Advancing Research on Mixtures:
New Perspectives and Approaches for Predicting Adverse Human Health Effects

Workshop Summary

NIEHS Workshop
September 27 & 28, 2011
Chapel Hill, North Carolina
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ACKNOWLEDGEMENTS

We would like to acknowledge the people who contributed to the success of this workshop.

Co-organizers:
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Contract Support:
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Overview: The NIEHS Workshop entitled “Advancing Research on Mixtures: New Perspectives and Approaches for Predicting Adverse Human Health Effects” was held on September 26-27, 2011 in Chapel Hill, NC (http://tools.niehs.nih.gov/conferences/dert/mixtures/). The purpose of the Workshop was to identify and focus on key issues that present challenges in mixtures research. The results from the Workshop will be used to inform the development of an NIEHS intramural and extramural mixtures research strategy and provide input to the scientific community for advancing mixtures research. The goals of this workshop were to:

- Identify and prioritize the knowledge gaps and challenges in mixtures research specific to each of the following disciplines: toxicology, epidemiology, exposure science, risk assessment, and statistics
- Obtain advice on integrating multidisciplinary capabilities to address critical topics in mixtures research
- Provide recommendations for research on key topics
- Inform the development of a long-term NIEHS mixtures research agenda
- Foster collaborations between extramural and NIEHS scientists

Day 1: The first day of the Workshop consisted of discipline-specific presentations from speakers in each of the areas listed above on the state-of-the-science and major challenges associated with mixtures research. These presentations were followed by breakout sessions consisting of discipline-specific groups tasked with developing and prioritizing a list of important knowledge gaps and research topics.

John Bucher, Ph.D., welcomed participants and provided a summary of previous NIEHS research on mixtures. Cynthia Rider, Ph.D., provided an overview of the Workshop format and goals. “Mixtures” (for the purposes of the Workshop) was defined broadly as any combination of chemicals that will contribute to our understanding of joint toxicity.

Glenn Ric, Sc.D., focused on the assessment of human health risk posed by exposures to environmental mixtures. He explained the various models available for conducting human health risk assessment of mixtures including component-based and whole mixture approaches. He used his work on disinfection by-products in drinking water as an example of a whole mixture case study. Rice discussed the toxic equivalency factor approach applied to dioxin-like chemicals as an example of a component-based risk assessment. He outlined the data needs of risk assessors including chemical analysis of complex mixtures, low-response region data, and high-throughput technology use and interpretation.

Paul Price, M.S., focused on the field of exposure assessment and specifically three issues in exposure science: lack of whole mixture data, lack of component data, and testing under the lamppost. He discussed the need for both monitoring and modeling in the characterization of real-world exposures. In terms of monitoring, Price discussed new sensor technologies that are being developed. Lastly, he described methods for prioritizing mixtures for study including the maximum cumulative ratio.

Earl Gray, Ph.D., discussed his work on chemicals that target androgen signaling in the developing male rat. He described the current paradigm that focuses on applying dose addition exclusively to chemicals with the same mechanism of action. His work suggests that this is too narrow a definition and that dose addition should be applied to chemicals that target a common signaling pathway or tissue.
David Christiani, M.D., discussed his work on metal mixtures associated with neurodevelopment. Specifically, he mentioned his work within the Harvard University Superfund Research Center, where he is evaluating birth cohorts that will be followed into childhood in the U.S., Bangladesh, and Mexico City. He has found interactions among metals and other contaminants. Discussion following his presentation revolved around the different definitions of mixtures terminology in epidemiology and toxicology and the need for increased cross-disciplinary understanding.

Chris Gennings, Ph.D., focused on methods for predicting the toxicity of an “unknown” mixture based on the toxicity of a reference mixture. She discussed examples of testing for sufficient similarity of whole mixtures for both data rich and data poor scenarios. Methods for determining sufficient similarity were based on equivalence testing used by FDA to determine that generic drugs are similar to their name brand counterparts.

Following the Day 1 presentations, participants formed discipline-based breakout groups to discuss current challenges in mixtures research specific to their field. The epidemiology group highlighted the need for proper exposure characterization, translation between epidemiology and toxicology, and prioritizing mixtures for study. The risk assessment group focused on the need for more individual chemical data, development of methods to group compounds, and the potential use of high through-put screening (HTS) in mixtures research. The statistics group explored case studies that could benefit from statistical methods development including data mining, developing whole mixture reference libraries, and scenarios involving mediation of “unknown” mixtures. The biology/toxicology group discussed topics ranging from in vitro to in vivo linkages and understanding dosimetry to predictive models of mixture toxicity. Lastly, the exposure science group discussed the need to approach exposure characterization from both a chemical perspective and a disease perspective, consideration of temporal and spatial variables, inclusion of non-chemical stressors in exposure analyses, and diversity of populations.

Rider presented the findings from a Request for Information (RFI) conducted prior to the Workshop to gain insight into key mixtures challenges. Major themes included identification of interactions, mixture model development and validation, the need for better exposure characterization, temporal considerations, use of HTS and omics technologies in the study of mixtures, and inclusion of network/systems approaches. These themes were used in development of focus areas for Day 2 discussions.

Day 2: Presentations covered novel approaches for addressing mixtures challenges, such as cross-discipline experimental design considerations, Environment-Wide Association Studies (EWAS), and multi-pollutant epidemiological assessment tools. Following the presentations, multidisciplinary breakout sessions were held to address key topics, develop a priority matrix scheme consisting of timeframe and scientific impact, and propose suggested approaches to evaluate the most highly ranked research questions.

