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Supporting systems science through *in silico* applications: A focus on informing metabolic mechanisms

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Abstract

Despite the advent of new *in silico* methodologies to characterize exposure and pharmacokinetic properties of environmental chemicals, numerous limitations remain in the understanding and predicting of metabolic processes. Metabolomics has arisen as means to better inform underlying mechanisms of metabolism and biological effects following chemical exposure. Metabolomics is applied for numerous purposes, including clinical diagnoses of disease, biomarker discovery, toxicological studies, and environmental monitoring. One of the primary challenges involved in metabolomics is identification of detectable metabolites, due to lack of chemical information in databases or reference spectra. Several computational tools, such as metabolite-predicting software and *in silico* spectral libraries, have been developed to address such issues. Herein, application of such tools is discussed to support environmental monitoring and nontargeted chemical analyses or approaches that are based on pathway frameworks designed to better inform chemical effects within living systems. As these tools advance over time and are able to provide more accurate prediction of metabolites, the list of identifiable chemicals within a given exposure medium will continue to grow. This, in turn, will improve confidence in exposure-based and hazard-based decision-making practices.

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Metabolomics, Environmental monitoring, Nontargeted analyses, Adverse outcome pathway, Aggregate exposure pathway, Pharmacokinetics.

In silico predictions of pharmacokinetic behaviors to support risk assessment

Ensuring chemical safety often requires information regarding the relationship between external concentrations and biologically effective internal concentrations [1]. This relationship is influenced by pharmacokinetic (PK) behaviors such as absorption, distribution, metabolism, and elimination (ADME). Because a large number of chemicals in production or currently on the market lack comprehensive exposure and PK information, a significant effort has been taken to predict these chemical-specific characteristics through *in silico* means. Many computational exposure tools require inputs of ingredient concentrations, exposure duration, consumer activity, and product use to generate their predictions [2–5]. Ingredient information for products can be found in repositories such as the United States Environmental Protection Agency's (US EPA) Chemical and Product Database [6,7]. Information regarding exposure routes and duration to certain population groups can be found in the US EPA's Exposure Factor Handbook [8], which is supported by an online tool that provides advice on exposure assessment methodologies (https://www.epa.gov/expobox). In addition, several databases are available that provide information about consumer activity and product use. Some examples are the European Food and Safety Authority's Comprehensive European Food Consumption Database (http://www.efsa.europa.eu/en/food-consumption/comprehensive-database) [9] and the US EPA's Consolidated Human Activity Database (https://www.epa.gov/healthresearch/consolidated-human-activity-database-chad-use-human-exposure-and-health-studies-and) that include product information and demographic metrics such as age. Despite this wealth of available information, exposure predictions can sometimes span an order of several magnitudes for some chemicals [10] as human behaviors vary widely and influence exposure variability more so than chemical information. In seeking to avoid unreliable exposure estimates, some studies have used physicochemical properties to predict exposure routes and the most susceptible exposure groups from among the general population [11,12]. Physicochemical properties are also often the basis behind predictions of absorption and distribution. For example, the number of hydrogen bond donors/acceptors and the octanol–water partition coefficient of a chemical can be used to estimate its oral bioavailability [13], and properties such as molecular weight and water solubility can be used to predict a chemical's ease of absorbance across the skin

[14]. Distribution across biological barriers such as the brain or placenta can also be predicted by input of physicochemical descriptors like molecular weight, polar surface area, and the octanol–water partition coefficient into quantitative structure activity relationship (QSAR) models [15–19].

