White Paper Prepared for the ACC LRI on Application of Non-Targeted Exposure Analysis in Risk Assessment: Opportunities and Challenges[1]

Executive Summary

Estimating the risk of environmental chemicals leading to human health effects requires characterization of two quantities: the concentration at which the compound causes effects and the exposure to the compound in the environment. Embracement of new approach methodologies and recent technological advances have increased the rate at which we can evaluate the concentration-response function that describes how a compound impacts a biological system. High-throughput screening methods make it possible to screen large numbers of chemicals for potential biological activity in less time using significantly fewer resources. Methods for estimating the environmental concentration of a substance—a necessary input parameter for calculating exposures—have lagged behind, but are slowly catching up. Until recently, the majority of these efforts have focused on estimating exposures using chemical-specific exposure pathway information or inferring exposures from biomarker levels in the population (e.g., NHANES). These traditional approaches are targeted (i.e., they are designed to measure concentrations of a defined set of pre-determined known chemical molecules). Recent emergence of the concept of the "exposome" and the hypothesis that chemically-mediated health outcomes are a function of aggregate exposures to a plethora of compounds has stimulated development and application of more screening-level approaches for exposure estimation. In one approach, non-targeted exposure analysis (NTEA), environmental or biological samples are analyzed to uncover, and postulate potential structures of, as many compounds as possible present in complex matrices that represent sources of human chemical exposure (house dust, personal samplers, etc.). These non-targeted approaches hold significant promise for more rapid estimation of the complex chemical exposures associated with different locales and populations. However, NTEA methods are typically only able to tentatively characterize molecular structures and usually do not quantify concentrations. Follow up studies, in which the suspected chemical structures are more directly investigated, are needed to generate definitive structural confirmation of presence and to quantify concentrations. Nevertheless, the rapid evolution of the scientific concepts, technologies, and applications of NTEA create both challenges and opportunities for the chemical risk assessment community. Here we discuss the current state of NTEA, its promise to aid different levels of chemical safety decisions, and a number of emerging challenges and pitfalls that impact its utility.

Rapid advances in mass spectrometry approaches have been the key enabling technology behind NTEA. Coupled with other separation techniques—principally liquid chromatography—mass spectrometry is used to differentiate chemical constituents of a sample. Whereas conventional exposure measurements target a single or a small number of compounds in an environment, NTEA is potentially free from presumption of hypothesis. In concept NTEA results in a survey of the environment without bias; in reality decisions made throughout the design and execution of the experiments have a sizable impact on the outcome. Most significantly, we find that decisions made in sample collection (e.g., the use of silicon wristbands versus other

[1] September 20, 2019. This white paper was prepared by ScitoVation scientists with support provided by ACC LRI. The contributing scientists included Jeffrey R. Enders, Martin B. Phillips, Harvey J. Clewell III, Rebecca A. Clewell, Miyoung Yoon and Patrick McMullen (editor). The authors thank Elin Ulrich, Mark Strynar, and Jon Sobus for helpful discussions related to this work.

passive samplers, whether particulates are fractionated from air before analysis, etc.) and sample analysis (e.g., the separation technology employed, the database of compounds used to compare mass spectra against) have the potential to significantly impact findings. The sensitivities of separation and measurement technologies (i.e., mass spectrometry in various modes, liquid chromatography, gas chromatography, etc.) to detect compounds are largely dependent on the physical-chemical properties of the compound. The potential of each of these experimental factors to introduce systematic bias and influence study outcomes makes inferring quantitative exposure levels, or even definite identification of analytes incredibly nuanced. The field continues to evolve, but presently is primarily suited for hypothesis generation and falls well short of being able to be used as part of a quantitative risk assessment.

Although NTEA currently appears to be of little value for quantitative exposure assessment, its value could be greatly increased in the future through:

- Evaluation of an approach's domain of applicability in terms of chemical properties and the ability of the sampling media and device to accurately reflect human exposure needs to be conducted. Defining domains of applicability of various sampling tools used to provide inputs for NTEA analysis and also identifying a way to augment the current sampling and collection practices based on a combination of approaches, both for the environmental exposure data and prediction tools.
- Development of in silico prediction methods such as quantitative structure property relationships (QSPR) for chemical transfer from the environment to the sampling devices versus the human body should be the first step in that direction. The generation of experimental data with currently available devices will be required to support the development of such computational prediction tools.
- Understanding human exposure factors influenced by lifestyle and individuals' physiological conditions is also critical in making risk-based decisions based on NTEA.
- More concerted efforts to build database resources for the NTEA community would contribute to reducing potential biases pertaining to the specific datasets used for data and chemical structure interpretation.

Background

Exposure data are necessary to understand the human health impacts of chemicals. The exposome, as it was coined by Wild in 2005, is "all life-course environmental exposures (including lifestyle factors), from the prenatal period onwards"[1]. Under the new paradigm for toxicity testing[2, 3], advances have been made in moving towards the use of *in vitro* and computational approaches, as evidenced by multiple initiatives in North America[4-6] and the European Union (EU). The ultimate aim of these initiatives is to increase both the efficiency and the human relevance of toxicity testing for chemical safety assessment. High throughput screening (HTS) with *in vitro* toxicity assays have made it possible to screen a large number of chemicals for potential biological activity in less time with significantly fewer resources than traditional *in-vivo* tests. The United States Environmental Protection Agency's (USEPA's) ToxCast margin of exposure analysis has demonstrated the utility of HTS data in risk assessment for prioritization when converted to *in vivo* equivalent exposures[7-10]. These efforts however, revealed the huge information gap in human exposure. It is becoming clear that in moving towards *in vitro/in silico* based risk assessment, technologies for rapid screening of biological activity are much farther along in development than methods for rapid estimates of human exposure. Reliable human exposure information is critically required to coherently integrate and apply *in vitro* and *in silico*-based data to risk and safety assessment. Substantial efforts are ongoing in government, academia and industry to rapidly estimate human exposure to large numbers of chemicals[11-16]. However, until recently, the majority of efforts have focused on estimating exposures using chemical-specific exposure pathway

information or inferring exposures from biomarker levels in the population [e.g., National Health and Nutrition Examination Survey (NHANES)]. These approaches are targeted; they estimate the exposure to a known chemical. In a regulatory landscape where rapid screening for chemical prioritization is becoming common practice, these one-chemical-at-a-time approaches are not sufficiently high throughput. Further, targeted approaches are unnecessarily limiting in scope, as they only seek to evaluate known chemicals of interest, but only scratch the surface of the exposome of a person or a population.