Linda Birnbaum, Ph.D., Director of the National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP), provided an introductory presentation to begin Day 2 of the workshop. She expressed her enthusiasm for the study of complex exposures, which include both chemical and non-chemical stressors. Birnbaum also provided a brief history of NIEHS’s success in the field of combined exposures by presenting examples of previously funded research, current efforts (e.g., DNTP), and future endeavors (e.g., development and utilization of comprehensive databases, the exposome, and the investigation of latent effects of combined exposures).
Paige Tolbert, Ph.D., discussed her work in developing strategies to advance multi-pollutant epidemiologic research in the context of the complex mixtures of air pollution at the Emory/Georgia Tech Southeastern Center for Air Pollution and Epidemiology. This Center has a multitude of ongoing studies on health effects in commuters, asthma development, and acute health outcomes in a number of cities across the United States. Tolbert discussed the various analytical and experimental approaches currently being used in these studies to determine which components of air pollution contribute to the health effects being studied.

Andreas Kortenkamp, Ph.D., discussed the dose addition model as an extremely powerful tool for approximating mixture effects. He provided two examples of applying toxicological methods to epidemiology studies. In one example, relative potency factors derived from toxicology studies could be used to weight component chemicals in deriving an aggregate exposure estimate. In the second example, a whole mixture approach could be used by testing the activity (e.g., estrogenicity) of human samples from epidemiology studies and then identifying the active mixture components through chemical analysis. Throughout his talk, Kortenkamp emphasized the need for collaboration among the various disciplines.

Chirag Patel, Ph.D., discussed his methods to conduct environment-wide association studies (EWAS), which utilize approaches adopted from “omics” research. Patel analyzes the expansive data available in the public domain to look for associations between environmental factors and health effects in order to develop testable hypotheses. He discussed an example of this approach in which he identified environmental exposures that may be contributing to endpoints associated with cardiovascular disease (e.g., cholesterol levels) and Type II diabetes.

Following the Day 2 presentations, participants congregated in multidisciplinary breakout groups to address the specific mixtures topics developed based on the RFI. The first group discussed “Modeling Mixture Toxicity: Constraints of Extrapolation.” Participants discussed several issues including testing the interaction threshold, assessing the maximum cumulative ratio hypothesis, building a database to indentify interactions, exploring application of sufficient similarity approaches, and the need for statistical methods development. The second group was assigned the topic of “Exposure Assessment: Making Sense of Biomonitoring Data.” They discussed goals including developing methods to generate and analyze high-density exposure data and databases for consolidating exposure information. The third group addressed “Epidemiology: Reconciling Epidemiological and Toxicological Approaches to Mixtures” and covered translation of tools from toxicology to epidemiology and vice versa. The fourth group discussed “Chemical Interactions: Predicting the Unpredictable,” focusing on improving in vitro assays, using sufficient similarity techniques, and integrating across data types to understand interactions. The fifth group was tasked with “Mixtures Across Time” and discussed incorporating temporally-relevant features (aging) into animal models and approaches for assessing exposure over time.

Rick Woychik, Ph.D. provided closing remarks and discussed the development of the NIEHS 2012–2017 Strategic Plan. Major themes of the Workshop highlighted by Woychik included: relationship of external to internal dose, application of innovative approaches to mixtures, a need for databases to house diverse mixtures-related data, use of a systems-based perspective in mixtures research, and evaluation of the total exposure scenario.
The National Institute of Environmental Health Sciences (NIEHS) organized a workshop called “Advancing Research on Mixtures: New Perspectives and Approaches for Predicting Adverse Human Health Effects” (also called the NIEHS Mixtures Workshop) that was held September 26-27, 2011 in Chapel Hill, North Carolina.

The goal of the NIEHS Mixtures Workshop was to identify and focus on key issues that presented challenges in mixtures research (for this workshop, “mixtures” included combined independent exposures). The NIEHS used the results from the workshop to inform the development of an intramural and extramural mixtures research strategy. The workshop also provided input to the scientific community for advancing mixtures research. The summary document presented information from plenary and breakout group sessions that communicated results of the workshop to the greater scientific community.

Specifically, the workshop goals were to:

- Identify and prioritize the knowledge gaps and challenges in mixtures research specific to each of the following disciplines: toxicology, epidemiology, exposure science, risk assessment, and statistics
- Obtain advice on integrating multidisciplinary capabilities to address critical topics in mixtures research
- Provide recommendations for research on key topics
- Inform the development of a long-term NIEHS mixtures research agenda
- Foster collaborations between extramural and NIEHS scientists

The audience for the workshop consisted of invited participants and registered observers. The participants were invited based on their expertise, and were the primary discussants in the breakout session working groups. Observers also contributed to working group discussions, provided written comments during the meeting, and provided input during the question and answer periods.

This document summarizes the presentations the speakers gave during the plenary sessions, and variations in the style of the presentations are reflected in the text for each talk. This document also provides a synthesis of the breakout session discussions.
Day 1 of the workshop began with a series of presentations that provided overviews of relevant areas (epidemiology, statistics, biology, exposure, and risk assessment) within mixtures research from a discipline-specific perspective that included knowledge gaps and future directions of mixtures research. After lunch, discipline-based breakout groups addressed questions related to describing the state of the science in mixtures research. All attendees reconvened for summaries of the breakout sessions that are presented later in this document.

WELCOME – JOHN BUCHER, Ph.D., DABT, DIRECTOR OF THE DIVISION OF THE NATIONAL TOXICOLOGY PROGRAM (DNTP)

The conference, “Advancing Research on Mixtures: New Perspectives and Approaches for Predicting Adverse Human Health Effects,” was a collaborative effort of three divisions of NIEHS, the Division of Extramural Research and Training (DERT), the Division of the National Toxicology Program (DNTP) and the Division of Intramural Research (DIR). The primary organizers of the workshop were Cynthia Rider and Danielle Carlin. In addition, numerous people that included the organizing committee from NIEHS, as well as representatives from other organizations helped to put the workshop together.

