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Mixtures Research at NIEHS: An Evolving Program

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Abstract

The National Institute of Environmental Health Sciences (NIEHS) has a rich history in evaluating the toxicity of mixtures. The types of mixtures assessed by the Division of the National Toxicology Program (DNTP) and the extramural community (through the Division of Extramural Research and Training (DERT)) have included a broad range of chemicals and toxicants, with each study having a unique set of questions and design considerations. Some examples of the types of mixtures studied include: groundwater contaminants, pesticides/fertilizers, dioxin-like chemicals (assessing the toxic equivalency approach), drug combinations, air pollution, metals, polycyclic aromatic hydrocarbons, technical mixtures (e.g. pentachlorophenol, flame retardants), and mixed entities (e.g. herbals, asbestos). These endeavors have provided excellent data on the toxicity of specific mixtures and have been informative to the human health risk assessment process in general (e.g. providing data on low dose exposures to environmental chemicals). However, the mixtures research effort at NIEHS, to date, has been driven by test article nominations to the DNTP or by investigator-initiated research through DERT. Recently, the NIEHS has embarked upon an effort to coordinate mixtures research across both intramural and extramural divisions in order to maximize mixtures research results. A path forward for NIEHS mixtures research will be based on feedback from a Request for Information (RFI) designed to gather up-to-date views on the knowledge gaps and roadblocks to evaluating mixtures and performing cumulative risk assessment, and a workshop organized to bring together mixtures experts from risk assessment, exposure science, biology, epidemiology, and statistics. The future of mixtures research at NIEHS will include projects from nominations to DNTP, studies by

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extramural investigators, and collaborations across government agencies that address high-priority questions in the field of mixtures research.

Keywords

combined exposures; cumulative; multi-pollutant; co-exposure; stressors

1 Introduction

The National Institute of Environmental Health Sciences (NIEHS), located in Research Triangle Park (RTP), North Carolina, is one of the 27 Institutes and Centers of the National Institutes of Health (NIH). The NIEHS is comprised of three divisions: the Division of Intramural Research (DIR), the Division of Extramural Research and Training (DERT) and the Division of the National Toxicology Program (DNTP). The mission of the NIEHS is to reduce the burden of human illness and disability, by understanding how the environment influences the development and progression of human disease.

Recently, the NIEHS developed its 2012–2017 Strategic Plan (http://www.niehs.nih.gov/about/strategicplan/) to prioritize research activities. Although the topic of mixture is relevant to many of the Strategic Plan goals, Goal 4 of the plan entitled “Combined Exposures” specifically focuses on elucidating human health effects associated with chemical and nonchemical stressors. This goal is in recognition that humans are exposed to multiple chemicals including man-made and natural chemicals, throughout their lifetimes. Therefore, it is necessary to consider the action of diverse exposures including chemical (man-made and natural) and non-chemical stressors that can vary widely in populations, in order to gain a better understanding of the effects of the environment on human health. It follows that mixtures have been and will continue to be the focus of research projects in both the extramural and intramural NIEHS community.

1.1 History of Mixtures Testing at DNTP

The National Toxicology Program (NTP) is an interagency, government research program that was established in 1978 as a cooperative effort to coordinate toxicology testing programs within the federal government. The goals of the NTP are to strengthen the science base in toxicology, develop and validate improved testing methods, and provide information about potentially toxic chemicals to health regulatory and research agencies, scientific and medical communities, and the public. The NTP is headquartered within the DNTP of NIEHS, and encompasses NTP mission-relevant activities at NIEHS, along with the National Center for Toxicological Research (NCTR) of the Food and Drug Administration (FDA) and the National Institute for Safety and Health (NIOSH) of the Centers for Disease Control and Prevention (CDC). Together, the NIEHS and NTP are key contributors to supporting and conducting research to assess the human health effects of agents in our environment.

Assessing the potential hazard posed by mixtures has been a key area of interest for the NTP for many years. The earliest work by NTP on mixtures was in the early 1980s-90s,

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spearheaded by Dr. Raymond Yang, and focused on understanding the effects of well-defined “complex mixtures” of compounds that are frequently found as groundwater contaminants near hazardous waste sites (NTP 1993a). Those studies focused on a complex mixture of organic compounds and metals, the dose levels of which were environmentally relevant based on a USEPA survey. A second set of companion studies focused on a pesticide/fertilizer mixture (NTP 1993b) that was representative of groundwater contaminants in California and Iowa.

These studies in some way set a tone for the coming decades of NTP mixtures research in that they highlighted some of the governing principles that are considered when approaching such problems, namely: “environmental” relevance of the mixture; simplification of complex exposure scenarios through the attainment of good exposure data to develop tractable study design; and use of defined mixtures to explore interactions within mixtures and mixed exposure scenarios. In addition, these projects provided important empirical datasets, which informed the mixtures risk assessment process. Subsequent NTP projects addressing mixtures were built on this foundation.

In addition to these projects, NTP has been engaged a series of component-based mixture projects, including: assessing dose additive interactions within mixtures of dioxins and PCBs that bind the aryl hydrocarbon receptor (AhR) and are included in the dioxin toxic equivalency factor (TEF) scheme; effects of phthalate mixtures on reproductive tract development in collaboration with Dr. Earl Gray (US EPA/NHEERL); polybrominated diphenyl ether (PBDE) technical mixtures; and chemical studies to support risk assessment of mixtures of water disinfection byproducts.

Some of the projects that came later addressed a variety of mixtures and mixed exposures including: combinations of AIDS therapeutics (AZT, Lamivudine (3TC), Nevirapine and Nelfinavir mesylate) to assess the impact of combination therapy on the hazard posed by AZT; whole mixtures testing of a botanical extracts used in dietary supplements including aloe vera extracts, ginseng, kava kava, ginkgo biloba extract, green tea extract; technical mixtures used as flame retardants; and mixed exposures to fumes created during welding.