In contrast to these targeted approaches, non-targeted exposure analysis (NTEA) does not adhere to the mantra of: form hypothesis, test hypothesis, reconcile hypothesis against collected data to draw conclusions. Such hypothesis-driven approaches can limit the scope of the study by requiring an idea of what the result may be before the study begins. This can lead researchers to focus only on the analytes they think are most important and potentially miss the very abundant but otherwise unknown analytes. Often, exposure studies are prioritized based on a prior knowledge of chemical potency and toxicological effects. Chemicals with biological activity in traditional hazard identification studies are then studied to evaluate other components of risk-based decisions, including dosimetry and exposure. This workflow either intentionally or unintentionally biases exposure analysis toward chemicals that have undergone traditional effects studies, and neglects chemicals with little toxicological effect data. In other words, this workflow ignores the important role of exposure in determining human risk—prioritizing compounds through biological activity alone. Instead, NTEA seeks to collect all information and then use it to form the initial hypothesis, thereby negating this initial bias.

NTEA is increasingly being used in an effort to identify and characterize chemicals in environmental and biological media to efficiently generate quantitative exposure data for a growing number of chemicals. Many of these chemicals remain largely unexamined as demonstrated by a number of recent works presented at the Non-Targeted Analysis Workshop held by US EPA in 2015, "Advancing Non-Targeted Analyses of Xenobiotics in Environmental and Biological Media". Notable advances have been made in characterizing environmental chemicals found in house dust[17].

NTEA has great potential to unravel the complexity of the exposome and fill in the information gaps for human exposure as the field moves forward with improved methodologies for risk-based decision making for chemical safety. Rapid technological advances will continue to drive experimental initiatives to the edge of what is possible. However, caution must be exercised as NTEA is still in its infancy, and its results can be extremely complicated and nuanced. As such, the utmost care must be taken to ensure that data analysis and interpretation are transparent. The studies should remain unbiased, to the extent possible, so that the most useful data can be made available for human health assessments, and to avoid unnecessarily alarming the general public. Most importantly, it should be acknowledged that NTEA data for chemical profiles alone generally cannot be directly used for quantitative exposure assessment.

What is Non-Targeted Exposure Analysis (NTEA)?

Non-targeted exposure analysis is intended to collect a large amount of information about chemical concentration without prior bias or prioritization of analyte or analyte class. In this way, non-targeted analyses are hypothesis-generating as opposed to following the canon of the scientific method. In the literature, non-targeted and untargeted are at times used interchangeably, however, untargeted is predominantly used in the metabolomics/flux analysis literature. Though both terms are used to describe a hypothesis-generating analysis, the term "non-targeted" will be used throughout this work.

The "streetlight effect" (Figure 1) illustrates how what we find is influenced by where we are best able to search. Targeted analysis can allow quantitation of ever-smaller concentrations of substances in environmental and biological media. However, it is implicitly understood that these methods fall far short of being exhaustive. Non-targeted analysis is a way of bypassing the observational bias inherent in past methodologies. The goal of these methods is to capture as wide a range of exposures as possible. In general, non-targeted methods prioritize breadth over more typical considerations such as quantitation, structural identification, and/or biological relevance.

Figure 1. Cartoon illustrating the "Streetlight Effect."

Other forms of exposure analysis exist that are principally hypothesis-generating, but do not fall under the category of "non-targeted exposure analysis" as we have defined it. These include environment-wide association studies (EWAS) used for epidemiological analyses. These techniques do not necessitate the use of any non-targeted forms of analysis, but instead require subject-matched estimates of exposures and measurements of health status and/or outcome. An example of this type of study by Patel et al. uses data from various targeted blood-borne screening techniques (i.e., NHANES data) to generate hypotheses regarding the causes of type 2 diabetes[18]. The blood-borne screening data in NHANES is neither non-prioritized nor hypothesis generating; rather, these measurements were taken for a specific purpose (i.e., the evaluation of the nutritional and health status of the US non-institutionalized population) and were acquired using traditional, validated analytical methods specifically designed to measure for substances of interest. For these reasons, EWAS and related studies will not be covered in this work. The reader is directed to other notable works for further information[19-23].

Why do NTEA?

While initiatives such as ToxCast[24, 25] have made progress toward assessing potential biological activity of the universe of chemicals in commerce, less progress has been made to estimate population-level exposure to these chemicals. Several models generate estimates based on NHANES biomonitoring data, notably ExpoCast[11, 26], the United Nations Environment Program and Society for Environmental Toxicology and Chemistry toxicity model (USEtox)[27], and the Risk Assessment Identification and Ranking (RAIDAR) model[28]. However, NHANES is not comprehensive in terms of its chemical toxicant coverage (the 2009-2010 cycle measured concentrations of 363 unique chemical substances[29] compared to the 7,000-30,000 chemicals in commerce). As was noted by Wambaugh, et al.: "expanded monitoring data are needed to better characterize actual exposures. For the majority of chemicals, where resources, such as NHANES data, are not available, new and more flexible approaches are needed to quantify population-level chemical exposures"[11]. NTEA is uniquely suited to support this growing need (Figure 2). As a matter of fact, there has been a rise in

the use of non-targeted exposure analyses over the last decade in an effort to map human chemical exposure (Figure 3).