Mixtures research has always been extremely challenging. The DNTP has had a long history of mixtures research. Ray Yang, Ph.D., conducted some of the first systematic attempts to study mixtures within the DNTP, including studies with drinking water contaminants and pesticide mixtures. Later, Nigel Walker, Ph.D., led a considerable effort to validate the use of toxic equivalency factors (TEFs) with dioxins and dioxin-like compounds for predicting cancer effects using rodent bioassays. The DNTP also evaluated the toxicity of many complex chemical mixtures. For example, through an interagency agreement with the National Institute of Occupational Safety and Health (NIOSH), the DNTP had measured complex occupational exposures (e.g., cellulose insulation installation, welding fumes, metalworking fumes, and asphalt paving fumes) and characterized the toxicity of these mixtures in animal studies. The DNTP has also invested a great deal of effort in herbals, such as those available at local pharmacies and health food stores, which are inherently complex mixtures.

As technology advanced, specifically with genetically modified animals, high throughput assays, omics, etc., toxicology studies could reveal more information in a shorter time span. With rapid advances because of the Toxicology in the 21st Century initiative, we had an opportunity as scientists to advance mixtures research significantly at a new pace. Given current technologies, scientists more rapidly assessed chemical interactions with an expanded chemical universe and across a wider biological space.

The workshop came at a critical time, and allowed mixtures research to move forward by gathering information from the various disciplines, determining the most impactful mixtures questions, and discussing the best approaches to address those questions.
INTRODUCTION TO WORKSHOP – CYNTHIA RIDER, Ph.D., DABT, TOXICOLOGIST, DNTP (CO-ORGANIZER)

The first item for the workshop that needed to be addressed was the definition of the term “mixture.” “Mixture” can be defined and interpreted in many different ways. Rather than providing a set definition for mixtures, the underlying goal for the workshop was to understand the contribution of complex environmental exposures to human disease. In that context, a mixture was a co-exposure that helped in understanding how chemicals acted jointly and contributed to human disease. This definition included whole complex mixtures, independent exposures to discrete chemicals, as well as exposures at different times. In essence, if it helped to understand joint toxicity, it counted as a mixture.

There were many mixtures conferences last year, which indicated that there was critical level of interest in mixtures. There is a rich history of mixtures research in scientific arena. A quick literature search for the term “mixtures” yielded approximately 55,000 articles. Furthermore, exposure characterization has improved and there are now large databases available cataloging this information. Risk assessment is also progressing in parallel from a single chemical focus to cumulative and community-based risk assessment that included non-chemical stressors. Therefore, this is a great time to build on the past and inform the next phase of mixtures research and cumulative risk assessment. This workshop provided an opportunity to discuss previous efforts and determine how best to proceed in mixtures research.

For this workshop, the first plenary session included researchers who provided an overview of current thinking in their discipline, as well as the major issues. Later, discipline-based breakout sessions were convened to create prioritized lists of the major issues from the various disciplines represented at the workshop. Following report-back from the discipline-based breakout groups, results from the NIEHS request for information (RFI) were presented. In summary, the first day involved primarily brainstorming and sharing perspectives on major issues in mixtures research.

On the second day of the workshop, discussion moved from brainstorming to addressing major topics identified on the first day. The second day plenary talks covered innovative approaches to studying mixtures. Multidisciplinary breakout sessions followed the talks, which focused on topics that were identified through discussions at NIEHS, the RFI results, and consultation with leading experts in the field. Finally, the results of the breakout sessions were shared in open discussion with all workshop participants.

Through this workshop, we hoped to gain insight into major topic areas in mixtures where targeted research would make a significant contribution to forward progress. Two other goals of the workshop were cross-disciplinary engagement of experts through communication and collaboration on mixtures, and generating ideas for improving current and future mixtures research projects.

At this workshop, there were invited participants, registered observers, and chairpersons. The intent was to maintain a small discussion group environment, and therefore it was not possible to include everyone as participants. The major part of the discussion during the breakouts took place among the participants. Comments from observers were welcomed during discussions or submitted in writing.
Exposure Science Overview

Paul Price, M.S., is an authority on developing models to assess exposure to multiple chemicals and, therefore, has an understanding of the data required for accurately predicting human exposure. He is currently investigating how certain constituents are responsible for the observed effects of complex mixtures. He is a risk assessment leader in the Chronic Risk Assessment and Statistics Group at Dow Chemical Company. His current areas of research include mixtures risk assessment and the use of simulation modeling to investigate variation in dose and response in humans. Prior to joining Dow, he worked at the U.S. EPA, the American Petroleum Institute, and most recently, the Lifeline Group, where he built software to perform aggregate and cumulative exposure assessments.

As stated previously, the goals for the workshop were to identify issues that presented challenges for mixtures research, obtain advice for how to integrate multiple disciplines and capabilities, and provide recommendations for mixtures research. In adherence to the goals of the workshop, this talk focused broadly on the field of exposure assessment and specifically, on three important issues in exposure science:

1) Although the preference for risk assessors is to use whole mixture data, there is a significant challenge in testing whole mixtures because complex mixture permutations changed over time. Due to this complication, component-based approaches are often used, which led to the second issue:

2) Available component data are lacking for component-based mixture assessment.

3) Finally, the impact of biases in selection of test mixtures in toxicology studies will be discussed.

Lack of Component Data

Strategies for working with mixtures when data are lacking were discussed. In component-based assessments, there is a bottom-up approach, in which the toxicity of the mixture is understood through understanding the toxicity of each of the components. When there are large data gaps (most of the mixture components are lacking toxicity information), exposure assessments can help in prioritizing components for study.

There are several techniques for estimating the toxicity of chemicals that aim to identify an exposure level below which there is little probability of harm. One technique used to estimate toxicity is called the Threshold for Toxicological Concern (TTC), and it is considered to be a very useful tool in mixtures research. Using only the structure of a chemical and toxicity data from structurally related chemicals, chemicals lacking toxicity data can be binned into categories (e.g., potentially very toxic, potentially toxic, and relatively non-toxic). This tool is a work in progress, and it continues to be redefined. Researchers are exploring expansion of TTC and improving the development and application of...
structure/activity relationships. Other techniques are being built that use mechanism of action information to estimate the toxicity of chemicals for which toxicity data is not available. Finally, Richard Judson, Ph.D., of the National Center for Computational Toxicology (NCCT) at the U.S. EPA has just published a paper that describes a framework for using high throughput in vitro data and pharmacokinetic modeling to estimate doses of a chemical that are likely to meaningfully disrupt a biological pathway, which he refers to as the biological pathway-altering dose (BPAD) (Judson et al. 2011).