Building on this history of mixtures work, recent events have brought into focus the need for a comprehensive strategy on assessing mixtures and mixed exposures. Recent environmental disasters such as the Deepwater Horizon oil spill in the Gulf of Mexico and increased efforts by regulatory agencies to handle risk assessments of chemicals that may act through similar mechanisms of action highlight a desire to provide the public with a more comprehensive assessment of the hazards posed by real-life exposures.

1.2 History of Mixtures Research by NIEHS Extramural Scientists

The NIEHS and DERT have a long history of funding research in the area of mixtures (Table 1). The first NIEHS dedicated mixtures grants were funded in 1998 as a response to the Request for Applications (RFA: ES-98-002) titled “Chemical Mixtures in Environmental Health”. This RFA, developed in collaboration with the U.S. Environmental Protections Agency (US EPA), encouraged innovative experimental approaches and computational, statistical or predictive strategies that focused on the mechanistic basis for chemical

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interactions, related health effects, and the development of biologically relevant risk assessment models for human exposure to chemical mixtures. Research resulting from the RFA included: development of targeted microarrays to screen chemicals for activity and mechanism (Bartosiewicz et al. 2001); use of a monitor compound to probe low dose interactions among chemicals (Vogel et al. 2002); and development of computer modeling tools for predicting the effects of complex mixtures (Liao et al. 2002).

Another avenue for conducting mixtures research has been the Superfund Research Program (SRP), which continues to support research on the biological effects and remediation of mixtures related to Superfund sites. From 1995–2000, Dr. Raymond Yang, from Colorado State University, was supported to formulate new risk assessment methodologies for chemical mixtures by coupling physiologically based pharmacokinetics/pharmacodynamics (PBPK/PD) and experimental toxicology with statistical/mathematical modeling. The CSU Center and SRP also jointly hosted a meeting at Colorado State University in 2001 entitled “Application of Technology to Chemical Mixture Research” (Suk et al. 2002). The specific recommendations that came from that meeting included: development of a rational approach for identifying chemicals and chemical mixtures based on credible exposure assessment, utilization of -omics and high-throughput technologies as tools to determine the interaction between environmental exposures and genes, the need to understand the mechanisms of interactions at the quantitative level for confidence in risk assessments for chemical mixtures, and the importance of interdisciplinary collaboration, of which SRP Centers are one such example where biomedical researchers work together with ecologists, engineers, etc., to address complex problems found at Superfund sites.

At approximately the same time, researchers at Texas A&M University (Center Director: Dr. Stephen Safe) were characterizing the toxic potential of complex mixtures of polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs). Their methodology utilized a variety of cultured cell types and measures of a range of toxic endpoints (e.g., genotoxicity, developmental toxicity, immunotoxicity, and enzyme induction) to provide a comprehensive evaluation of the health risks associated with PAH and HAH mixtures. The results from their experiments suggested that a risk assessment for a complex PAH mixture based solely on benzo(a)pyrene (BP) or BP-equivalents of identified PAHs may not accurately predict the mixture's genotoxic or immunotoxic potential (i.e., the complex mixture was more potent than predicted based on BP content or BP equivalents), which was attributed to either unidentified active PAHs present in the complex mixture or potential greater than additive interactions (Chaloupka et al. 1993; Chaloupka et al. 1995; Harper et al. 1996).

2 An Evolving Program

Mixtures science is in the midst of another wave of development, as evidenced by the flurry of mixtures-related conferences and workshops in 2011 and 2012. Through several decades of consistent work on mixtures, the field of seemingly infinite and intractable problems (Borgert 2004) has begun to shrink in number and scope as we hone in on the key questions. Previously, the concern over mixtures was centered on the idea that potential interactions among chemicals presented a confounding problem. There was a general belief that

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interactions could occur between any two compounds, the magnitude of these interactions was unpredictable, and the more chemicals present, the greater the potential for interactions. Now, it has emerged that within the body of mixtures research identifying greater than additive effects, interactions may be less prevalent and of a lesser magnitude than was initially believed (Boobis et al. 2011). This suggests that identifying interactions of public health concern may be a more tractable problem than initially thought. Additionally, methods are being developed to identify mixtures of concern through tools such as the maximum cumulative ratio (Price and Han 2011). These lines of study have helped to shape the current field of mixtures questions which center around: determining what characteristics of chemicals are important for grouping into cumulative risk assessments, testing the boundaries of low-dose additivity to determine when this model breaks down, and developing methods to assess complex mixtures, such as approaches based on sufficient similarity of unknowns to reference mixtures.

Given that NIEHS has many intramural and extramural scientists that are interested in the study of mixtures, it was identified that a coordinated NIEHS-wide mixtures strategy across both intramural and extramural divisions would be beneficial to make effective use of NIEHS investments in this research area. In order to begin the process of developing such a strategy, representatives from the three divisions of the NIEHS (DERT, DNTP, and DIR) came together to discuss how to move forward. The first phase involved gathering information on the major challenges and data gaps in mixtures research. NIEHS put out a Request for Information (RFI) in the spring of 2011 to gather broad-based input on the key challenges to mixtures research. The responses to the RFI provided the basis for identifying focus topics (Table 2). These focus topics represent research areas that require attention from the scientific community. Subsequently, NIEHS coordinated a multidisciplinary workshop in order to focus attention on the key topics.

2.1 NIEHS Workshop

The NIEHS workshop titled “Advancing Research on Mixtures: New Perspectives and Approaches for Predicting Adverse Human Health Effects” took place September 26–27, 2011, in Chapel Hill, NC. Experts from risk assessment, exposure science, biology/toxicology, epidemiology, and statistics gathered to discuss current challenges in mixtures science and prioritize research goals. During the first day of the workshop, speakers oriented participants by providing discipline-based perspectives on the state-of-the-science and major challenges associated with mixtures research. Following these presentations, participants were placed in discipline-based discussion groups to further develop a list of key research topics. The focus on discipline-based concerns was designed to identify overlap between fields, as well as discipline-specific challenges with which other groups may not be familiar.