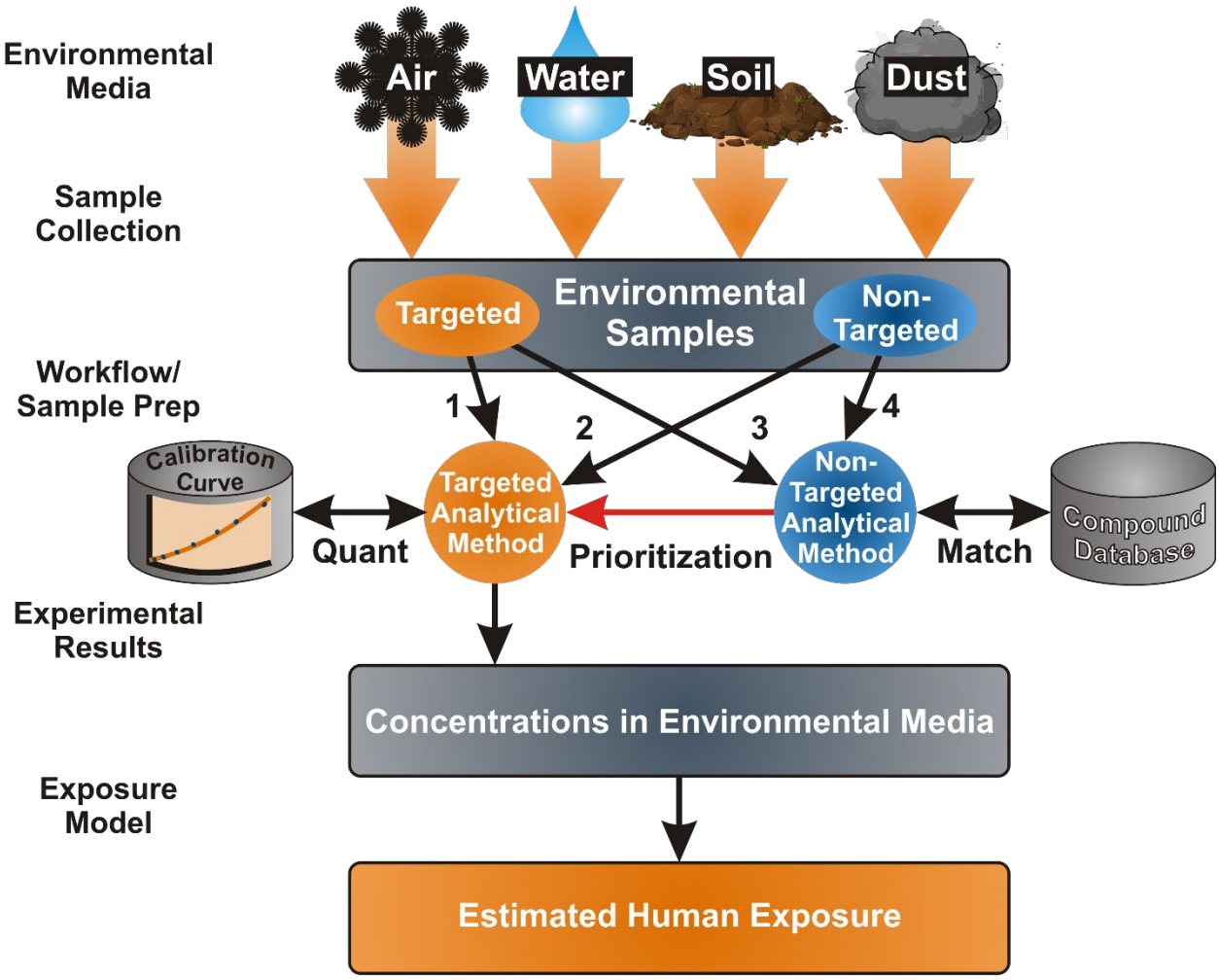

Figure 2. Overview of Non-Targeted Exposure Analysis. Solid black arrows indicate traditional analysis, and red arrows indicate new approaches using NTEA.

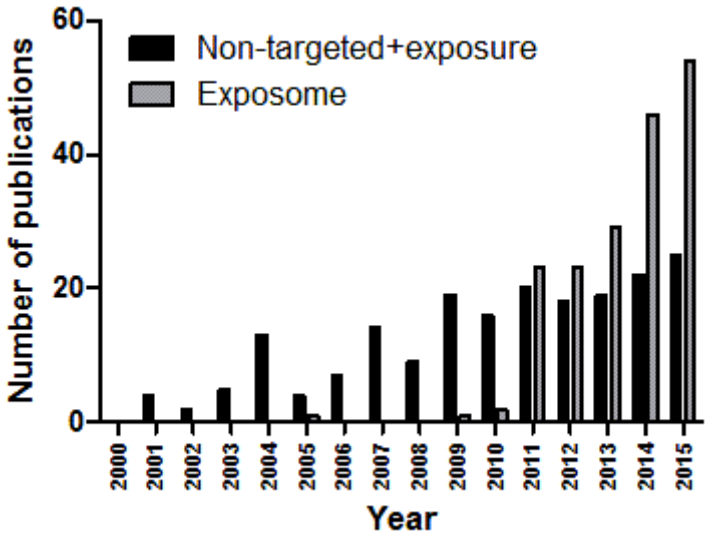

Figure 3. Number of publications related to NTEA over time.

A general overview of NTEA intended to support human exposure assessment is shown in Figure 2. Samples are collected from environmental media, such as air, water, soil, or house dust. These environmental samples can be considered to be on a spectrum from targeted to non-targeted based on how the sample was collected and the degree of effort to preserve all potential analytes in the collection process. The samples are then worked up, which can also affect what analytes are retained in further steps (e.g., fractionation or extraction work-ups). Both targeted and non-targeted samples can be analyzed by targeted or non-targeted analytical methods (arrows 1-4). Traditionally, targeted analytical methods have been preferred (arrows 1-2); new efforts are being made to expand the use of non-targeted analytical methods (arrows 3-4). As more compounds are run through non-targeted methods, they can be added to compound databases that will then increase confidence in future analyses. Results from quantitative analytical methods are used to estimate concentrations of analytes in environmental media, while those from non-quantitative methods are used for prioritization of future studies. Exposure models can then be used to translate environmental concentrations to estimated human exposures. The prediction of internal human dosimetry from analytical results may be attainable in the future using personal dosimeters if designed carefully to capture all the aspects of human ambient exposure. Other supporting data and computational modeling would be used in conjunction with NTEA generated qualitative exposure information. The necessary supporting data would include environmental media concentrations and their time trends. Also included are the computational modeling capabilities to simulate transfer of those chemicals identified in NTEA from environmental media to sampling devices, and then from the device to human body.

What technologies support NTEA?

The goal of NTEA is to detect and/or quantify the major components of a sample; however, this is extremely difficult using a single analytical method. The majority of techniques available to the analytical scientist are predicated upon a targeted model, since the high level of specificity typically required comes at the cost of breadth. Techniques that detect signals from many or all components of mixtures are capable of balancing specificity against breadth. Some examples of methods that are closer to achieving this balance are nuclear magnetic resonance (NMR), inductively coupled plasma (ICP) based techniques, and scanning-mode

spectroscopic techniques (UV, IR, fluorescence), especially when coupled with separation methods such as chromatography (liquid or gas), extraction (liquid-liquid, solid phase [SPE]), and/or capillary electrophoresis [CE]). Many of these techniques are capable of detecting a very wide breadth of chemical classes and are known to provide reliable quantitation under the right conditions. The most promising methodologies for NTEA at the present time involve a combination of liquid chromatography and mass spectrometry (LC-MS). The analytical orthogonality of the chemical affinity separation power of chromatography paired with the mass-based detection of mass spectrometry (especially when using high resolution mass spectrometry technologies) has allowed researchers to achieve significant gains in mapping the human chemical exposome through NTEA studies.