Consider a situation where a mixture had 78 compounds, 30 of which had oral Reference Doses (RFDs) or other measures of permitted dose and 48 compounds do not. The risk assessor then had to decide what to do with the rest of the compounds in the mixture. Techniques described above can be used to come up with preliminary and conservative estimates of toxicity for the 48 chemicals with missing values. A simple additive model can then be used to estimate the total toxicity if the sum of the toxicities is acceptable then no additional information is needed. If the estimate of the total toxicity of the mixture raises a concern, then an estimate of the contribution of the uncharacterized compounds to total toxicity can be made. If the uncharacterized fraction did not contribute anything to the total toxicity, the missing toxicity data is not necessary and the risk assessor can focus on the 30 chemicals that are driving the toxicity concerns. If the uncharacterized fraction does contribute significantly to the mixture toxicity, then additional toxicity data will need to be collected. Therefore, using a risk framework and known exposure levels helped identify when additional toxicity data should be collected and when these screening approaches were adequate.

Testing Under the Lamppost

The next issue, from an exposure science perspective, was the common practice of toxicity testing under the lamppost. What I mean by this is that mixtures typically investigated do not reflect the mixtures that occur in environmental samples or pose the greatest problems for risk assessors. It was widely recognized that in order to get data to determine whether toxicity was dose additive, response additive, or interactive (synergistic or antagonistic), dose-response data was needed. To get dose-response data, we have to dose animals at the levels that cause frank effects in a significant number of animals. It was very costly to conduct chronic mixture studies in animals, so toxicologists routinely perform studies of acute endpoints observed in one-day or seven-day studies. Such studies must use very large doses in order to observe effects. As a result, the studies only report interactions that occur at doses where acute frank effects occur. Under current regulatory frameworks such doses are not permitted. Instead what are permitted are mixtures where each component is at or below the RFD. Thus the bulk of the literature on mixture toxicity is not directly relevant to the most important question in mixture risk assessment: Do concurrent exposures to multiple chemicals at doses below the RFDs post a risk?

The solution to the discrepancy between levels that were in the environment and levels tested in traditional toxicity tests, as Glenn Rice pointed out in the previous talk, lay in developing in vitro strategies and techniques that allowed the use of more doses, lower doses, and PBPK models from human cells. Then, PBPK models were used to relate the concentration-response data in the in vitro studies back to external doses.
Challenges of Working with Many Permutations

Much of the remainder of the talk addressed permutations in mixtures research. Someone once said that trying to figure out the toxicology of a mixture by testing one complete mixture after another is like trying to understand the Sahara Desert by investigating one grain of sand at a time. It is very expensive and not very enlightening.

If you estimate the number of possible combinations that are out, they are truly frightening. For example, for a group of three chemicals, there are three possible unique pairs; for a group of 10 chemicals, 45 pairs; for the 10,000 compounds that were in commerce, 50 million pairs. The situation gets more complicated when combinations of three chemicals or more were added. There truly are, for all practical purposes, an infinite number of mixtures to evaluate.

The question is: how can exposure assessment help on this issue? It turns out that in the real world, most of the possible combinations do not occur or they occur extremely rarely. The goal of exposure assessment is to help understand which patterns of co-occurrence of mixtures exist and which ones do not. There are two ways this can be accomplished: modeling or monitoring.

Once toxicologists, epidemiologists, and others have that data on chemical co-occurrence, exposure and risk assessment frameworks can be used to help prioritize which individual chemicals and which combinations of chemicals are of the highest priority for study. In this way, exposure assessment can help focus on the field of mixture toxicology.

As was stated previously, not all combinations are equally probable. The combinations of chemicals that we interact with (largely anthropogenic chemicals, but also naturally occurring chemicals in food, air, and water) are driven by the chemistry of fate and transport. In effect, chemicals that degrade quickly are rarely encountered because they do not last long enough for us to encounter them. The ones that are persistent, conversely, tend to be encountered more frequently. For example, the chemicals that accumulate in our food tend to be encountered; the ones that do not accumulate in our food are not.

The combinations of anthropogenic chemicals that we are exposed to are driven by their use. If the uses are exclusive (i.e., when one chemical is used, others cannot be
used), then they will not co-occur. If, on the other hand, they come from processes that co-occur, then you have a higher probability of exposure to the combination. Therefore, co-occurrence is not random and the probability of encountering any possible combination is not uniform.

The next question is how to identify that small fraction of the virtually infinite number of possible combinations? The graph in Figure 10 shows interesting patterns emerge when you analyze this monitoring data (Tornero-Velez et al. 2012). The white bars show the percent of total contribution on a mass basis of pyrethroids in surface wipes from daycare centers. If you look at all of the samples, you find that every one of the pyrethroids is represented. This suggests that many possible combinations of pyrethroids could occur.

For samples from sites with the higher pyrethroid loadings (top 25% total loadings), a much smaller number of pyrethroids occur, thus there are few possible mixtures. Sites with the highest pyrethroid loadings (top 10%; black bars) had a further reduction in the number of individual pyrethroids driving the exposure. The important point of this analysis is that the pattern of exposures is different for low exposures than for high exposures. This is a very important principle that is observed over and over again - people who have high exposures are not being exposed to the same patterns as those who have low exposures. One need that exposure assessments can fill is to help identify the patterns that are associated with the people who have the highest exposures. The mixtures that are associated with the highest exposures should be given priority for testing.

Modeling

The goal of exposure assessment is to find out when and where compounds occur and when those compounds really are the drivers of toxicity. How do we determine these co-occurrences? One of the ways to do this is modeling. A strategy for determining co-occurrences was inherent in the development of modeling software that I was involved in while working for The Lifeline Group. The starting place for this strategy is to determine why a chemical was present (i.e., what was the chemical’s purpose). For example, pyrethroids tend to be present in specific combinations because they work well together. Therefore, there are certain combinations that are expected.

