA, 2004

^a This study was conducted using tributyltin.

^b This limit is for a group of organotin compounds including dibutyltin dichloride. EFSA considered the effects dibutyltin, tributyltin, dioctyltin, and triphenyltin to be similar enough to warrant a group limit, and derived the limit from a 2 year chronic study with tributyltin and shorter studies with all four compounds individually.

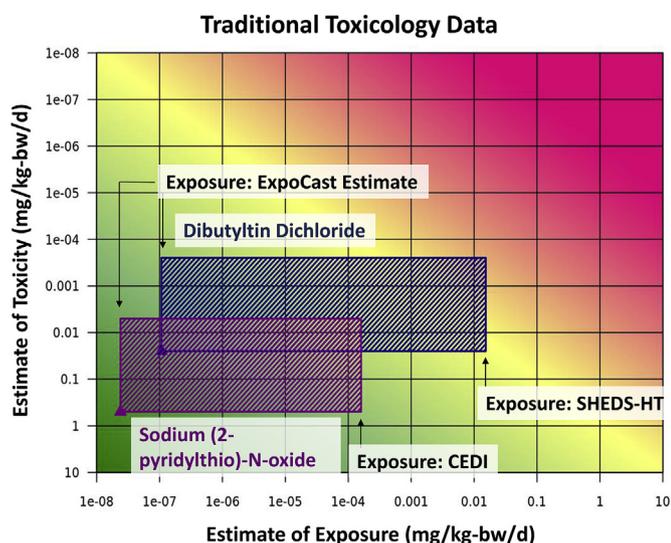


Fig. 2. RISK21. Estimates of exposure from Table 1, and the most sensitive NOEL/NOAEL value from Table 2 were used to develop a RISK21 plot using the RISK21 webtool. The ExpoCast estimates of exposure are used as the lower estimate of exposure for both DBTC and SPO, and the upper estimates of exposure are the CEDI value for SPO and the SHEDS-HT value for DBTC. Estimates of toxicity (a NOEL of 0.5 mg/kgbw/d for SPO and a NOAEL of 0.025mg/kgbw/d for DBTC) were plotted as point estimates with an uncertainty factor of 100 to define the reference dose or tolerable daily intake established limit for each compound. The top of the colored boxes per chemical denote the reference dose or the tolerable daily intake, and the bottom of the box is the dose given to the animal used to determine that limit.

emerges as a most sensitive target group to DBTC. The cytotoxicity center for DBTC was 1.07 μM , with a cytotoxicity limit of 0.26 μM , and 12 assays remain after cytotoxicity filtering (Fig. 3 A, C). The assays remaining after cytotoxicity filtering are listed in Table 3. For both chemicals, there is no group of most sensitive assays, and many of the assays after cytotoxicity filtering are loss-of-signal assays. For SPO, many of the active assays after cytotoxicity filtering are BioSeek assays investigating inflammatory and angiogenic endpoints or Tox21 nuclear

receptor assays, and for DBTC many of the active assays after cytotoxicity filtering are Tox21 nuclear receptor antagonist assays. However, the BioSeek assays were not run for DBTC, and the difference in the types of assays hit may be influenced by the assays run for each chemical in ToxCast.