Use of predictions to aid in characterization of clearance

Absorption and distribution can influence the ability of an active chemical to reach its intended biological target once in the body. Clearance can also impact the ability of an inactive parent chemical to reach its target in an active form, by mediating disappearance of that parent through transformation or excretion. Clearance can occur through biliary clearance and fecal excretion, via transport-mediated processes occurring along the apical and basolateral membranes of hepatocytes. Because bile is not easily collected in healthy individuals, estimation of biliary clearance is difficult despite several *in vivo* and *in vitro* methodologies, such as fecal recovery and sandwich-configuration hepatocytes [20,21]. Predictions of biliary clearance for poorly metabolized compounds are based on physicochemical properties such as molecular weight and polarizability [22]. Renal clearance also remains a major route of elimination, via both active transport and passive reabsorption mechanisms. Renal clearance can be predicted through knowledge of unbound chemical fraction in the plasma, dipole moments and electrical charge, molecule size, and physicochemical properties such as lipophilicity [23]. Studies conducted to predict these types of clearance mechanisms have resulted in the development of the Extended Clearance Classification System for the purposes of drug discovery, which is based on passive membrane permeability and physicochemical properties [24,25]. Advanced knowledge of a chemical's mechanism of clearance (i.e., hepatic, renal, or biliary) allows for better predicting its clearance rate via that mechanism, as the training set in the model can be constrained to chemicals only undergoing that specific mechanism [26]. Other routes of elimination can include exhalation, perspiration, and loss through breast milk, with these pathways also heavily dependent on chemical-specific properties such as volatility or lipophilicity.

It should be noted that a majority of clearance models have been developed using primarily pharmaceuticals within the training set, because of a greater availability of data for such compounds; applying these models to estimate clearance mechanisms or rates for environmental chemicals may result in domain of applicability issues. Often, clearance of environmental chemicals occurs primarily through phase I and phase II metabolism or a combination of renal and metabolic processes [10], and so determination of total clearance may provide the best estimate for such chemicals. In its simplest form, total clearance can be derived through knowledge of a chemical's volume of distribution (V_d), half life ($t_{1/2}$), and body weight (BW) according to the following equation:

$$CL_{total} = \frac{\ln(2)V_d}{t_{\frac{1}{2}}}\mathrm{BW}$$

Volume of distribution can be predicted using properties such as a chemical's lipophilicity, ionization state, and tissue–plasma partition coefficient at a given pH [27,28], and half-life has been extrapolated in humans based on observed half-life in rodents and a linear equation consisting of two constants and the chemical's octanol–water partition coefficient [29].

Opportunities and challenges associated with metabolomics approaches

The study of metabolites in cells, tissues, organs, or biofluids, especially those generated in response to a xenobiotic stressor, is known as metabolomics [30,31]. Metabolomics has arisen as a critical application of systems biology to better characterize the mechanisms underlying metabolism. One significant advantage of metabolomics is that a multivariate profile of metabolites can be generated upon biological perturbation, thus aiding in biomarker discovery. Such applications have been used in aquatic toxicology [32,33], drug discovery [34], clinical diagnoses of disease [35], and identification of metabolites. Analyses can involve use of nuclear magnetic resonance spectrometry, because of its nondestructive nature and high reproducibility of data [36]. More recently, in an effort to apply approaches with a higher sensitivity to resolve a greater number of compounds, gas chromatography or liquid chromatography, coupled with mass spectrometry, has risen in use [37]. Regardless of the method, it is combined with multivariate statistical approaches to better understand the complexity involved in the analysis of hundreds to thousands of low-molecular-weight metabolites [38]. Despite the numerous powerful technological tools and applications that are available to support metabolomics approaches, identification of metabolites remains a significant challenge. This limitation is often due to sparse or limited data available for a compound's structure, metabolic pathway, or reference spectra.

Using *in silico* tools to inform metabolic mechanisms in support of environmental monitoring and nontargeted chemical analyses