To continue with the pyrethroid example, when there are two insecticide products containing pyrethroids that are from two competing companies, and Company A uses one pyrethroid, while Company B uses another, people are unlikely to buy both and spray them simultaneously. Therefore, a negative correlation between those two pyrethroids would be expected.

In modeling this scenario, information on the product composition is captured and incorporated into the design. Then, information about human behavior (e.g., how much people use) is added. For example, some people spray pesticides frequently, some do not. Information about where these pesticides are used and how they are applied are factored in. Based upon these processes, you can make a prediction about when you expect things to co-occur in low doses and at high doses.

Modeling is useful because it is a way of taking monitoring data and using it in an enhanced way to show how it would apply in different scenarios, and as such, it does not provide an either/or answer.
Modeling and monitoring studies often come from a perspective of taking monitoring data and using modeling to identify how to most effectively use that data.

Historically, use of software models has been limited for a variety of reasons: 1) they are expensive to develop; 2) they have had too little data and too many assumptions, especially in the earlier versions; 3) methods for evaluating the reliability of modeled predictions were not available; and 4) computer capacity was an issue, to some extent (e.g., EPA’s first cumulative organophosphate risk assessment required a roomful of computers running day and night for three weeks to complete all the necessary iterations).

However, the main reason that these models have been under-utilized is that they have been sold as “the answer.” It has been perceived that such model could generate the number that would be the final answer. This is a misuse of the models. Models are best used when they help to integrate and to leverage data to answer questions that cannot be addressed by direct measurements. There is a real future for the use of simulation models based upon real-world data to help estimate when co-occurrence and/or high doses are likely.

**Advances in Monitoring**

There have been many advances in monitoring, which allows for a discussion about “toys.” Modeling chemical exposures? Yes, there is an app for that. The photo (right) shows a little device attached to the port of an iPhone® that can detect in real time exposure to ammonia, chlorine, and methane. It is the size of a postage stamp. When plugged into the iPhone, it will automatically download the real-time concentrations and send them (via network or WiFi) to a computer where the data is recorded.

This tool is only one example of available technology. We now walk around with sophisticated electronics that have almost become a part of our anatomy. That opens up the opportunity to do real-time measurements of exposure in a way that we never could before. Now, we just need to harness these new capabilities to collect much more detailed information on chemical exposures.

What about monitoring inside the body? The photo (left) shows a monitor that is a flexible, plastic monitor. The little black dots are batteries, and the large square on one side is the antenna to transmit data on blood pressure, body temperature, and heart rate, e-mailed via Bluetooth® to an iPhone® that you carry with you to upload to your computer.

If this is not invasive enough, another tool has been developed to detect nano-particle florescent dyes that...
are injected under the skin. These dyes’ ability to fluoresce are sensitive to different concentrations of sodium and glucose present. A fluorescing source of light on the outside of the skin and a photo detector can be used to make real-time measurement of internal concentrations of glucose and sodium. And an iphone uploads pictures to show the intensity of the frequency for real-time monitoring of chemical exposures.

In summary, one day, we will have the ability to design studies to not only collect and measure single samples but also collect longitudinal data on chemicals to which people are exposed. The potential here is to look at not only anthropogenic chemicals, but also to follow physiological changes that reflect stressors in an individual’s life. Therefore, the future of monitoring is very exciting.

Using Risk Assessment to Guide Toxicologists

Risk assessment can be used to help guide toxicologists to the key components and the key interactions that need to be studied in environmental mixtures. It is not just toxicology that drives the importance of doing a cumulative risk assessment; it is also the exposures. I will first review some terminology.

Chemical risk assessment has traditionally been done in a screening fashion. A hazard quotient (HQ) is simply the monitored dose divided by the permitted dose (RfD). If the HQ is less than one, the dose is less than the RfD. The Hazard Index (HI) is the sum of the HQs. Assuming that all of the chemicals operate in an additive fashion, then, the HI should remain less than 1 in order to protect human health (i.e., there would be a low level of concern). HI values that are greater than 1 would elicit a higher concern. If you have a mixture that gives you an HI greater than one, the mechanisms of action should be compared to decide whether they should be added together or not.

Below is a very simple example of how exposure is important in determining when you need to perform a cumulative risk assessment. In this example, a new tool, the Maximum Cumulative Ratio (MCR), is introduced that can help in this evaluation. For this example, there are two individuals who have cumulative exposures to five chemicals. We do an HI assessment and find that both individuals have 3.5 as their HI, which is an unacceptable value. The HQs for individual one are 0.6, 0.7, 0.8, 0.7, and 0.7 and for the second individual, the hazard quotients are 3.2, 0.25, 0.4009, and 0.001. The data are shown in Figure 13. These individuals present very different challenges for risk managers.

![Figure 13](image-url)
For the first one, classic chemical-by-chemical risk assessments would have concluded that Person 1 is adequately protected from harm, and all of the chemicals are below their safe doses. However, the sum of the HQs leads to an HI over 1 and is a concern. In this case, traditional chemical-specific risk assessments would have failed. For the second person, the chemical-specific risk assessment would have identified the problem as a chemical one because it is an unsafe dose, and the other chemicals are not contributing significantly to the problem.

From a risk assessment perspective on mixtures, it is important to determine whether people are exposed to scenarios comparable to person #1 or person #2. Additionally, in scenarios like that of person #1, how much is information is missed when a chemical-by-chemical assessment is performed? Are we losing a fraction of the hazard, or twofold, five-fold, 10-fold?

The MCR offers a very simple way of discriminating between the two examples. The MCR is defined as the measure of cumulative toxicity divided by the highest toxicity that comes from any one chemical in the mixture. It is the HI divided by the largest of the HQs.