Most non-targeted studies rely on a database that is used to infer chemical identities from the predicted chemical formulas of features detected[30]. This database is essentially a long list of chemical names and chemical formulas. After data acquisition, a software package for spectral processing (typically available from instrument vendors) is used to compare the "hits" (i.e., a chemical mass signal with sufficiently reasonable abundance and acceptable chromatographic peak shape) detected in the sample with the database entries based on exact mass, as well as a number of other potential features. In most cases, the software will return a score that may serve as a confidence rating of the chemical identity assignment based on all user-specified criteria. Aside from exact mass, the other two most notable criteria used to score an assignment include isotopic spacing (a product of the atomic make-up of the chemical species) and isotopic distribution (an artifact of the isotopic abundances of the atoms in the chemical; this is most useful when the substance of interest includes halogens). Retention time on the LC column is traditionally included as a means of judging a hit assignment in targeted analyses; however, in non-targeted studies this value is not as useful since the retention times of potential substances are not known ahead of time and are very difficult to predict based on chemical structure or chemical formula alone (though significant gains have been made in this regard[31-38]). Some databases may include fragmentation data (e.g., METLIN[39]) which can greatly increase confidence in hit assignments. Matching hits from a mass spectrometry experiment against a database to try to assign specific structures or chemical identities to the generated molecular formulas is a technique generally and loosely referred to as "suspect screening." In order to leverage data in existing databases, attention should be paid to the analytical method including mass spectrometer acquisition parameters used to generate the data in the database. The more closely these can be matched, the more easily the results will be comparable.

Non-targeted analysis requires not only specialized analytical methods to detect and characterize all the various components of a sample, but also ways to collect samples that are as representative as possible of their surroundings in the first place (see Figure 2). Ideally, these collection efforts will also cover a wide variety of environmental media (i.e., air, dust, water, etc.). These efforts can focus on any scale, from the individual, to a household, to a watershed, to the planet. Figure 2 illustrates various sample collection scenarios that are applicable for NTEA. For example, pathway 1 would be to use an air sampler that fractionates particulate matter and collects particles <1.6 μm. This fraction can then be checked for mold toxins[40]. Both sampling and analysis are optimized for specific analytes. Pathway 2 is probably the most common form of analysis. For instance, water at a drinking water purification plant is sampled. The sample itself contains everything that is introduced into the distribution system, but due to the targeted nature of the analysis, only certain analytes are measured and reported. NTEA includes pathways 2-4. Pathway 3 might fractionate particulate matter in air as before, but might analyze for all components present, not just mold toxins. House dust analysis is an example of pathway 4—dust will pick up a large number of chemicals present in the home. The analysis can then proceed to systematically identify the major components in the dust. Samples for NTEA may be collected directly from the environmental media, or indirectly collected

through 'exposure tracking devices' or 'passive sampling devices.' Those devices are intended to collect exposure information at an individual-or personal level. However, interpretation of the data from those passive sampling devices are less straight-forward than those from environmental media due to the lack of exposure models for these devices that translate analyte concentrations to magnitude, frequency, and duration of exposure.

Current Efforts in NTEA

Current efforts in collection

Collection of individual-level exposure data to inform personal health decisions has been substantially increased raising questions about the implications of the data and how to best communicate results to stakeholders{National Academies of Sciences, 2017 #109}. Recent advances in personal sensor technologies and other emerging tools to track daily patterns in exposure and behavior, augmented by the availability of personal environmental exposure data to people and communities, have significantly contributed to this trend.

Among these personal exposure tracking devices, the use of silicone wristbands has gained attention for the potential to track an individual's exposure to environmental chemicals. These efforts were designed around the ubiquitous silicone wristbands, a cultural trend largely popularized by the Livestrong Foundation. Many different types of compounds can be recovered from silicone, including polycyclic aromatic hydrocarbons (PAHs), consumer products, personal care products (PCPs), pesticides, phthalates, and other industrial compounds with octanol-water partition coefficients (log K_{ow}'s) ranging from -0.07 to 9.49 (caffeine to tris(2-ethylhexyl) phosphate)[41]. The Anderson group at Oregon State has published studies on this, giving 92 children wristbands for 7 days[42]. The final sample size was 72 after taking into account some subjects not returning the wristbands and subjects excluded for deviating from the protocol. The authors looked for 41 compounds: 35 brominated diphenyl ethers (BDEs), 4 organophosphorus flame retardants (OPFRs), and 2 other brominated flame retardants. Twenty of the compounds were above the lower limit of quantitation (LLOQ). Seven BDEs and four OPFRs were detected in >60% of samples. Associations were found between amount of flame retardants absorbed by the band and a number of factors including: a) age of the house where the child lived, b) vacuuming frequency, and c) family context. This study demonstrates the utility of a passive sampling method for individual exposure. Since these wristbands pick up a wide range of analytes, they constitute non-targeted sample collection; because the authors paired this with targeted detection methods, it is an example of a type 2 analysis (Figure 2). There is now a movement to combine this type of personal exposure tracking device with non-targeted analysis (type 4, Figure 2). A recent case study with 28 adult volunteers from the Environmental Defense Fund was conducted applying the suspected screening analysis approach. The volunteers wore silicone wrist bands for one week and the bands were analyzed qualitatively for a suite of 1,400 chemicals[43]. Out of this list, 57 chemicals were detected from several chemical classes, including PAHs, pesticides, plasticizers/phthalates, fragrances, preservatives, and flame retardants, with an average of 15 chemicals in an individual band.

While silicone bracelets will pick up a wide spectrum of analytes with a range of log K_{ow}'s, there are still limitations to this sampling method if intended for NTEA. For instance, although PAHs can also be extracted using silicone rubber, log K_{ow} had a major impact on desorption rate from sediment to silicone[44]. Other polymers have been used that have different physicochemical properties; passive sampling of PAHs using low-density polyethylene tubing has been used at Superfund sites to concentrate environmental PAHs[45, 46]. Volatile compounds are unlikely to be retained in a polymer matrix, and alternative chromatographic

methods (e.g., gas chromatography) are likely to be required for detection/quantitation of these substances in any case.

Another case study of non-targeted sample collection and analysis was conducted by a group at the EPA who used house dust to assess indoor exposure and the accumulation of outdoor contaminants indoors[17]. The details of this study are described later in this paper.