One recent application of metabolomics has been the identification of metabolites in biological and environmental media [39–41] to aid targeted and nontargeted environmental monitoring strategies. Traditional

monitoring of an environmental or biological medium involves extensive analysis of tens to hundreds of select compounds [42,43]. These compounds are often chosen primarily because of *a priori* knowledge of their presence in that particular medium and of adverse biological responses they might elicit in humans or other organisms upon exposure. As technical instrumentation has advanced, the lowering of detection limits and increase in measurement capabilities has enabled identification of a greater number of compounds in environmental and biological media over the years. This, in turn, has led to emerging data that can be used to support conventional and novel exposure tools and models, thus allowing for risk assessment to transition from a primarily hazard-based approach to one that equally considers chemical exposure prior to health response [44]. As a result, there has arisen an impetus to investigate exposure for the hundreds to thousands of chemicals in existence in order for exposure science to keep pace with 21st century toxicity testing. To achieve this goal, one strategy has involved development of nontargeted monitoring of animal tissue or biofluids to complement the more traditional targeting of select compounds [45]. This approach can allow for identification of heretofore-unknown compounds within a given environmental medium and lead to hypothesis generation in regards to what their presence might mean to organisms inhabiting that medium. It should be noted that the metabolites extracted from an animal's tissue can be derived through its own metabolic mechanisms or can be taken in as preformed compounds that have been generated through prior microbial or physicochemical processes (e.g., photolysis or hydrolysis). As metabolomics is a study of chemical and biological processes at a system level, its application to environmental monitoring of xenobiotic stress response is obvious. Metabolomics approaches are relevant also to identification of metabolites produced through microbial mechanisms occurring outside of an organism, because those metabolites are generated through biological processes. Although metabolomics is a means to investigate biological systems, the primary outcome of such an approach is characterization of metabolic mechanisms, and as such, the tools involved in this discipline can also be utilized to aid in the identification of metabolites present in abiotic media such as air, water, and dust [46–48].

Nontargeted analysis of abiotic media involves attempting to match spectra or formula determined experimentally against known compound formulas and masses found in online chemical databases such as ChemSpider (http://www.chemspider.com/Default.aspx), PubChem (https://pubchem.ncbi.nlm.nih.gov/) or the United States Environmental Protection Agency's Computational Toxicology Dashboard (CompTox; https://comptox.epa.gov/dashboard), as well as vendor or in-house databases that may or may not be available online. However, unmatched spectra or formulas can often result in a lack of detection of many chemicals that might actually be present in a medium, because of the absence of their empirical information in these databases [49]. One possibility is that the parent chemicals themselves may be absent from the searched chemical database. More often, it is the case that, despite the dual biological and chemical nature of metabolites, their information is also absent from these databases.

Given the large number of chemicals present throughout the environment and the much greater number of multiple transformation products for each chemical, it is no surprise that structures or masses are often not available for these 'dark matter' of the metabolome [50]. Fortunately, there are numerous computational tools that have been developed in the effort to facilitate identification of metabolites. Many of these platforms predict only sites of metabolism, whereas others extend their capabilities to also predict metabolites generated through any one of the many known metabolic pathways, especially from cytochrome P450–mediated processes. A few examples of such platforms include Meteor Nexus (Lhasa Limited), QSAR Toolbox (Organization for Economic Cooperation and Development), MetabolExpert (CompuDrug), MetaPrint2D-React [51], and ADMET Predictor (Simulations Plus, Inc.) One common downside with such *in silico* tools, however, is that all possible known reaction schemes are inputted into a library and so will result in prediction of any hypothetical metabolite known to be generated from that particular scheme. Another limitation is that library reaction schemes for a platform may be based on nonhuman metabolic mechanisms (e.g., rodent or microbial metabolism) [52]. Many of these *in silico* tools contain proprietary data, and recently one open-source platform was developed to predict a reasonable number of metabolites for single chemicals or chemical batches through knowledge of six reaction systems including both human and soil/aquatic microbial metabolism, with the reaction libraries obtained from empirical data within literature [53].