OEDs were calculated from the ToxCast AC_{50} values, and the calculated OEDs were plotted alongside the NOAEL and LOAEL levels from the animal data for both chemicals (Fig. 4). For both compounds, OEDs were calculated for several different AC_{50} values to get the range of possible OEDs, including the cytotoxicity center, the cytotoxicity limit, and the 5th, 25th, 50th, 75th, and 95th percentile AC_{50} values for all active assays. For SPO, the OEDs lined up well with the values from the ToxCast data. The LOAEL falls between the cytotoxicity limit and cytotoxicity center OED values, indicating that bioactivity in the ToxCast assays occurs at concentrations that align with doses seen to cause effects in animal bioassays (Fig. 4 A). For DBTC, the relationship was not as clear. In lieu of measured TK parameters, when the most conservative rTK estimates were used (no hepatic clearance and 95.5% of the chemical bound to plasma proteins), IVIVE-based calculations to estimate OEDs from bioactivity in ToxCast were well below doses needed to cause effects in the animal bioassays (Fig. 4 B). When the rTK estimates were varied to include different possible plasma protein binding levels and hepatic clearance levels that span the range of hepatic clearance values measured *in vitro* for over 400 chemicals for this method (Fig. 4 C), it was found that the calculated OEDs for any given ToxCast parameter shifted by three orders of magnitude. Thus, for DBTC, the lack of TK data limits the conclusions that can be drawn from the data, and future work to generate rTK parameters could significantly improve estimates.

4. Discussion

This study presents case studies of two indirect food additive chemicals for which *in vitro* HTS data from ToxCast were compared to *in vivo* toxicity data using the RISK21 risk assessment-based decision framework. The RISK21 framework is a problem-formulation based exposure driven risk assessment tool that visually represents risk by graphically plotting exposure and hazard data. One of the main challenges facing incorporation of HTS data in chemical risk evaluation is the

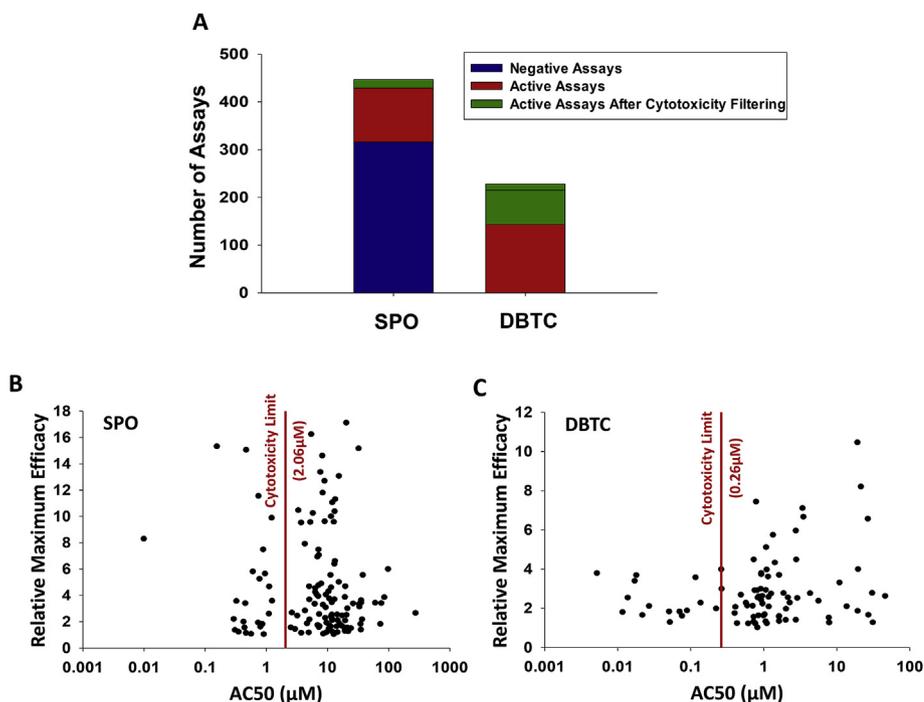


Fig. 3. Summary of the ToxCast data for DBTC and SPO. Assay-specific data for both chemicals were pulled from the ToxCast Dashboard. A) ToxCast hit calls were used to define negative and active assays. Cytotoxicity filtering was done by applying the cytotoxicity limit as a cutoff value wherein assays having an AC_{50} below the cytotoxicity limit remained active after filtering. B, C) All assays determined to be active for either compound were plotted as AC_{50} versus the modTp (maximum efficacy of modeled concentration-response curve) parameter from the ToxCast data, for each assay, with the cytotoxicity limit plotted as a straight line.