Landfill leachate is a good example of a non-targeted sample collection. Leachate contains not only the chemicals that have been directly disposed (which in turn depend on the timeframe of landfill operation, local manufacturing activity, and geographical patterns of product use and disposal), but also environmental degradates, purification byproducts, and microbial and fungal metabolites. In one study, leachate was analyzed for 70 different per- and polyfluoroalkyl substances (PFAS) using orthogonal LC-MS/MS[47]. This study used large volume injection to compensate for the dilute nature of the mixture and the chromatographic separation used two zirconium-modified diol guard columns to retain PFAS followed by a C_{18} column to separate the analytes. This study found 36 previously-unanalyzed PFAS, with 3-perfluoro propanoate (5:3 FTCA) being the most concentrated. A previous study (a standard targeted analysis of 24 PFAS) showed a perfluoroalkyl carboxylate compound as the most abundant; the fluorotelomer carboxylates were not even looked for[48]. This finding is important because it shows that other analyses may have missed previously unknown members of a class of compounds known to have significant human toxicological relevance. In the absence of more non-targeted approaches, scientists could be missing major constituents of environmental mixtures.

Not all exposure estimates require measurements of chemicals in environmental media. Passive sampling methods using bar code readers tied to ingredient databases for consumer products have also found use in this field. Bennett et al. sent surveyors to visit subject's homes, scan all consumer products and weigh them, and attach accelerometers to the two most commonly used products[49]. The surveyors came back to reweigh each bottle a week later, add any new products, and get the accelerometer data. The bar codes were translated into a list of consumer chemicals and personal exposure profiles were developed. This study avoids questionnaires (response bias) and recall bias by consumers[49]. These approaches avoid some of the bias inherent in analytical chemistry-based techniques, although they are susceptible to other sources of bias (e.g., trade-protected ingredients lists) and are applicable to individuals and require large sample sizes to allow extrapolation to populations.

To aid interpretation of the data collected from these new exposure tracking devices, efforts are being made to incorporate patterns of exposure and other personal behaviors such as dietary patterns and daily activities. One example is the incorporation of exposure tracking into smartphones, fitness trackers, or GPS trackers[50]. It is expected that non-targeted analysis will increasingly be applied to collecting personal exposure data to link to personal exposure tracking tools with a goal of generating a personal exposome.

Current efforts in detection

The breadth and diversity of compounds present in the human chemical exposome renders a truly non-targeted analysis experimentally impossible as there are no forms of chemical analysis capable of detecting all chemicals with equivalent efficiency. In practical terms, any single technique provides data on only a subset of the vast chemical landscape. For example, if the non-targeted detection is performed with positive mode high performance liquid chromatography-time-of-flight mass spectrometry (HPLC-TOFMS) then only those chemicals that are of moderate polarity (retain and elute from the chromatographic column) and able to be positively ionized (using ESI, for example) are able to be detected. Even then it is widely recognized that not all analytes are detected with equivalent ease[51].

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) has been used to great effect in the non-targeted analysis of environmental exposure samples[33, 37, 52-58]. Reverse-phase chromatography is the most typical form of LC utilized and both time-of-flight (TOF) and Orbitrap mass spectrometry are commonly used. Analyses using LC-HRMS generally provide a great deal of chemical breadth since all analytes that even moderately adhere and elute from a reverse-phase column and are ionizable using electrospray ionization (ESI) can potentially be detected. Kern et al.[33] used a reverse phase C_{18} column with an Orbitrap instrument to test water samples for transformation products (TPs) in a non-targeted fashion using a data-dependent acquisition/scanning mode. Once exact mass hits were cataloged, these hits were compared against a large database of potential structures including not only known TPs but also computationally predicted TPs. Tengstrand et al.[56] used non-targeted reverse-phased liquid chromatography paired with a QTOF to demonstrate the sensitivity of this workflow for detecting unknown contaminants in food products. Deyerling and Schramm[54] used a retention time index method to estimate the partition coefficient (logP) of detected unknowns in their reverse-phase HRMS non-targeted method (Figure 4). Bade et al.[37] expanded upon this idea by using artificial neural networks (ANNs) to aid in their prediction of retention time for potential unknown analytes. Their workflow included using an all-inclusive ion fragmentation mode (as mentioned earlier). Ouyang et al.[57] used two-dimensional LC coupled with HRMS to increase the detection capacity of the system by providing additional chemical separation space[57]. This study analyzed house dust and dryer lint using this non-targeted arrangement.

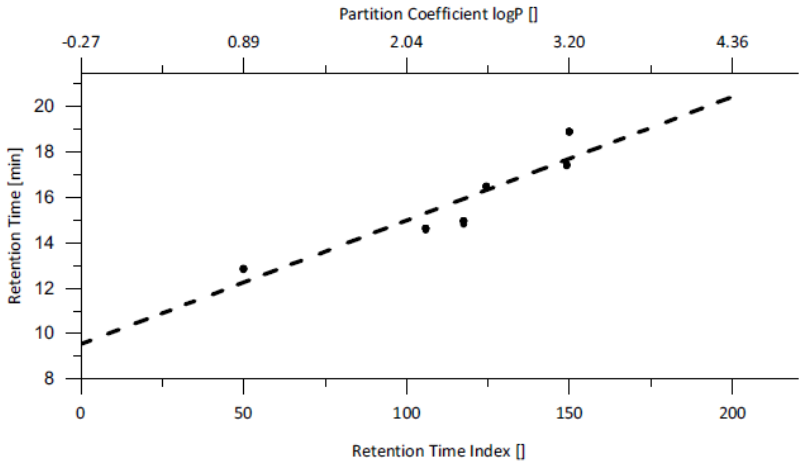

Figure 4. Demonstrated use of retention time from reverse-phase HPLC to estimate the logP values for unknown detected compounds in a non-targeted method[54].

In another study, wastewater effluent was analyzed for 20 specific PFAS but also for total oxidizable precursors (TOP)—while this does not provide structure information, it does help the researcher understand how close they are to detecting all supposed analytes[59]. Non-targeted methods such as TOP could meet a crucial need; namely, indicating how much of the mass of a complex mixture has been accounted for by standard targeted analyses, and prompting the researcher to develop new methods to detect the remaining components until a certain mass threshold has been met. Another technique, particle-induced gamma-ray emission (PIGE) can detect fluorine atoms in biological tissues including bone[60]. This is quantitative but does not give the structure of the compound from which the fluorine originates. While not as broad as other methods of non-targeted analysis, both TOP and PIGE still detect compounds for which more specific methods do not yet exist and can indicate whether a mixture deserves further analysis. These methods could

be useful for NTEA of fluorine-containing compounds, such as PFAS and fluorine-containing pharmaceuticals[60].