Using the MCR in the example shown in the graphs above, person #1 has an MCR value of 4.4 because the highest HQ for an individual chemical is 0.8 and the HI is 3.5. Person #2 gets an MCR value of close to 1. When the MCR is large, a cumulative risk assessment should be conducted. Mixtures with large MCR values are important to evaluate using cumulative risk assessment, whereas mixtures with MCR values that are close to 1 are not as important to evaluate in that way.

The MCR has some very interesting properties. A value of 2 indicates that one chemical is providing 50% of the toxicity, and a value greater than 2 indicates that no single chemical is the dominant source of toxicity. A value of 1.1 indicates that 90% is coming from one chemical. The Pareto principle can apply here: it is the old 80/20 rule that 80% of your sales will come from 20% of your clients. The Pareto principle is a phenomenon that has been observed to occur in a wide variety of settings, and measurements, and it implies that if MCR values follow this principle approximately 80% of the hazard would be due to the presence of 20% of the chemicals in the environment.

HI and MCR can be combined to come up with some rules as to when a cumulative risk assessment should be conducted. A grid can be used (Table 1) that shows HI less than 1, HI greater than 1, MCR greater than 2, and MCR less than 2. If the HI is less than 1, there is low concern about cumulative toxicity. The mixture is not toxic, regardless of whether the low toxicity is being driven by one chemical (MCR<2) or many chemicals (MCR>2). Alternatively, an HI greater than 1 and an MCR less than 2 indicates that you have a mixture that is a problem, but it is a problem because there is one component that is driving the toxicity. In this case, a single chemical problem is masquerading as a mixture problem. For this scenario, a chemical-by-chemical risk assessment would have been sufficient to identify the problem.
The last category is the one that raises a red flag in risk assessment. In the last category, there is an HI greater than 1, signaling a significant hazard, and an MCR greater than 2, indicating that many chemicals are contributing to the hazard. This category demands a cumulative risk assessment (Figure 14).

Now, a real world example will be used to further illustrate the MCR and compare it with the HI. To the right is a plot of the MCR values for 3,000 pesticide mixtures in a U.S. Geological Survey database showing MCR plotted against the HI. In this mixture, there are 5–30 pesticides detected in every sample, and a mean of about nine. All MCR values were less than four. The cloud is somewhat dense at the bottom, indicating that the mean MCR was well below two. Furthermore, there is a decline in MCR values as toxicity increases. If you take the same data set and look at the ecological criteria, the same pattern occurs.
Another dataset that was analyzed in this manner was from ground water data from a USGS survey on drinking water wells (Figure 15). This example is more indicative of a complex mixture than the previous example. The mixtures observed in this survey on average contained 30 chemicals with detectable levels. The last example (Figure 16) shows data from a small survey done in the European Union (EU) on polar compounds in rivers, where the number of chemicals in the samples ranged from 5 to 30 (with an average of 25).

In all of these patterns, we see that the MCR is generally much smaller than the total number of chemicals present, and it tends to go down with higher toxicity. That trend is very important because it suggests that the more toxic mixtures are not more toxic because they have many more chemicals than mixtures with low toxicity, but they are more toxic because there are a few chemicals present at unusually high concentrations. It will be very important to determine if this pattern occurs universally in mixtures. If it occurred in the environment, then we hope to be able to look at these mixtures and identify small numbers of driving chemicals and small numbers of interactions that will be much more tractable to investigate.

Another example with higher MCRs (Figure 17) shows monitoring data for dioxins, furans, and PCBs that have been normalized through their TEQs. These values came from the National Health and Nutrition Examination Survey (NHANES) data. In this plot of MCR versus total TEQ for individuals <45 years old, the mixtures generally have higher MCR values than the mixtures in the previous examples. The mixtures in the two earlier studies had most MCR values below 2. In this example, MCR values range from 2 to 7, strongly suggesting that for dioxins, furans, and PCBs, you need to do a cumulative risk assessment because there are multiple components that contribute to the total hazard. For people >45 years old, there is an even more pronounced effect such that almost all individuals’ total exposure on a TEQ basis is driven by the contributions of multiple compounds. Our ongoing research suggests that...
when MCR values are large, the mixtures tend to contain compounds with similar toxicity and similar concentrations, and these compounds are highly correlated with each other.

The last point is that the MCR is not necessarily a function of how many components are in the mixture. If we were to graph the pesticide data from the earlier example by the number of compounds that were detected in each sample, we have some mixtures that have as few as five and some as many as 20 compounds. We find that the number of detected compounds predicts toxicity very well. There is a two-order-of-magnitude increase in predicted toxicity as the number of compounds detected goes up – the more compounds detected, the more toxicity. However, if you plot MCR against the number of detections, you find that MCR values do not increase very much at all until you get to the very end, and then it is not clear whether the increase is statistically significant. The propensity of a mixture to be driven by one or two compounds occurs in both mixtures that have five components and mixtures that have 20 components.
There is ongoing research that is trying to put all of these decision points together (Figure 18). The World Health Organization (WHO) has issued guidance for approaches to mixtures, and there is a new report that came out this summer. Andreas Kortenkamp, Ph.D., who will be presenting at this workshop, has just completed a major review of mixture toxicity (Kortenkamp et al. 2009). We tried to capture that work in a flow chart (Figure 18) as a part of a decision-making process that, at the end of which, we will
identify the compounds and the interactions that are the drivers for even these complex environmental issues.

In conclusion, more research is needed on exposure, not just toxicity. If exposure and toxicity are integrated, a much better understanding of risks posed by mixtures will be gained, and can help focus research efforts in toxicity studies.
L. Earl Gray, Ph.D., has been instrumental in advocating the biological perspective in mixtures research, shifting focus from mechanisms of individual chemicals to target pathways and tissues affected by multiple chemicals. His work on mixtures that target androgen signaling has been extensively used by regulatory agencies in deciding how to group chemicals for cumulative risk assessment. Gray is Research Biologist and Team Leader with the Abnormal Development Team in the Reproductive Toxicology Division of the U.S. EPA.