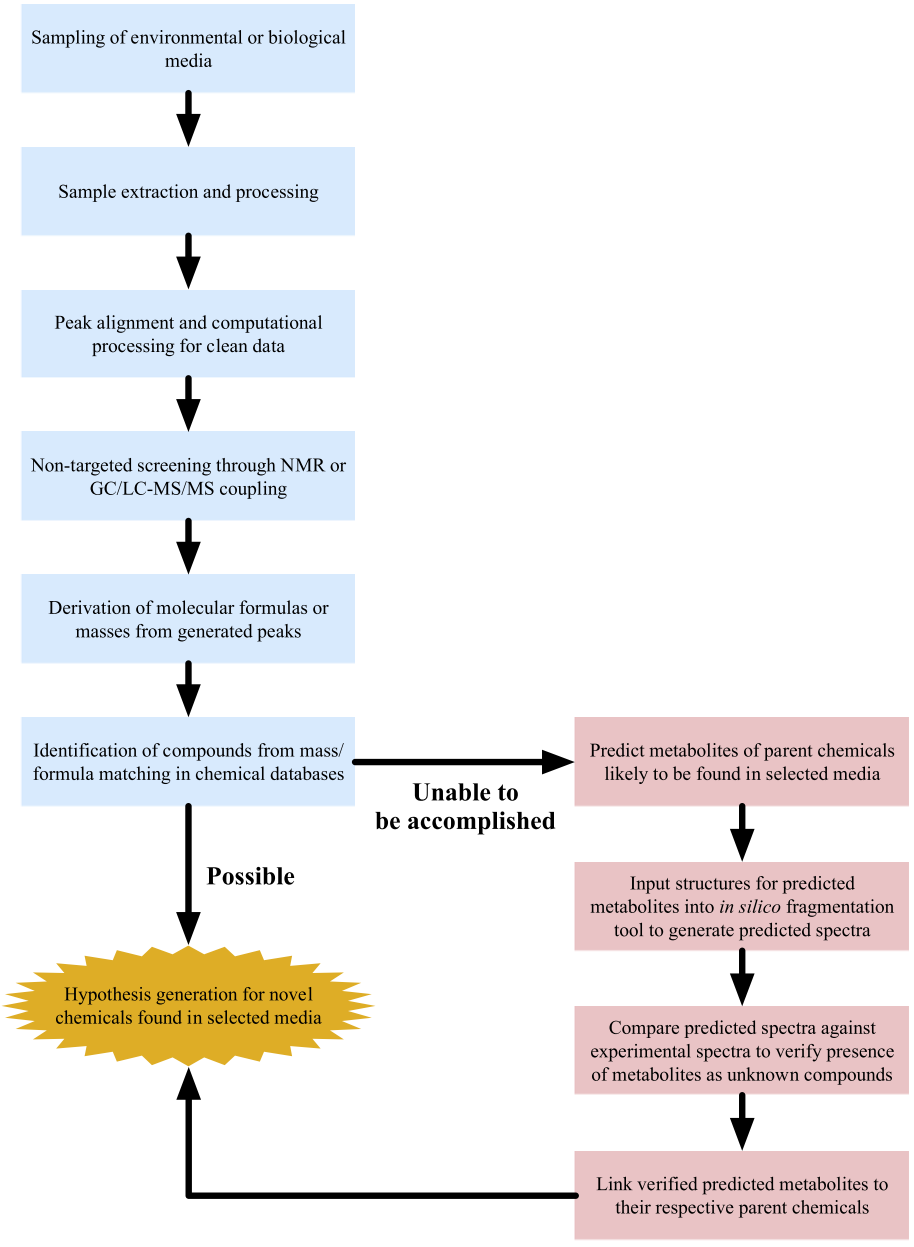
The presence of metabolites predicted by any of these platforms can be verified by comparing experimental spectra generated from extracts of the select medium to reference spectra contained in online databases. Some examples of these spectral libraries include METLIN [54], the Mass Spectral Library developed by the National Institute for Standards and Technology (http://nistmassspeclibrary.com/), and the Golm Metabolome Database [55]. Unfortunately, it is often the case that spectra for these metabolites will not be available, just as their general chemical information is not available within chemical databases. As a result, several *in silico* tools have been developed that contain predicted spectra based on how a chemical is expected to fragment in a mass spectrometer [56]. Such spectral predictive

tools include LipidBlast [57], MetFrag [58], MS-Finder [59], and the Competitive Fragmentation Modeling for Metabolite Identification tool [60]. The combined use of *in silico* applications that predict metabolites and generate spectra for those putative metabolites can complement nontargeted analysis (Figure 1). By combining both biological screening and nontargeted chemical analysis that apply similar technological tools, chemicals within multiple media types can be more effectively monitored and managed [61].