On the second day of the workshop, speakers presented views on innovative approaches for addressing mixtures issues. Presentations covered cross-discipline experimental design considerations for epidemiologists and toxicologists, using global gene expression tools to agnostically assess environmental exposures, and novel mixtures approaches being used in epidemiology. These talks were followed by multidisciplinary breakout sessions. Each group was given a key topic on which to focus. A priority matrix was provided to the groups

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and they were tasked with ranking research topics. They were also asked to propose general strategies for testing highly ranked issues. There were several cross-cutting themes that emerged from the workshop (Table 3).

A workshop report is currently in progress and will be posted to the website in the Fall of 2012 (http://tools.niehs.nih.gov/conferences/dert/mixtures/). Background materials, as well as slides from presenters and breakout sessions can also be found on the meeting website.

3 Current Mixtures Research at NIEHS

3.1 Mixtures Challenges at DNTP

There are several mixtures challenges that are particularly relevant to NTP studies. These include: chemical analysis and sufficient similarity issues related to complex mixtures inherent in work with plant extracts such as herbal supplements, study design considerations for nominations of chemical classes or multiple co-occurring chemicals, determining what kind of data would be most useful to federal partners performing human health risk assessments, and adapting high-throughput screening methods to the study of mixtures. The examples presented below include diverse test articles, questions, and approaches, but share the potential to elucidate the basic biological principles of joint action among chemicals. In current and future NTP projects, research with mixture-related test articles will serve a dual-purpose: 1) address the identified data gaps and 2) provide an opportunity to develop and test a mixtures hypothesis of interest. In addressing the first purpose, considerations of human relevance of route and biological response are important. However, in developing testable mixtures hypotheses, a diverse array of mixtures ranging from simple, defined mixtures (e.g., 2–3 component drug combinations) to highly complex mixtures (e.g., herbals or PAH-containing environmental mixtures) will be useful in elucidating biological principles of mixture toxicity and informing our understanding of real-world mixtures.

3.1.1 Herbals—Herbal dietary supplements are regulated by the FDA under the Dietary Supplement Health and Education Act (DSHEA) of 1994. As such, the contents of these supplements are not regulated in the same way as drugs intended to treat, cure, prevent, diagnose, or mitigate disease. The DSHEA legislation requires premarket approval for new dietary ingredients and prohibits manufacturers from introducing products posing “significant or unreasonable risk” (FDA 1994). In effect, although specifications for purported active ingredients may be provided by bodies such as the US Pharmacopeia, compliance to these specifications is voluntary. Therefore, products on the marketplace often display a wide range of constituent concentrations that often differ significantly from label claims (Draves and Walker 2003; Edwards and Draper 2003; Fransen et al. 2010; Harkey et al. 2001; Ruparel and Lockwood 2011). This lack of consistency in content found in products available on the marketplace presents a considerable challenge in test article selection for NTP studies with herbal products.

A major mixtures question inherent in NTP studies with herbal extracts is how to determine whether a complex mixture is chemically and biologically representative of other complex mixtures. This is a question of establishing that complex mixtures are sufficiently similar to one another. Development of methods to assess sufficient similarity would aid in the

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selection of test article and extrapolation of results to similar products in the marketplace. Assessment of sufficient similarity would include both an analytical chemistry component and a biological component. The analytical chemistry portion of this evaluation would likely require comparison of chromatographic fingerprints (Xie et al. 2006) along with quantification of marker constituents between samples. The biological comparison of complex mixtures will require case-by-case development. It will be important to develop a testing paradigm that is not cost- or time-prohibitive (e.g., in vitro assay(s)), but is demonstrably linked to the adverse outcome(s) identified in the definitive study with the test article. The identification of early biomarkers of effect (e.g. gene expression changes) during the definitive toxicity or carcinogenicity studies would facilitate development of short-term indicators that could be used in this capacity. In addition to the measurement of the chemical and biological "fingerprint" of the complex mixtures, statistical methods and criteria for including samples as sufficiently similar or excluding samples as significantly different must be established. Although the issues surrounding toxicity testing of herbals are considerable, they offer an excellent opportunity to develop methods for determining sufficient similarity across chemical and biological space.

3.1.2 Complex nominations—As awareness of exposure to multiple pollutants grows, NTP has increasingly received complex nominations. These nominations include classes of compounds (e.g. ionic liquids, flame retardants, sunscreen agents, and perfluorinated compounds) as well as combinations of chemicals that may co-occur (e.g. pharmaceuticals and pollutants in water sources, combination AIDS therapeutics, and mold mixtures). Complex nominations often require innovative approaches and special design considerations. Some examples of current complex nomination projects are discussed below.

3.1.2.1 Flame retardants: One of the more challenging class nominations is the flame retardants, which were nominated to NTP by the Consumer Product Safety Commission (CPSC). This class includes chemicals such as antimony oxide (AO), tris(2-chloroisopropyl) phosphate (TCPP), and aromatic phosphates. The aromatic phosphates in particular offer an opportunity to utilize a chemical class nominated for toxicity assessment to generate and test mixture hypotheses and/or develop a blueprint for approaching future complex class nominations. The aromatic phosphates include tert-butylphenyl diphenyl phosphate (BPDP), isodecyl diphenyl phosphate (IDDP), isopropylated triphenyl phosphate (IPP), 2-ethylhexy diphenyl phosphate (EHDP), triphenyl phosphate (TPP), and tricresyl phosphate (TCP). These compounds are used in commercial formulations that contain other active ingredients.