For most of the chemicals that we have worked on, currently risk assessments are based on an individual chemical assessment. There are now efforts within the U.S. EPA and the Consumer Product Safety Commission to consider cumulative assessments on phthalates and possibly other chemicals that disrupt similar biological systems. We are doing research to try to decide where, in this particular hierarchy, we believe that it is appropriate to group chemicals for cumulative assessment. The data that I will show you argues for disruption of the common pathway through multiple mechanisms of toxicity, and possibly disruption of common target tissue through different pathways, but we do not have much data to support the latter yet, so I will not going to talk about that further.

To rephrase, what I think the key question our research is trying to address is: should chemicals that disrupt sexual differentiation of the reproductive tissue of the male rat by different molecular mechanisms of action in different tissues be included in the common mechanisms group? Traditionally, the default answer for this is no, and the model used by some risk assessors has been response addition, especially for the pesticides. The assumption here is that if you add all the chemicals by a response addition below the no-effect levels, you have no adverse effects.

The significance of this being an incorrect assumption is that, if dose addition really applies for all these chemicals, then you add them up below the no-effect level, you have reproductive tract malformations. The studies that I will talk about are designed to try to discriminate between using chemicals that disrupt reproductive development to discriminate between which one of these is appropriate. So these are not dose-response studies; these are not low-dose studies.

We are operating in a dose response range for the effective chemicals that we are studying, but I would add for some of the chemicals we study, like the phthalates, the exposures vary over five orders of magnitude within the human population. Within an individual, they can vary by an order of magnitude within days or across days.

There is a mixture theory proposed by some scientists for how to use mechanisms of toxicity in order to group chemicals with similar and dissimilar mechanisms of toxicity. Under dose addition, if I mix chemicals A and B, and they are two phthalates or two androgen receptor antagonists with common mechanism of toxicity, then those chemicals would be modeled and predicted well by dose additions. However, if I were to mix a phthalate with an androgen receptor antagonist, these are different mechanisms of toxicity and would be modeled by response addition, meaning zero plus zero equals
zero. Data from our studies do not support the utility of response addition for modeling chemicals with
different mechanisms of toxicity that disrupt the same tissue through the same pathway. When we do
these studies, we model a variety of effects and we use different simple models to compare dose
addition, response addition and integrated addition. These basic equations come from Dr. Gerry
LeBlanc, Ph.D., at the Computational Approach to Toxicity Assessment of Mixtures (CATAM) website at
North Carolina State University (http://service004.hpc.ncsu.edu/toxicology/faculty/leblanc/web1/index.html).

What end points should you model in a cumulative assessment? Historically, these have been based on
adverse effects. They could be what you consider an irreversible phenotypic alteration or a transient
phenotypic alteration and adverse effect. There is a course to use endocrine growth factor in genomic
changes for individual chemical assessments, and we heard how nice it would be to use high-throughput
in vitro screening systems or mixtures and also in silico models have been proposed. The question has
been proposed on how adequate screening is – how can it be used for mixture research?

More appropriately, the limitations of current systems must be addressed before we can determine
their future utility. Limitations that must be considered include: 1) all in vitro systems currently lack the
ability to metabolize chemicals, 2) some of the high throughput batteries have a limited biological space,
and 3) many key pathways are absent. Therefore, we are unable to predict anything about some classes
of chemicals in vitro at this point in time, so we need to address them. The phthalates are an example.
They have to be metabolically activated in the pathways, and they are not generally represented well in
vitro at this point in time.

There are some cases in eco-toxicology when the in vitro assays or individual chemicals in mixtures do
provide useful predictive information about the effects in the field. The effects of estrogens in mixtures
and effluences is an example of that, where in silico models, we are going to have to realize that
reproductive development is an extremely dynamic process where there are interconnected pathways
involved that change daily in the rat. This is going to be difficult to model in silico or to reassemble from
in vitro assays.

In terms of the endocrine growth factor and genomic changes, I think we can use these in some cases,
but they need to be causally linked to what we would call an adverse effect. I will show some data for
some of the phthalates, and I think some of these are being considered for some of the ongoing risk
assessments.
When we do the studies – and I will talk about some of our data now – we model every end point that we measure. We measure a variety of end points in the male offspring from a postnatal study, so this takes six to nine months, and this is after exposure to a phthalate or an androgen receptor antagonist. And we also, in some of the studies at least with the phthalates, are looking at fetal endocrine and genomic end points at the end of sexual differentiation, and model those as well.

We used logistic regression models of individual effects of some of the chemicals we have worked with to assess relative potency for the chemicals for different endpoints (Figure 19). This is a generic phthalate of the potency of like dibutyl phthalate, but if you look at these different effects, the relative potency for chemicals to one another is not consistent across all the end points. This is because the phenotypes and modes of action of the phthalates and the androgen receptor antagonist are different.

The relative potency factor, for example, for producing hypospadias for vinclozolin and a phthalate is not the same as a relative potency factor for causing gubernacular agenesis because the androgen receptor antagonists do not cause gubernacular agenesis. Therefore, a single TEF approach is not going to work for different classes of chemicals with different mechanisms of toxicity, while I do think that it will work fairly well within a particular class, like within the phthalates, where they operate through a common mechanism of toxicity.

What we are trying to do is screen phthalates for their effects on fetal endocrine and genomic alterations. We screen chemicals at a single high dose, and then for those that are positive, we do dose response studies on the testosterone production by the testes, and then the genes. We use a custom-designed PCR plate that contains genes for these pathways designed by Vicki Wilson, Ph.D., and Bethany Hannas, Ph.D., in our group with some input from Paul Foster, Ph.D., at the National Toxicology Program.

We modeled all of those genes and among them, there are about ten genes that are consistently affected by the phthalates whereas none of them are affected by the androgen receptor antagonists. So we are modeling the testosterone and the gene expression from those ten genes to try to determine
which one might you use in a cumulative assessment. Is that the right thing to do? Furthermore, is one more sensitive than testosterone, or are these more sensitive than looking at the malformations?