Expanding the utility of systems-based pathways in risk assessment through *in silico* characterization of metabolism

The Mode of Action framework was developed to aid in human risk assessment by establishing the relationship between a biological effect and exposure to a specific chemical or chemicals [62,63]. This was later followed by the chemical-agnostic Adverse Outcome Pathway (AOP) framework, which was developed primarily for ecosystem risk assessment and then expanded to human

Figure 1



Workflow of *in silico* approaches (red boxes) to complement nontarget screening (blue boxes) for identification of detectable compounds within biological and environmental media. NMR, nuclear magnetic resonance; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry.

risk assessment applications [64–66]. Both frameworks operate by organizing chemical knowledge and data across a series of biologically connected events within a living system to allow for informing mechanism-based outcomes relevant to human and ecological health. The practicality of the AOP framework lies in its underlying premise that induction of the first key event within the series (i.e., the molecular initiating event) will eventually lead to a final adverse biological outcome, regardless of the chemical involved. More recently, this premise has led to the suggestion that the AOP framework can be integrated with a newly proposed Aggregate Exposure Pathway (AEP) framework. The AEP framework is designed to follow a chemical or nonchemical stressor from its site of origination to its site of biological action [67,68]. It can be assumed that, given a sufficient concentration, the site at which a chemical acts is analogous to the site at which the molecular initiating event of an AOP will be triggered. Thus, integration of these two frameworks allows for better understanding the entire source-to-outcome continuum for a given chemical [69].

Often, the action of one chemical at a specific site is insufficient for triggering a molecular initiating event, and so an AOP might not be assumed possible for that chemical. However, multiple chemicals acting at the same site can lead to an adverse outcome, and so it is critical that all those potential chemicals are considered. Many times, these chemicals will include active metabolites of an inactive parent compound [51]. Predicting and verifying these metabolites through the aforementioned *in silico* approaches allows for identifying additional chemicals that can perturb a given biological target. Alternatively, these metabolites may act upon other molecular sites and lead to different adverse outcomes that might not otherwise be identified if not accounting for those metabolites.

Conclusions

Numerous technological tools have been developed to address the growing number of chemicals released to market on an annual basis [70] and to advance the field of exposure science [44]. A lack of available chemical-specific data for many chemicals has resulted in development of *in silico* methodologies to investigate exposure and PK characteristics for evaluating their safety risks. While a majority of PK behaviors can be predicted through knowledge of physicochemical properties, characterization of clearance, especially metabolic mechanisms, remains a significant challenge. Investigating the metabolome of a living organism exposed to environmental chemicals can provide insight into resulting phenotypic biological effects, and metabolomics tools, such as metabolite prediction applications, offer great potential for environmental monitoring. The integrated chromatography–mass spectrometry approaches and tools used for metabolomics studies are also easily transferrable to nontargeted analysis of abiotic media for investigators wishing to refine their chemical detection practices. Many major obstacles remaining for metabolomics to meet its true potential, such as lack of spectral reference libraries and inability to identify detectable metabolites, are being addressed through development of *in silico* tools such as metabolite-predicting software and predictive spectral libraries. Complementing nontargeted environmental analysis with bioanalytical screening will help to identify chemicals that previously may have been unable to be detected in a given medium, even if their presence is likely. The Toxic Substances Control Act involves regulation of novel or existing chemicals (https://www.epa.gov/tsca-inventory), and the ability to detect and identify chemicals that were previously unable to be identified in a particular medium can significantly aid in facilitating regulation of such chemicals. These *in silico* tools that help to advance exposure science and that help to fill data gaps in a chemical's AEP framework can also help to improve confidence in risk-based decisions applying knowledge of an AOP.

Disclaimer

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Conflicts of interest statement

Nothing declared.

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* of special interest
** of outstanding interest

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