As with other complex mixtures, chronic toxicity testing of each active ingredient alone, as well as the representative commercial formulations in which they are present would be infeasible. The testing of commercial products is further challenged by the dynamic and proprietary aspects associated with commercial formulations. In the case of aromatic phosphates, a tiered approach will be used to assess the individual class constituents identified as high production volume chemicals in medium- to high-throughput assays and select representative compounds will be further assessed in robust toxicity/carcinogenicity studies. The testing of individual chemicals during the initial phase could inform the

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development of mixtures-related hypotheses that would be relevant to complex mixtures (e.g., other commercial formulations or environmental mixtures) in general.

For example, a downstream goal of this work could be to examine the biological effects associated with each of the individual aromatic phosphates and compare those to a commercial formulation or multiple commercial formulations in order to determine whether an individual constituent or the formulation is responsible for the observed toxic action. This kind of work would help inform the process of prioritizing individual chemicals or mixtures for study. However, significant challenges include: 1) selecting an appropriate combination of medium- to high throughput assays that reflect the biological targets of this class and 2) interpreting results from individual constituents versus commercial formulations. Regarding the first challenge, a careful analysis of available literature provides the basis for identifying toxicity targets and selecting appropriate assays. The utility/sensitivity of the selected screening panel will only be definitively evaluated following in vivo studies conducted in the second tier of the program. The latter challenge would likely involve use of analytical methods to identify patterns across biological space (e.g., pathway analysis and principal component analysis).

3.1.2.2 Polycyclic aromatic hydrocarbons (PAHs): The PAHs are another example of a complex class nomination. As discussed above, the PAHs offer multiple challenges with respect to accurately predicting the toxicity of complex mixtures based on the limited data available for commonly monitored, well-characterized PAHs (see section 1.2). There has been a great deal of interest in PAHs recently with the Deepwater Horizon oil spill and the release of the Environmental Protection Agency's (EPA's) draft "Development of a Relative Potency Factor (RPF) Approach for Polycyclic Aromatic Hydrocarbon (PAH) Mixtures". A Scientific Advisory Board (SAB) that reviewed the EPA PAH document suggested developing a whole mixtures approach along with the proposed RPF approach. Furthermore, they recommended requesting that NTP test 12–15 reference PAH mixtures (e.g. coal tar, coke oven emissions, diesel and gasoline exhaust, etc.) in 2-year rodent carcinogenicity studies to inform the development of a future whole mixtures risk assessment approach for PAHs.

This class of chemicals offers many challenges, as well as exciting opportunities to greatly impact the approaches used to assess complex mixtures. The first challenge is in selecting appropriate test articles. Many of the sources of PAH mixtures produce dynamic outputs. Additional challenges involve the chemistry of complex mixtures. For example, extracting complex mixtures from a source or acquiring enough of a sample to use in a 2-year bioassay are not trivial considerations. Additionally, detailed chemical analysis would be required to determine whether other components often present in complex PAH mixtures (e.g. heavy metals, dioxin-like compounds) could contribute to any observed toxicity. Lastly, as discussed with herbals, methods for determining sufficient similarity would need to be developed for this class, as these methods are not one-size-fits-all.

Future PAH work should be aimed at strengthening the database for the current RPF approach used to predict human health risk associated with exposure to PAH-containing mixtures, while concomitantly moving forward to develop whole mixture-based approaches

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for assessing risk. Once a whole mixture framework is established for predicting PAH mixture toxicity, a comprehensive comparison of the RPF method and whole mixture method could be conducted. Results of this comparison would be extremely informative in guiding future complex mixture risk assessment directions and identifying pressing data needs.

3.1.2.3 Pathway-based nominations: Pathway-based nominations represent a recent development that is congruent with an overall trend in toxicology moving from a chemical focus to a focus on disruption of biological pathways (NAS 2007). This trend is especially relevant to mixtures research and cumulative risk assessment considering the complexities associated with assessing the effects of multiple chemical (and non-chemical) stressors on a pathway. The shift to a pathway focus was recently recommended by an NAS committee tasked with evaluating whether a cumulative risk assessment should be performed for phthalates (NAS 2008). From a risk assessment perspective, the challenge then emerges of how to decide which chemicals (or stressors) to include in a cumulative risk assessment based on pathway disruption.

Theoretically, only chemicals that contribute to dose additive toxicity should be included in a cumulative risk assessment because chemicals that act independently (in a response additive manner) will not contribute to mixture toxicity below their no observed adverse effect level (NOAEL). Therefore, the question can be re-framed as: do chemicals that target a common pathway contribute to dose additive mixture toxicity? Dose additivity is an accepted model for chemicals that share a common mechanism of action and has been used for cumulative risk assessments of chemical classes, such as organophosphates. However, the question of whether chemicals, which act at different points along a pathway through different mechanisms of action abide by a model of dose additive toxicity, remains an active area of research. Research to date has focused almost exclusively on endocrine disrupting chemicals (Crofton et al. 2005; Kortenkamp and Faust 2010; Rider et al. 2010). Current and future projects at NTP will be dedicated to probing the limitations of using target biological pathways to determine which chemicals will conform to a model of dose additive toxicity.

There is a tendency toward simplification (i.e. grouping chemicals according to class) based on the overwhelming prospect of identifying all chemicals that could disrupt the target pathway of interest. Fortunately, in a risk assessment context, only chemicals that are likely to co-occur require examination. This significantly narrows the chemical universe but requires an extensive exposure assessment.

3.1.2.4 Co-exposure nominations – AIDS therapeutics: A third category of mixture nominations based on likely co-occurrence is exemplified in the combination AIDS therapeutics project. These studies, performed in collaboration with NCTR, aim to determine whether co-administration of AIDS therapies increases incidences of cancer in rodents over levels elicited by a single compound. Hazard associated with pharmaceuticals is typically the purview of manufacturers, which are required to assess potential drug-drug interactions through screens for enzyme induction/inhibition. However, the requirement for assessing specific combination therapies (e.g., AZT + Nitazoxanide) is less clear and NTP can continue to play a vital role in addressing this data gap.