Strengths and limitations of current efforts

Collection strengths and limitations

When designing a NTEA method, one of the most important things to consider is which matrix to analyze, or in other words how to collect one's samples. This choice is easily the most important factor in designing an NTEA experiment of this type. The choice of matrix will have a major impact on the outlook or perspective of the study. Matrices for NTEA include environmental media and biological media. In this paper, we focused on the use of environmental media. For the details of current efforts on the use of biological media to inform human exposure, refer to human biomonitoring and biomarker data for human exposure assessment or reconstruction[61-64]. In addition, personal exposure tracking devices that are increasingly popular in public are currently used in lieu of environmental or biological media, whether that assumption is appropriate or not. For example, there is misbelief that chemical profiles detected in personal exposure sampling devices such as silicone wrist bands are a reflection of blood profiles.

House dust represents an ideal matrix for method development and for linking environmental concentrations to exposures at various receptors. House dust picks up a wide variety of chemicals present in the home, meaning the results are likely to have wide applicability. This is in contrast to methods that rely on production volume or other factors that may represent a combination of far- and near-field exposures. In addition, there are well-established methods in exposure science that can estimate exposure to dust using age and activity data—vacuuming and cleaning activities lead to inhalation exposure in adults, while hand-to-mouth behavior in infants and toddlers causes oral exposure. Dust can be easily collected from the home, its various components separated using extraction and/or chromatographic methods, and each component systematically identified and eventually quantitated.

A more complex situation arises when considering silicone bracelets. Silicone wrist bands are easy and comfortable to wear allowing for tracking of personal exposure for an extended period. These bracelets, like house dust, pick up a wide variety of chemicals. However, they are limited in terms of the type of chemicals that can be collected, and largely favor lipophilic organic chemicals. In addition, the high affinity of these chemicals to silicone may complicate extraction and detection from silicone sampling devices and lead also to a skewed representation of the chemical profiles favoring compounds in that favored space. In addition, the link between a concentration in a bracelet and an exposure is more complicated, given that the profiles of chemicals in the passive device would only an indirect reflection of what people are exposed to. Interpreting the results of these studies is therefore more complicated. It is a challenge to obtain the individual's exposure conditions and human exposure factors affected by one's life style in parallel with the samples collected from exposure tracking devices such as silicone bands.

Detection strengths and limitations

By far the greatest strength of these methods, especially those using LC-HRMS technologies, is the broad coverage of the chemical landscape. There is no other analytical platform currently available that is better equipped to handle the breadth and complexity of NTEA samples. Various software packages are available that evaluate a complex mass spectrometric dataset and generate a prioritized list of highest scoring hits for follow up confirmation and/or quantitation studies[65]. Instrumentation is well supported by numerous vendors who are constantly advancing their technologies and capabilities. Nevertheless, there are some

limitations that should be considered when using LC-HRMS techniques for NTEA studies. A brief discussion is provided below.

Chromatographic separation

The retention and detection of analytes depends on them having moderate polarities. If a compound is too hydrophilic it will likely be lost during washing of the column, or will elute too quickly form the column, preventing detection with the mass spectrometer. On the other hand, if a compound is too hydrophobic, it may adsorb too tightly to the extraction cartridge or column and avoid detection by the mass spectrometer. Both of these situations, however, can be avoided with proper vetting of the non-targeted method/preparation (using quality control compound mixtures with highly variable chemical characteristics) and optimization of the LC gradient.

LC-HRMS requires that the compounds be soluble in aqueous solvents. Analytes that are not miscible with water can result in false negatives. This scenario has reasonable potential to occur in exposure analysis samples where there is a large incidence of airborne pollutants. If this class of compounds is suspected, the study would be better served through separation with gas chromatography (GC) rather than liquid chromatography. GC can still be connected in series with HRMS detectors and is therefore useful for NTEA studies. It should be noted, however, that while most gas-phase compounds are not amenable to liquid chromatography, most liquid-phase compounds are not amenable to gas chromatography without some form of chemical derivatization to improve volatility. These derivatization reactions can be difficult to perform on many classes of compounds at once and may not be available for all classes of compounds in an exposure sample. This necessity for prior knowledge of sample composition makes gas chromatography a non-ideal choice for NTEA when it can be avoided.

Mass spectrometry detection

Mass spectrometry is dependent on analytes being positively or negatively ionizable, typically either through protonation/deprotonation reaction schemes or charged species adduction. Chemical analytes comprise the entire range of possibility in this regard; some analytes do not ionize in positive or negative mode, some are moderate ionizers, while others are superb ionizers. This quality of a chemical compound is very difficult to predict a priori. In the context of non-targeted analysis this can cause type II errors or misinterpretations if trying to infer quantitative values. Although one hit may produce a more abundant signal than another hit, it is not necessarily at a higher concentration in the sample. Analyte quantitation must only be evaluated using quantitative methods involving a calibration curve preferably made from certified reference standards of the compound being analyzed.

Compound database

One ongoing need is for better libraries of compounds for mass spectrometry, of chemical constituents of consumer products, etc.[66]. By adding results to compound databases, scientists can leverage each other's work to dramatically accelerate the identification of components of complex mixtures. One of the examples of building resources for the non-targeted testing community includes US EPA's DSSTox Chemical Database. This database is carefully curated and quality controlled to ensure that chemical identifiers (e.g., CAS number, common name) match up with each other and to the correct structures. Another advantage of DSSTox is that all the data are public, since the database is maintained by a governmental agency.

Directions of future efforts

Future instrumental directions

The efficiency of chemical exposome research will continue to be driven by the availability of ever advancing technological support. The types and scale of studies being carried out today would have been nearly impossible to achieve 5-10 years ago. The incorporation of state-of-the-art technology must be a constant consideration in environmental toxicology in lieu of the continued use of the "old" and "familiar" tools of the trade.

Though major improvements in LC are unlikely since the science has remained largely unchanged for decades, additional improvement of mass spectrometric technologies are frequent and often of major impact to the field. Incremental gains in resolution and sensitivity will continue to improve the quality of chemical exposome data and results, while incorporation with mass spectrometry aligned technologies could drastically increase the detection capacity of these techniques. Addition of technologies such as ion mobility (through either drift tube designs by Agilent[67-71], traveling wave technologies from Waters[72-74], or asymmetric waveform technologies from SCIEX[75-77]) could potentially have an enormous impact on NTEA. Ion mobility technologies have the potential to add an additional slightly-orthogonal dimension to the data without an increase in analysis time or an inordinate increase in the cost of instrumentation resulting in supplementary structural information via drift time measurements. Having the extra dimension of ion mobility separation also allows for mobility correlated fragmentation detection which has the potential to improve the interpretation of data arriving from all-inclusive ion modes (i.e., SWATH, MS^E, AIF, All Ions MS/MS).