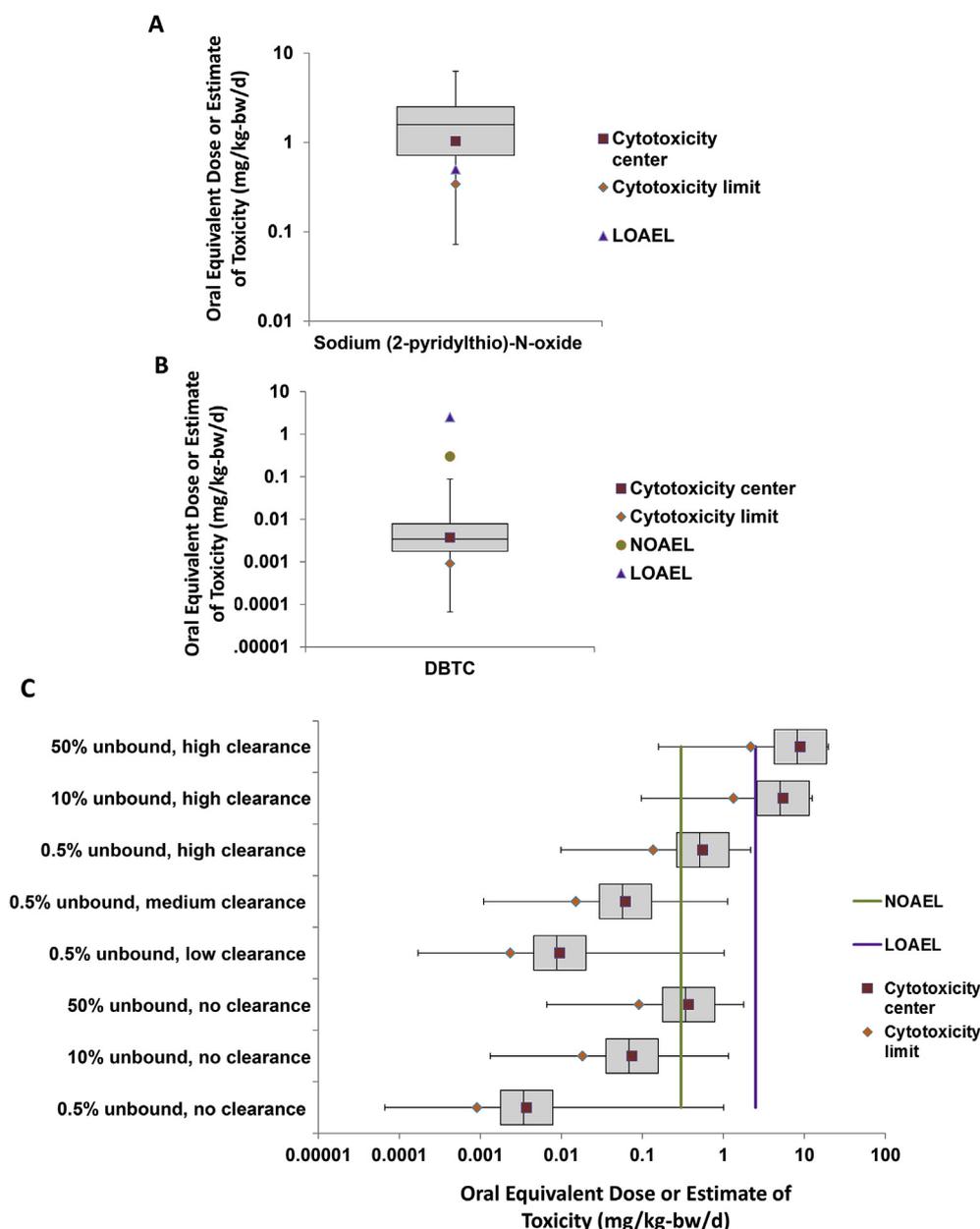


Fig. 4. Comparison of ToxCast and Traditional Toxicology Data. ToxCast assay AC_{50} values were converted to oral equivalent doses (OED) and plotted as a distribution across the 5th, 25th, 50th, 75th, and 95th percentiles for all active assays for SPO and DBTC. The LOAEL/NOAEL values from the *in vivo* animal data, the cytotoxicity center, and cytotoxicity limit OEDs are plotted as point estimates. A) OEDs for SPO were determined using available toxicokinetic data. B) OEDs for DBTC were determined using the worst-case toxicokinetic parameter estimates. C) For DBTC, a range of toxicokinetic parameter estimates were used to determine OEDs from the AC_{50} values for the 5th, 25th, 50th, 75th, and 95th percentiles of all active assays, the cytotoxicity limit, and the cytotoxicity center. The *in vivo* NOAEL and LOAEL data are plotted as point estimates shown as green and purple lines respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ability to relate an *in vitro* bioactive concentration to an external dose. Our study directly addressed the challenge of converting concentrations from *in vitro* assays into comparable units of doses given *in vivo*. For one chemical used in this study, SPO, conversion of concentrations that elicit bioactivity in ToxCast to OEDs showed good correlation to *in vivo* animal-derived LOAELs. For the other chemical, DBTC, this was not the case. However, while SPO had available TK data from animal studies, DBTC did not have *in vivo* TK parameters. As an alternative, we utilized worst-case TK parameter estimates for IVIVE with DBTC, and found that this resulted in the concentrations that elicited bioactivity in ToxCast mapping to OEDs well below those shown to cause effects in animal studies. This is similar to what would be done on a first round risk assessment