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3.1.3 Need for better understanding of Risk Assessor Data Needs—Another major challenge for NTP moving forward is in communicating *a priori* with end-users of NTP data in order to provide the greatest impact for improving public health through research. Two examples of this need for enhanced communication can be drawn from the previously discussed herbals and PAHs.

Two issues that frequently arise with herbals during the data interpretation phase are test article selection and human relevance. In terms of test article selection, collaboration with FDA to determine which suppliers hold the greatest market share and to develop criteria for excluding samples from selection would provide confidence that the test article selected is representative of market products. The issue of how NTP data relates specifically to humans is more difficult to address. Since NTP is not a regulatory agency it is not charged with specifically conducting risk assessments, that require an integration of both hazard information, dose response and exposure data. However, NTP does explore the mechanisms of toxicity or tumorigenicity for comparing across species in order to provide risk assessors with data that will help in extrapolating from rodents to humans.

The PAHs illustrate a challenge that will help to define the path of future mixtures work at NTP. It is widely held that risk assessors prefer toxicity data from the complex mixture of interest. When this is not available, data from a sufficiently similar mixture is preferred. Only when the above options are unavailable, is a relative potency factor approach used. However, in practice, data from the complex mixture or a sufficiently similar mixture is rarely available. Therefore, the RPF method may be the rule, not the exception at this time. This begs the question, should research effort be focused on strengthening individual chemical data for use in an RPF approach, or would effort best be spent on beginning to develop complex mixture reference libraries and sufficient similarity assessment approaches.

These two paths represent dichotomous research tracks that would have little overlap. A serious conversation between data-users (risk assessors) and researchers is needed to clarify to which path resources should be dedicated. If, for example, there is agreement that the RPF approach is protective of human health and is the approach that will be realistically used for the next several decades, then effort dedicated to building individual chemical databases would be a reasonable use of resources. However, if the sufficient similarity approach is heavily favored by risk assessors and it is held that the RPF approach should be phased out in the next decade, effort would be better spent, at this time, developing and strengthening methods to determine sufficient similarity and building complex mixture toxicity databases.

3.1.4 High-throughput Screening and Mixtures—Evaluating the toxicity of individual chemicals and mixtures is resource intensive, and time consuming. There are 10s of thousands of chemicals in commerce, which when combined could result in limitless mixtures. It is clear that the present toxicity-testing paradigm cannot address the data needs for understanding the potential risks associated with exposure to these individual chemicals, let alone the numerous mixtures of these chemicals. In recognition of these challenges, the DNTP developed a vision and roadmap of toxicity testing in the 21st century that called for

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the development of high-throughput screening (http://ntp.niehs.nih.gov/files/NTPrdmp.pdf). Shortly after the release of its roadmap, the NTP and the US EPA funded the NRC to develop a long-range strategy for toxicity testing. These reports serve as the basis for the concept of "Toxicity Testing in the 21st Century" (NAS 2007).

A key concept in the 2007 NAS report is the development of high-throughput screening (HTS) assays to interrogate toxicity pathways. A toxicity pathway is a cellular response pathway that, when sufficiently perturbed, is expected to result in an adverse health effect. The present HTS technologies can evaluate hundreds of thousands of chemicals per week per assay. Application of the HTS approach to mixtures has promise to resolve the resource limitations that has plagued the fields of mixtures research and mixtures risk assessment.

The HTS approach to toxicity testing is an adaptation of HTS approaches used in drug discovery by the pharmaceutical industry. There are some challenges to adapting this approach to environmental toxicology. In drug discovery, the goal is to identify possible drug candidates for further evaluation by identifying potent and efficacious chemicals with the occurrence of false negatives being of little concern. With these goals in mind, drug discovery approaches to HTS typically use only a single concentration of test chemicals. In contrast, the use of HTS in environmental toxicity testing is aimed at flagging any chemical with potentially toxic activity for further evaluation. Therefore, the occurrence of false negatives is a far greater concern than false positives in this context. Additionally, for environmental toxicity testing initial information about the dose-response of chemicals in the HTS system is desirable for interpreting results and informing further testing.

In order to adapt and develop HTS methods from drug discovery to environmental toxicology, significant effort is required, which is beyond the capabilities of any single institute. Therefore, the NTP, EPA, the National Institutes of Health Chemical Genomics Center (NCGC), and the FDA have established a collaborative research program designated Tox21, which has developed quantitative HTS (qHTS) methods that perform 15-point concentration response curves in HTS assays for toxicity pathways (Inglese et al. 2006). Additional work is needed including the development of tools to translate in vitro concentrations to internal doses in vivo and identification of the causes of false negative and positive HTS responses.

Presently, the Tox21 program is in Phase II of data collection, where over 8,000 compounds will be evaluated in over 50 qHTS assays. These assays were chosen based on information from *in vivo* toxicological investigations, experience from Phase I qHTS efforts, advice from basic researchers, and nominated assays. These assays target pathways related to nuclear receptor activation and inhibition and stress pathways such as oxidative stress, DNA damage, hypoxia, inflammation, and heat shock. A small mixtures pilot project that will evaluate whether the present qHTS approach is applicable to mixtures toxicity testing has been included in the Phase II initiative. The pilot project will also evaluate the interactions of up to 80 chemicals in over 130 mixing ratios. For all individual chemicals and mixtures, 15-point concentration response curves will be generated for each assay. All data will be made publically available on PubChem as well as in the NTP Chemical Effects in Biological

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Systems (CEBS) database. http://www.niehs.nih.gov/research/resources/databases/cebs/index.cfm.