Technological breakthroughs come not only through vertical incorporation but also through lateral incorporation. One such possibility along these lines is the integration of NMR technologies into the LC-HRMS workflow. A predominant shortcoming of LC-HRMS is the inability to conclusively infer chemical structure or atomic linkages. LC-NMR, which has found a foothold in various fields[78-84], could be an ideal complement in this regard. NMR-MS has been complexed in series[85-88], primarily through the use of flow splitting, and also in parallel[89] with a mass spectrometer to achieve these chemical structural benefits for analyzing complex mixtures in other fields. Notably, LC-NMR-MS has been used to investigate environmental exposure samples using non-targeted workflows[90].

Proposition for ideal collection and detection methods

Recommended guidelines for collecting samples for a NTEA study

In order to preserve the greatest variety of analytes, there are some general guidelines when collecting non-targeted samples (Figure 2). The best material for sample storage is glass; this is minimally reactive with most analytes. For certain analytes, silanized glass may be called for, where the hydroxyl groups of glass have been passivated, while for glass-reactive chemicals (e.g., some acids), plastic may be required. Whenever using plastic, the possibility of leaching should be considered. The best way to deal with this issue is to split samples and store them in both glass and plastic; however, it is recognized that this is not always realistic.

Sample storage time should be monitored and noted, since the length of storage can greatly impact both what is detected and its concentration. As mentioned above, components of the storage container may leach into the sample. Similarly, analytes may adsorb to the surface of the container. They may also degrade over time. Keeping the temperature as low as possible and minimizing exposure to light and freeze-thaw cycles will minimize degradation of most analytes. All storage conditions (time, temperature, container material, number of freeze-thaw cycles) should ideally be recorded in case discrepancies are noted post-analysis.

An important consideration is the pH of the sample during collection and storage. Changes in pH due to storage buffers or other additives can cause partitioning, ion trapping, hydrolysis, etc.[91] In general, extremes should be avoided; a pH range of 6-8 is a good target. Analytes that are not stable at physiological pH (e.g., undergo degradation or hydrolysis) are unlikely to be relevant from a human exposure perspective.

How representative a given sample is depends strongly on spatial sampling procedures. For water samples, it is usually sufficient to sample at a particular location (e.g., wastewater treatment plant, drinking water treatment plant, well water, point-of-use tap water) and noting that location. For other matrices, like house dust, a sampling plan should be developed and uniformly applied to ensure that the sample is as representative as possible. Food poses particular problems, since sourcing, processing, transport, kitchen preparation, and incorporation into meals all impact the actual exposure to chemicals in or on food items. The FDA has guidelines on measuring pesticide residues on food items; these are a good resource when developing sampling plans[92]. The matrix will also affect the type of analytes that might be detected. Volatility, lipophilicity, acid/base chemistry, and water solubility will especially affect what is likely to be present in a sample. Volatile chemicals require special care, necessitating immediate freezing and other specialized handling procedures. Water solubility is obviously of primary importance when interpreting the results of water sampling.

Matched environmental samples (e.g., house dust, water, ambient air, soil, surface wipes) will give the most coverage of chemical space for a particular location. These matched samples will also provide clues as to the environmental fate and transport of analytes. Time series sampling will give insight into seasonal and/or long-term trends in analyte concentrations. Repeated measures, with or without spatial sampling, will help with the estimation of variability in analyte concentration. Most of these considerations are outside of the scope of most non-targeted analyses, however, and are not necessary if simple cross-sectional data are required.

Recommended guidelines for detecting samples for a NTEA study.

When performing NTEA studies it is important to fully disclose any potential bias in the analysis, especially as it pertains to any sample cleanup/preparation that may have occurred. Sample cleanup is most commonly used to remove inorganic salts such as in the case of solid phase extraction (SPE). While adept at removing salts, SPE also tends to remove highly polar compounds. If this is not properly tracked and documented, it can have significant analyte or compound class-level ramifications on relative levels of resulting intensities. Another common sample cleanup technique is protein crashing where cold organic solvent is added to the sample before centrifuging to remove protein or other biological marcomolecules. One potential bias of this approach could occur if analytes are preferentially bound to this protein or macromolecule material. The potential health impact of exposure to these drugs could be substantial despite it not showing up in analysis due to being protein-bound.

The sensitivity of non-targeted chemical analysis methods should be properly vetted by running mixtures of compounds at varying concentrations (a.k.a., a quality control, QC). This can most easily be done by making a high concentration stock solution which includes chemicals similar to the compounds expected from the samples being analyzed and running various dilutions of this mixture. The more chemicals that are added to the mixture the more rigorous the validation, but ideally these compounds should span a wide range of hydrophobicities (K_{ow}'s), molecular weights, functional groups, chemical classes, etc. The detection method used should be optimized using this QC sample at relatively low concentrations. Reporting of the composition of this mixture as well as the method's ability to detect the standards within (including thresholds used, i.e., intensity and accuracy thresholds) and at which concentrations would best be reported in

the materials and methods section or supplemental information of accompanying publications along with other validation information.

Once samples are analyzed, the next step is likely comparison to a database of chemical formulas to match these with the exact masses detected during the study. This is often referred to as "suspect screening"[66]. In the case of exposure analyses, databases such as the ToxCast database, the Inventory Update Reporting and Chemical Data Reporting (IUR), EPA's Aggregated Computational Toxicology Online Resource (ACToR), and the Crop Protection Research Institute (CPRI) database would be useful repositories currently available. It is recommended that either new databases to support NTEA should be built or the key information required for NTEA such as chemical name, chemical formula, and CAS number would need to be added to the existing databases to help increase utility of the NTEA and move the field forward. It should be noted that any database may inherently have biases in chemical spaces depending on the purpose of the database intended. However, those intended uses of specific databases can help prioritize chemicals to be explored using NTEA.