where, in the absence of data, worst case estimates would be used to see if an adequate decision could be made using those data. Comparing predictions from the worst-case scenario estimates against those derived using less conservative TK input parameters revealed a shift in OED potency of three orders of magnitude for DBTC. These OED values range from well below the dose shown to cause NOAEL/LOAEL effects *in vivo* to slightly above the LOAEL dose level, indicating that the

more conservative estimates would result in lower levels relative to current data, and that refinement of the TK parameters has the potential to bring OEDs calculated from the ToxCast data into line with doses shown to cause effects *in vivo*. This finding underscores the importance in generating or refining uncertain TK information to ensure productive comparisons can be made between *in vitro* and *in vivo* datasets, as well as the potential utility of *in vitro* TK estimates for IVIVE.

As seen with DBTC, without adequate TK data IVIVE is challenging, but this difficulty can start to be overcome by employing worst-case estimates. Many chemicals besides DBTC are lacking toxicity data including TK data, and gaining these data would be of great significance for this type of work (Judson et al., 2009; Neltner et al., 2013). Work is ongoing in the estimation of TK parameters using *in vitro* assays as well as in the development of predictive models (Ingle et al., 2016; Wetmore, 2015; Wetmore et al., 2013). Expansion and refinement of these datasets and tools will be of great importance for studies such as this one. For our study, we wanted to show what would result from using rough estimates for IVIVE, as the most conservative values are often used in risk assessment when more specific data are not available

Table 3
Assay remaining after cytotoxicity filtering.