Although it is clear that in vivo studies on this number of chemicals and mixtures would be nearly impossible, the qHTS approach has some limitations and challenges, which include typical in vitro system issues. For example, how do in vitro concentrations relate to in vivo exposures? How can the 3-day or shorter cell exposure be extrapolated to chronic in vivo exposures? In addition, how can we relate in vitro changes to complex diseases and adverse outcomes? Other challenges and limitations unique to qHTS methods include the use of robotics by NCGC that limit, at this time, the chemical space that can be screened to non-volatile chemicals that are soluble in DMSO. There is clearly a broad range of chemicals that are of interest that do not fit into this category. Another challenge is identifying toxicity pathways. With only 50 assays available, not all toxicity pathways can be evaluated, potentially leaving out important pathways. Therefore, future needs include identifying additional toxicity pathways and developing appropriate qHTS assays for them. Furthermore, present qHTS assays use a single cell type and query a single pathway at a time. Interactions between different cell types are not considered in the present battery of assays, which is considered a significant limitation of qHTS for mixtures research. In the NAS report “Phthalates and Cumulative Risk: The Task Ahead”, it was recommended that cumulative risk assessment should move beyond assessing chemicals based on a single mechanism of action (NAS 2008). In their recommendation, the NAS panel suggested that cumulative risk assessments should include chemicals that induce a similar adverse effect independent of their mechanism of action. Since the present screens use only a single cell and query a single pathway at a time, the assays do not lend themselves to the type of cumulative risk assessment recommended by the NAS.

Another limitation of the qHTS approach is the limited or lack of metabolism in the cell systems presently in use. The role of metabolism in detoxification and activation of environmental chemicals cannot be understated and metabolism is even more important in understanding the potential toxicity of mixtures. Metabolic interactions are a source of non-additive interactions, which cannot be evaluated in the present methods. There are in vitro systems that have metabolic capability, such as primary human hepatocytes and more recently the HepaRG cells. Efforts are underway to develop qHTS assays using these cell types. It should be noted that not all in vitro assays can be converted to qHTS assays. qHTS assays typically use approximately 1000 cells/assay. Some cell types do not grow well in these conditions and in other cases the signal to noise ratio drops dramatically when moving from the 96-well plate format to the smaller well plates used for qHTS.

qHTS approaches have tremendous potential to increase our throughput in toxicity testing for individual chemicals as well as mixtures. These efforts are in their initial stages and have some significant limitations for mixtures research and mixtures risk assessment, some of which have been discussed above. Undoubtedly, the pilot qHTS mixtures project will identify additional limitations. Assays that can query multiple cell types and multiple pathways are necessary in order to understand cumulative risks. These present limitations should not preclude the use of qHTS approaches in mixtures research and efforts to enhance this approach should continue.

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3.2 Highlights from Current Mixtures Research by Extramural Scientists

3.2.1 Superfund Research on Mixtures—For many years, the SRP has highlighted the need for continued "Mixtures" research in their annual solicitation for the P42 Center Grant Request for Applications, indicating its importance to the SRP and its stakeholders. For example, the SRP is currently supporting investigators such as Dr. Robert Wright (Harvard University School of Public Health) who has been conducting research on the effects of metal mixtures (i.e., lead, arsenic and manganese in water and soil) on neurodevelopment in children. Investigators within this Center include Dr. David Christiani who is conducting a systematic approach to studying gene-environment interaction to determine susceptibility, how metals induce toxicity, and provide biological insight for potential treatment and prevention measures. Another Harvard investigator, Dr. Brent Coull, is developing statistical design and analysis tools to improve the accuracy and reliability of site and exposure assessment for Superfund hazardous waste sites (i.e., spatial model-based approach for design and analysis and comparing it to existing design-based approaches that do not account for spatial correlation).

The SRP Center at Louisiana State University (Center Director: Dr. Barry Dellinger) has begun to study mixtures of environmentally persistent free radicals (EPFRs) associated with ultrafine particulate matter, contaminated soils, or the thermal treatment from wood-treating sites and the fly-ash produced from incineration of hazardous substances. At high concentrations in thermal treatment devices, the EPFRs promote the formation of new molecular pollutants, such as polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F). The SRP Center at Oregon State University (Center Director: Dr. David Williams) has been determining PAH atmospheric deposition and composition, evaluating the role of PAHs in cancer, and developing innovative detection tools (i.e., passive sampling devices) deployed at Superfund sites to measure PAHs. Finally, the Center at the University of Iowa has been evaluating different forms of PCBs.

Several of the NIEHS R01 awardees, as well as researchers funded through other grant mechanisms (K-awards, SBIR/STTR awards), are investigating mixtures research questions. Some of the topics covered by NIEHS grantees can be seen in Tables 1 and 4. These studies assess a variety of mixtures, some of which are complex. In fact, these mixtures studied by NIEHS grantees are relevant to the regulatory communities, which often deal with real-world mixtures (e.g., particulate matter). We are also funding different types of research studies that evaluate a wide variety of outcomes. For example, these include developing different models to assess cancer, alternative animal models, and in vitro approaches.

3.2.2 Deepwater Horizon Consortium—NIEHS, along with seven other NIH institutes, are also funding an extramural program focusing on research related to the oil spill through a U19 grant mechanism lead by Dr. Claudia Thompson. Academic institutions including, University of Florida, Tulane University, University of Texas (Galveston), and Louisiana State University have formed a network assessing a variety of long-term health effects of the oil spill, focusing on different populations including women and children. There is also a special focus on the in utero and developmental effects associated with the oil spill. In terms of characterizing exposure, members of the consortium are collecting seafood samples in the

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Gulf region and developing analytical techniques to measure polycyclic aromatic hydrocarbons and other chemicals. In addition to the cross-institutional collaboration, these universities will also work with the intramural NIEHS community including DIR and DNTP.