Applicability

Prioritization

Once hit identities have been confirmed, there are several follow up studies that are crucial for interpreting the risk associated with these hits by quantifying the identified chemicals (see the red arrow in Figure 2). Not all identified chemicals in a NTEA have risk-based implications. Contextualizing these identified species from a toxicity perspective is an important step to make the results more consumable to the general public. Given the constraints in resources and time, prioritization of the list for follow-up studies is necessary. From the perspective of a risk-based approach, prioritizing based upon toxicity and environmental prevalence is a reasonable tactic[17]. Assessing the potential toxicity of confirmed species is unquestionably the next step after MoE analysis based on/after NTEA, However, for a rapid decision making, a large scale HT bioactivity screening data such as ToxCast can be used to provide chemical's bioactivity data for the post-NTEA analysis. In this sense, NTEA has the potential to improve the HT *in vitro* assay based-prioritization strategy by augmenting human exposure information required for margin of exposure (MoE) analysis. It should be noted that at the same time, compounds with bioactivity from HTS are used for prioritization, inevitably adding potential biases in detection. Toxicity has no meaning if no measure of degree/concentration of exposure is considered. Therefore, subsequent to identification and toxicity contextualization of identified species, it is advisable to perform quantitative studies on the NTEA samples collected. Quantitation can be performed in a number of ways including use of the HRMS platform used for the non-targeted portion of the study, and there are a number of studies which have demonstrated this[93-97]. In the absence of available information to inform compound prioritization for targeted analysis after NTEA, other approaches such as the Threshold of Toxicological Concern (TTC) decision tree can be used to make a decision about the potential for toxicity of the chemicals from NTEA and subsequent targeted analysis. The TTC approach is a screening and prioritization tool for the safety assessment of chemicals when hazard data are incomplete and human exposure can be estimated. This indicates another potential application of NTEA in chemical prioritization[98].

Case Study – EPA house dust study

A number of studies have recently been published that assess human indoor exposure via house dust[57, 99-107] using everything from analytical techniques to computational approaches to meta-analysis of available literature. One of those, a study by Rager et al.[17], adheres very well to the proposed guidelines set forth in this

work. In their study, Rager et al. used a straight-forward workflow to analyze house dust for suspect contaminants using an LC-TOF/MS platform.

A total of 56 samples were obtained from the American Healthy Homes Survey (AHHS) and sieved to isolate particles <150 μm prior to analysis. A very basic extraction protocol was employed (methanol wash followed by a normal phase SPE cleanup) in an attempt to retain as many chemicals as possible. An average of 3185 features were detected per sample. A vendor-specific data deconvolution tool was used to negate background signals and condense all associated features (i.e., sodiated, adducted, isotopic features, etc.) down to the same parent mass. With a "cleaned" feature list in hand, the EPA's DSSTox database was used to match features to chemicals of interest by comparing neutral exact mass, isotope distribution, and isotope spacing. Most features did not match to a mass in DSSTox. The 978 unique masses that did match corresponded to 3228 unique chemical substances (this number is larger due to the presence of structural isomers with identical molecular weights but different connectivity between atoms). The authors note that a positive identification of a feature should be confirmed through the use of standards. The choice of which chemicals to confirm was decided based on the "priority" candidate's frequency of detection in the 56 samples, the abundance (from the chromatogram) of these hits, the potential for human exposure (using ExpoCast), and previously demonstrated *in vitro* bioactivity (using the ToxCast dataset). This information was summarized in the paper using the ToxPi framework. Eventually, the authors confirmed 33 unique chemicals. A search of the literature shows that 15 of the confirmed chemicals had not been previously published as constituents of house dust. This demonstrates the utility of non-targeted analysis for finding analytes that you don't know to look for (those not "under the streetlight"). This work also emphasizes the important step of following up hazard identification with risk assessment that takes into account exposure information such as abundance and detection frequency. Studies that include hazard identification without risk assessment may inadvertently lead others who do not understand this distinction to form conclusions that do not have substantial weight-of-evidence.

Conclusions

Putting NTEA results in a risk-assessment context

Current efforts in NTEA have focused on qualitative characterization of chemicals to which individuals may be exposed, with an emphasis on reliable identification of unknown components of a complex exposure matrix. However, little consideration has been given to putting the potential exposures into a quantitative risk assessment context. As such, these studies are most useful for the purpose of informing the design of targeted studies. Risk assessment has two principal components: hazard (toxic potential) and exposure. The value of NTEA is primarily in identifying chemicals for which there is a likelihood of human exposure. These chemicals can then be screened for toxic potential using available methods such as quantitative structure activity relationship (QSAR) modeling and read-across.

Estimation of exposure from NTEA results is more problematic. In some cases (such as analyses of media air, water, dust, etc.) qualitative identifications can be followed up by quantitative determination of the concentration of the chemical in the media. This quantification typically would require calibrating a standard curve for the response of the instrument versus the concentration of the chemical. The media concentration could then be used with standard exposure factors[108] to estimate potential human exposures. In other cases, quantification of NTEA results may be more problematic. For example, in the case of silicone bands, the concentration of a chemical in the band would be a complex function of a number of factors: absorption of vapors from air (which in turn is a function of average air concentration during exposure, duration of exposure, time since exposure, diffusivity of the chemical in silicone, silicone:air partition coefficient of the

chemical, stability of the chemical, etc.), absorption of liquids, and surface adsorption of particles. To some extent it might be possible to calibrate the relationship between band concentration and media concentration for specific compounds and exposure scenarios, similar to the calibration of an analytical method. However, due to the unknown time-dependence of the variation in personal exposures, the effort required for such a calibration would probably not be justified except for high-interest chemicals. A more realistic goal would be to identify the physico-chemical properties that would favor accumulation of a chemical in the band (e.g., high lipophilicity, low volatility), in order to provide context for the types of chemicals that are likely to be identified using a particular device.

Recommended path forward

This white paper discussed the inherent biases in both sample collection and detection in NTEA when interpreting NTEA data to potentially inform human exposure. The current state of the art in both collection and sampling techniques would justify caution in applying NTEA for chemical identification and characterization. Although NTEA currently appears to be of little value for quantitative exposure assessment, its value could be greatly increased in the future through additional experimental data collection and computational simulation. Evaluation of an approach's domain of applicability in terms of chemical properties and the ability of the sampling media and device to accurately reflect human exposure needs to be conducted. Development of *in silico* prediction methods such as QSPR for chemical transfer from the environment to the sampling devices vs. the human body should be the first step in that direction. The generation of experimental data with currently available devices will be required to support the development of such computational prediction tools. These studies will also provide evidence for what is possible and what the limitations are in the current practice of NTEA. This kind of evaluation will aid in defining domains of applicability of various sampling tools potentially used to provide inputs for NTEA analysis and also identifying a way to augment the current sampling and collections practices based on a combination of approaches, both for the environmental exposure data and prediction tools. Understanding human exposure factors influenced by lifestyle and individuals' physiological conditions is also critical in making risk-based decisions based on NTEA. In parallel, more concerted efforts to build database resources for the NTEA community would contribute to reducing potential biases pertaining to the specific datasets used for data/structure interpretation.

Acknowledgements

This white paper was prepared by ScitoVation scientists with support provided by ACC LRI. The contributing scientists included Jeffrey R. Enders, Martin B. Phillips, Harvey J. Clewell III, Rebecca A. Clewell, Miyoung Yoon and Patrick McMullen (editor).

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