Sodium (2-pyridylthio)-N-oxide		Dibutyltin Dichloride	
Assay	AC50 (μM)	Assay	AC50 (μM)
ACEA_T47D_80hr_Positive	0.4427	ATG_PPRES_CIS_up	0.1376
APR_HepG2_OxidativeStress_24h_up	0.8647	TOX21_AR_BLA_Antagonist_ratio	0.0272
APR_HepG2_StressKinase_24h_up	0.8041	TOX21_AR_LUC_MDAKB2_Antagonist	0.0896
BSK_CASM3C_Thrombomodulin_up	0.9144	TOX21_ARE_BLA_agonist_ratio	0.0052
BSK_hDFCGF_CollagenIII_down	1.2514	TOX21_Aromatase_Inhibition	0.1174
BSK_hDFCGF_IL8_down	0.4710	TOX21_ERa_BLA_Antagonist_ratio	0.0172
BSK_hDFCGF_IP10_down	0.7484	TOX21_FXR_BLA_antagonist_ratio	0.0181
BSK_hDFCGF_MCSF_down	0.6056	TOX21_GR_BLA_Antagonist_ratio	0.0220
BSK_hDFCGF_MIG_down	0.1566	TOX21_PPARD_BLA_antagonist_ratio	0.0118
BSK_hDFCGF_MMP1_down	0.8950	TOX21_PPARG_BLA_antagonist_ratio	0.0709
BSK_hDFCGF_PAII_down	1.1242	TOX21_TR_LUC_GH3_Antagonist	0.0138
BSK_hDFCGF_TIMP1_down	0.9571	TOX21_VDR_BLA_antagonist_ratio	0.0767
BSK_hDFCGF_VCAM1_down	0.7786		
TOX21_AR_BLA_Antagonist_ratio	0.2953		
TOX21_Aromatase_Inhibition	0.3265		
TOX21_FXR_BLA_antagonist_ratio	0.4553		
TOX21_PPARD_BLA_antagonist_ratio	0.3095		
TOX21_TR_LUC_GH3_Antagonist	1.1110		

(Embry et al., 2014; Pastoor et al., 2014). Our findings highlight that having even basic TK estimates such as *in vitro* measures of protein binding and liver clearance can greatly improve results and produce more accurate estimates.

For both chemicals, a mechanism of action was not readily evident based solely on the ToxCast data. While for some biological outcomes HTS assay coverage is robust, for others it is not, or data across the full assay battery may not be generated for all chemicals (Silva et al., 2015). If the chemical does not hit one of these well covered outcomes, it is difficult to determine the mechanism of action for the chemical, and to predict what toxicity would be seen *in vivo*. As demonstrated by other researchers, case studies done with chemicals with known mechanisms of action including endosulfan and methidathion, or ortho-phthalate chemicals, were able to match up some *in vivo* effects with ToxCast data, but were not able to match other *in vivo* endpoints, demonstrating that more needs to be done connecting *in vitro* bioactivity to *in vivo* outcomes (Pham et al., 2016; Silva et al., 2015). Both of the chemicals used in our study were rather potentially cytotoxic in the ToxCast assays, and had relatively few hits below the cytotoxicity limit. Chemical concentrations that cause cytotoxicity in HTS assays show a burst activity in a number of assays, which is believed to be associated with cell stress and cytotoxicity responses as opposed to a single specific biomolecular interactions (Judson et al., 2016). It is possible that these chemicals do not have a specific cellular target and mechanism of action, but cause general cell stress and cytotoxicity, thus leading to toxicity. In this case, these results from the ToxCast assays are informative if the cell stress and cytotoxicity is the means by which these chemicals cause toxicity; however, without more assays and improved interpretation of existing assays, this is impossible to ascertain with certainty.

One exciting finding from this study is that for SPO, even though a mechanism of action could not be found using the ToxCast data, the values aligned well with the animal data, and would give a similar risk assessment outcome. Whether this is true for other chemicals is uncertain, and could be evaluated by doing this type of analysis on additional chemicals in ToxCast or other HTS data sets.