3.2.3 Exposure Biology—The NIH Genes Environment and Health Initiative was originally a four-year effort that has been extended. Different institutes are working together to determine the link between our genes and the environment. The NIEHS has had the responsibility of leading the Exposure Biology Program, which is a part of this effort. The objective of this program was to develop wearable, easy-to-use sensors for detection of environmental chemicals and measurement of physiological parameters. For example, these sensors could capture data on physical activity and psycho-social stress (e.g. heart-rate). Some of these sensors that were developed under this program are being used in some of the National Children's Study centers. Researchers are beginning to try to validate their use in different epidemiological approaches. These sensors offer exciting promise for measuring more than one or two chemicals; some can measure 40 volatile organic compounds (VOCs). The ultimate goal in developing these new technologies will be to measure the totality of human exposures.

As we develop new, more sensitive analytical techniques, it will be important to move beyond "looking under the lamppost" - only measuring exposure to chemicals for which toxicity data is available. There are many chemicals that are not being monitored or studied, which should be considered. We are developing more comprehensive databases to capture human exposures. For example, NHANES is an incredible resource, which is being mined for the identification of chemicals associated with a variety of different health outcomes. However, there is an issue in interpretation of NHANES data because it is a post-hoc analysis. There is a need for ongoing longitudinal prospective studies to collect samples at different timepoints in order to support the epidemiological findings and determine causal relationships. Examples of current longitudinal studies include the NIEHS GuLF study, the National Children's Health Study, and the ongoing California Biomonitoring Program, which is aimed at determining baseline levels of environmental contaminants and observing temporal trends in exposure. The NIEHS Children's Environmental Health Centers, funded with EPA, have several children's cohorts for which subjects are followed from birth on. The largest longitudinal cohorts include the Framingham Study and the Nurses' Health Study. Those studies provide an incredible amount of information for researchers to use in understanding environmental effects on human health. It is also important to consider how toxicologists and epidemiologists can work together. For example, toxicology studies can provide support for the biological plausibility of the links between exposure and observed human health effects.

In considering the growing databases cataloguing human exposures, it is necessary to harness the power of this exposure data and relate it to human health. The exposome, as described by Drs. Steve Rappaport and Martyn Smith, provides a characterization of total exposure and the metabolome (Rappaport and Smith 2010). This perspective takes advantage of new approaches, including chemical analysis and metabalomic tools. The exposome holds promise for contributing to our understanding of the relationship between

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total exposure and disease and the development of biomarkers. However, determining the contribution of environmental contaminants against the background of signals resulting from nutrition, disease, and internal chemicals that vary from person to person (e.g., hormones, inflammatory signals) represents a huge challenge.

4 The Future of Mixtures at NIEHS

As mentioned in the introduction, the NIEHS 2012–2017 Strategic Plan includes the study of combined exposures and joint effects of both chemical and nonchemical stressors as research priorities. Specifically, effort will be dedicated to elucidating the principles of how chemicals interact with nonchemical stressors such as the microbiome, infectious agents, diet, and psychosocial/behavioral factors.

In implementing the strategic plan, NIEHS will develop a coordinated strategy to address many of the data gaps associated with combined exposures. This effort will require extensive cooperation and collaboration across divisions, disciplines, and other organizations. Currently, a scientific focus group has been formed with representatives from all of the NIEHS divisions. This group will coordinate efforts to address the Combined Exposure Goal 4 of the NIEHS Strategic Plan, as well as provide a resource for communication of other mixtures-related research at NIEHS. Activities will include presentation of regular webinars/seminars from the intramural and extramural community, development of future workshops, and discussion of specific mixtures research projects.

In addition, members of the combined exposure scientific focus group will actively contribute their expertise to mixtures efforts lead by other organizations (e.g., development of US EPA Cumulative Risk Assessment guidance) and provide opportunities for collaboration between intramural and extramural communities. For example, NTP scientists were invited to participate and present current projects at the April 23–24 2012 meeting entitled "Complex Mixtures and Exposures: Analyzing, Modeling and Predicting Fate and Effects at Multiple Levels of Environmental and Biological Systems" hosted by Superfund Research Program grantees at Dartmouth College and Boston University.

In conclusion, mixtures research is a priority at NIEHS and will continue to be in the future. In developing the NIEHS mixtures program, on-going combined exposure projects will utilize cross-disciplinary expertise to address key questions to advance the field of mixtures. Through this effort, scientists will develop, strengthen and validate predictive models of mixture toxicity; assess the joint action of stressors on signaling pathways and systems; and continue improving exposure assessments and chemical analysis.

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Table 1

Past NIEHS Mixtures Grant Portfolio

Project Number	Title	PI	Organization	Years
R01ES009681*	cDNA Microarray To Detect Cellular Responses To Mixtures	Buckpitt, Alan	University of California-Davis	1999–2002
P42 ES010344	Genetic/Epigenetic Susceptibility To Superfund Chemicals	Costa, Max	New York University School of Medicine	2000–2005
R03ES014725	The Effect of a Mixture of Pesticides on the Rat Cardiac Proteome	Crow, John	Mississippi State University	2006–2009
R03ES016433	Environmental Exposure To Metal Mixtures and Kidney Disease	Fox, Mary	Johns Hopkins University	2007–2010
R01ES009673*	Asbestos and NO2 In Environmental Lung Disease	Heintz, Nicholas	University of Vermont & State Agric College	1998–2002
R01ES009642*	Immunotoxicity of Dermal Permethrin & cis-urocanic acid	Holladay, Steven	Virginia Polytechnic Inst and State University	1998–2001
R03ES012929	Metabolic Effects of Chemical Interactions In Toxicity	Jones, Dean	Emory University	2004–2007
R01ES009683*	Biologically Based Cancer Risk Assessment For Mixtures	Luebeck, Georg	Fred Hutchinson Cancer Research Center	1998–2003
K99ES016806	Additive Effects of Mixtures Endocrine-active Compounds To Medaka	Rider, Cynthia	Duke University	2009–2010
R01ES009690*	Strategy To Identify Nonadditive Response To Chemical	Vogel, John	University of California-LLNL	1998–2000
R01ES009676*	Modulation of Benzene Metabolism By Exposure To Environm	Weisel, Clifford	UMDNJ-Robert Wood Johnson Medical School	1998–2002
R01ES015447	Mechanisms of Resistance Of Aquatic Vertebrate Populations To Mixtures	Wirgin, Isaac	New York University School of Medicine	2006–2010
R01ES009655*	Developing a Predictive Strategy For Chemical Mixtures	Yang, Raymond	Colorado State University-Fort Collins	1998–2004

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Table 2

Key mixtures topics discussed by multidisciplinary breakout groups at the NIEHS Mixtures Workshop (Sept 26–27), Chapel Hill, NC

Topic 1 Modeling Mixture Toxicity: Constraints of Extrapolation
Topic 2 Exposure Assessment: Making Sense of the Data
Topic 3 Reconciling Epidemiological and Toxicological Approaches to Mixtures
Topic 4 Chemical Interactions: Predicting the Unpredictable
Topic 5 Mixtures across Time

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Table 3

Cross-cutting themes that emerged from the NIEHS mixtures workshop.

- In vitro versus in vivo approaches
  - A combination of in vitro and in vivo approaches are required to move forward on mixtures questions
  - Mixtures projects that include both in vitro and in vivo endpoints are needed
- Cross-disciplinary effort
  - Better coordination between epidemiology and toxicology is recommended
  - Specific areas that require attention include:
    - Different mixtures terminology in epidemiology and toxicology
    - More use of potency data from toxicology in epidemiology studies
    - Development of better statistical methods for assessing multi-chemical associations to disease
- Systems-based approaches for studying mixtures
  - Better understanding of biological pathways is required to develop mixtures hypotheses
  - Innovative bioinformatics approaches for managing “data-rich” mixtures experiments are needed
- Sufficient similarity as a key approach
  - Whole mixtures approaches are preferred by risk assessors and require fewer assumptions
  - Sufficient similarity methods require development/validation and more case studies
- Need for both bottom-up and top-down approaches
  - Both component-based and whole-mixtures approaches will be required in the future
- Federated databases should be developed to manage mixtures data, including exposure, in vitro, animal, and human data
  - Searchable, user-friendly database that integrates across data types would be an invaluable resource
- Prioritization of chemicals/mixtures is needed
  - Examples of suggested approaches included:
    - NHANES data to identify combinations with high exposure potential
    - Environment-wide Association Studies (EWAS) to develop testable hypotheses
    - Maximum cumulative ratio to prioritize mixtures for cumulative risk assessments

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Table 4

Current NIEHS Mixtures Grant Portfolio

Project Number	Title	PI	Organization	Years
R01ES015028	National Assessment of The Mortality And Morbidity Effects of Tropospheric Ozone	Bell, Michelle	Yale University	2006–2011
P42ES013660	Reuse in RI: A State-based Approach To Complex Exposures	Boekelheide, Kim	Brown University	2005–2014
R01ES012054	Statistical Methods For Population Health Research on Chemical Mixtures	Dominici, Francesca	Harvard University (Sch Of Public Hlth)	2003–2011
K08ES017045	Effects of PCBs And PBDEs On Three Distinct Components of Response Inhibition	Eubig, Paul	University of Illinois Urbana-Champaign	2009–2014
R21ES018993	Disruption of Transition Metal Homeostasis By Cd: Implications For Aging	Fierke, Carol	University Of Michigan At Ann Arbor	2010–2012
R01ES015276	Empirical Determination of Sufficiently Similiar Complex Mixtures	Gennings, Chris	Virginia Commonwealth University	2007–2011
T32ES007334	Integration of Mixtures Toxicology, Toxicogenomics, and Statistics	Gennings, Chris	Virginia Commonwealth University	2000–2012
R15ES013706	Long Term Toxicity of Di- and Tri-Chloroacetate	Hassoun, Ezdihar	University Of Toledo	2007–2011
K99ES020364	Prenatal exposure to a mixture of EDCs, maternal thyroid function and child neuro	Horton, Megan	Columbia University Health Sciences	2011–2013
R01ES014864	Metal and Organochlorines Exposure: Impact on Adolescent Behavior and Cognition	Korrick, Susan	Brigham And Women's Hospital	2006–2011
P42ES007381	Superfund Basic Research Program at Boston University	Ozonoff, David	Boston University Medical Campus	1995–2012
R01ES010807	Molecular Mechanisms of Complex Mixture Toxicity	Puga, Alvaro	University of Cincinnati	2001–2015
R15ES016905	Mechanisms of Immunological Adaptation to a Harsh Chemical Environment	Rice, Charles	Clemson University	2009–2012
R00ES015428	Assessment of Psychostimulant Addiction Risk Following Developmental PCB Exposure	Sable, Helen	University of Memphis	2007–2011
P20ES018163	Novel Methods to Assess Effects of Bisphenol A & Phthalates on Child Development	Schantz, Susan L	University of Illinois Urbana-Champaign	2010–2012
R01ES015687	PCBs, PBDEs, Hearing Loss & Attention/Impulsivity: Mechanistic Studies in Animals	Schantz, Susan L	University of Illinois Urbana-Champaign	2006–2011
R43ES019041	System for Decontaminating Well Water for Drinking	Srinivas, Girish	Tda Research, Inc.	2010–2011
R01ES014930	Metal Mixtures and Neurodevelopment	Wright, Robert	Brigham And Women's Hospital	2006–2011
P42ES016454	Superfund Metal Mixtures Biomarkers and Neurodevelopment	Wright, Robert	Harvard University (Sch Of Public Hlth)	2010–2014