Another challenge is the lack of exposure data available for chemicals with any use associated with foods. From the RISK21 plot in Fig. 2, it is evident that the estimate of exposure needs refinement for both chemicals, especially DBTC, while the estimates of toxicity using traditional animal-based methods are adequate. For many chemicals, estimates of exposure are either non-existent, or are determined at a broad screening level (Wambaugh et al., 2014). This works well to

prioritize chemicals for further study, but is not always adequate from a risk assessment perspective. The exposure modeling done with DBTC demonstrates one method of dealing with this data gap. However, even when such exposure model predictions are available, they may be too uncertain or overly conservative for use in final risk evaluations. Efforts to calibrate such conservative models with monitoring data or to refine information on the use of various chemicals in food applications (and corresponding migration and consumption information) can reduce this uncertainty. Data gaps in exposure and differences in exposure prediction approaches are highlighted in this paper by the large distance between the ExpoCast prediction and the CEDI or SHEDS-HT exposure estimates for both compounds. This discrepancy is due to differences in how these two exposure estimates are generated. To predict likely exposure, ExpoCast uses the amount of the chemical produced and 4 chemical use categories: pesticide active use, pesticide inert use, chemical/industrial process use with no consumer use, and consumer use and chemical/industrial process use. The ExpoCast model used here (which provides the current ExpoCast exposures in the CompTox Chemistry Dashboard) uses a regression model based on 5 heuristics as predictors of likely exposure. It takes into consideration the production volume of the chemical, and if the chemical has use as a pesticide inert ingredient, a pesticide active ingredient, consumer and industrial/commercial use, and industrial/commercial but no consumer use (important note for this is that the consumer and industrial use and industrial but no consumer use categories do not include food uses). The model median and its uncertainty are estimated via a calibration against the NHANES population median and is thus reflective of the exposure to the chemical via all pathways for an average member of the population. It is not designed to be conservative per se, but rather the best model estimate for the true population median (with associated uncertainty). CEDI and SHEDS-HT, on the other hand, take a different approach to estimating exposure – both are predicting food-specific use by taking the known use of the chemical in food contact substances (FCS), the amounts allowed in the FCS, and then predicting migration from the FCS into the food and consumption of the food. These mechanistic pathway-specific models make numerous conservative assumptions (Section 2.2), and thus may reflect a worst-case estimate of the population median. The SHEDS model predictions are not (as presented here) calibrated against biomonitoring data (as are the ExpoCast values). A comparison of the SHEDS values with biomonitoring data in Biryol et al. (2017) demonstrated that the SHEDS-HT values were likely over-estimates by at least two orders of magnitude. Recent work has integrated multiple SHEDS models (as well as other mechanistic

exposure models) with NHANES biomonitoring data in an evaluation framework called the Systematic Empirical Evaluation of Models (SEEM) (Ring et al., 2019). These efforts will allow for the integration of food contact pathways into next-generation ExpoCast exposure values while simultaneously correcting over-conservative models for individual pathways. Future work should also address the data gaps in the mechanistic food contact pathway exposure models that result in the use of overly-conservative assumptions. Such improvements will increase the predictive power of the food contact models within the SEEM framework, and hopefully reduce the resulting uncertainty in future ExpoCast predictions.

The case of DBTC highlights additional considerations needed for chemical groups since there is no standardized way of dealing with related chemicals that can interconvert and have similar effects (Groh and Muncke, 2017). Some agencies consider all members of such groups individually, whereas other agencies consider members of the group together. Clarity in the best practices for dealing with these types of chemicals are needed.

This study shows the potential for HTS data to be used in toxicity testing and food safety analysis, while also highlighting the need for more toxicokinetic data and increased breadth of HTS assay coverage. Though specific mechanisms of action were not identified from the ToxCast data for either compound, for SPO the OEDs calculated from the ToxCast data correlated with doses shown to cause effects *in vivo* and would lead to similar risk assessment conclusions. OEDs calculated for DBTC varied greatly depending on the TK parameter estimates of plasma protein binding and hepatic clearance used for IVIVE, but were generally lower than or included doses shown to cause effects *in vivo*. Great strides have been made in generating HTS data across commercial chemicals, and equally important will be availability of TK and exposure data to facilitate use of these data in chemical safety assessments.

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Disclaimer

The views expressed in this paper are those of the authors and do not necessarily reflect the views of their affiliations or policies of the US Environmental Protection Agency.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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