One example of a study in which we mixed five phthalates together was designed so that the phthalates would contribute equally to the mixture, assuming that they acted to a dose addition (Howdeshell et al., 2008). We looked at the fetal testosterone production. In Figure 20, the black line is the observed reduction in testosterone from the mixture of five phthalates given during sex differentiation, and the testosterone production is measured at the end of sex differentiation. The red line is the dose addition line, so the dose addition provides an accurate prediction of the effect.

We also did a postnatal study in this study, shown in the top right panel of Figure 20, measuring the incidence of epididymal malformations in lower seminal vesicle agenesis in the males. The black line again is the observed effect, the red line is the dose addition, and the blue line is response addition. You can see that response addition is not predictive at all. We have nearly 100% malformed and they predict zero and dose addition provides a useful model, but this would be expected because they have a common mechanism of toxicity.

We have also modeled testosterone production for several of the phthalates in fetal phthalate screening experiments, where we had individual values. We found that individual phthalates vary greatly in their potencies for inhibiting testosterone production (data submitted for publication). We have used this information to predict how a mixture would work in the fetus on testosterone production and gene expression. Relative potencies for gene expression effects also varied between individual phthalates, and we found that cyp11-B1 was the most sensitive and the cyp11B-2 was the least sensitive. The relative potency factors seem to be consistent across the end points.

We are confident that testosterone production is causally linked to the malformations of the androgen-
dependent tissues. Some of the lesions are clearly causally related to these gene expression changes, and so it would seem reasonable to use lesions in an individual assessment and in a cumulative assessment if it was predictive of a mixture. However, what about the genes? Would you use these genes in a cumulative risk assessment? The most sensitive across the board is cyp11-B1. Would you use that gene expression for your risk assessment or would you be more comfortable with SR-B1 and STaR involved in steroid transport or would you just stick with testosterone production? Or would you say “whatever” and go back to the malformations, which have a higher ED50?

The testosterone production pathway and the genes that we are studying are shown in Figure 21. The genes in red are enzymes in the steroidogenic pathway that are reduced, and the small blue boxes are the steroid transport proteins that transport cholesterol and starch into the mitochondria. If you recall, the circled genes are significantly reduced. Cyp11b-1 was the most sensitive, which is interesting because Cyp11b-1 is not involved in androgen synthesis in the testes. Instead, it is involved in corticosterone synthesis, which in adults does not take place in the testes. However, fetal testes are not the same as small adult testes; there are structural and functional differences. For example, the fetal Leydig cell population is completely different from the adult Leydig cell population. Fetal Leydig cells die and are replaced by adult Leydig cells. Therefore, what is going on in the developing animal cannot be modeled by using adult tissues or even adult Leydig cells.

So would you use cyp11B-1 in your risk assessment? I think not, and the reason is that there are publications that have looked at cyp11B-1 protein and report that, even though the gene is expressed at low levels, there is no protein. The genes change, there is no protein, there is no possibility of any effect occurring from this, and therefore I would not consider using that in a risk assessment basis, even though it is the most sensitive gene.
We did a mixtures study where we mixed nine phthalates together using the potency factors determined from the studies we did that I showed you earlier, and you can see that the red line dose addition accurately predicts the observed effects on testosterone production. So this works pretty well. And then if you look at the sum of the ten genes that have been modeled again, the genes are all recently been modeled using the doses addition equation—so dose addition works very well for testosterone production and gene expression in the testes for these phthalate-induced reductions. That is not too surprising, as I said, given the common mode of action. However, what can we do about chemicals with diverse mechanisms of toxicity that disrupt the same tissues, the androgen-dependent tissues?

9-PE Mixture & Gene Expression

![Graphs showing gene expression data](Figure 22)
We performed several studies in which we mixed an androgen receptor antagonist with a phthalate (Hotchkiss et al. 2004; Hotchkiss et al. 2010) (Figure 23). One blocks the receptor so the hormone cannot bind it, whereas the other one does not affect the receptor, but rather lowers the level of testosterone. Therefore, it is the same pathway, but different mechanisms of toxicity. When you expose animals in utero to either butyl benzyl phthalate (BBP) or linuron (a pesticide) alone, there are no hypospadias in either group (Figure 23, left panel). However, when you add them together at equipotent doses (at half of their respective ED50s), you get 56% of animals with hypospadias (left panel). According to response addition, you would predict that 0% plus 0% should equal 0%, indicating that this combination is not response additive. Whereas, according to dose addition, the combination should result in around 50% effect. In another study (Hotchkiss et al. 2010), we mixed procymidone, a pure AR antagonist, which has a mechanism like flutamide, with dibutyl phthalate (DBP) and hypospadias incidence rose from 1.5% with procymidone alone to 49% (Figure 23, right panel). These examples demonstrate different mechanisms of toxicity affecting the same pathway in a dose-additive manner.

We thought that the procymidone/phthalate mixture was the clearest one where you have two distinct mechanisms of toxicity, and it was simpler. In a follow-up series of experiments with procymidone and DBP, a fixed ratio of the mixture was tested at different dilutions of the high dose (Hotchkiss et al. 2010) The observed data was compared to predictions from dose addition and response addition models to see which provided the better fit (Figure 24). The observed effect on hypospadias is shown by the black line, the dose addition prediction is shown by the red line, and the response addition prediction is shown by the green line. Response addition grossly under-predicts the incidence of hypospadias for this mixture. This finding indicates that these chemicals are not acting independently but are acting together.
to disrupt the common tissue through a common pathway. Note that at the dose that is 60% to 70% of the top dose, response addition predicts no effect but about 80% of the animals have hypospadias.

Therefore, dose addition is the most logical model for the data response addition, it does not explain the results. Dose addition is consistent with the biology of hormone action. These chemicals that we have talked about here – phthalates, procymidone, linulin – all acted on the fetal tissues by disrupting a common pathway, the androgen signaling pathway. What the tissues see is a reduction in androgen receptor bound to an androgen molecule, so in both cases, androgen receptor gene expression in the tissue is attenuated. The androgen receptor antagonists does that by blocking androgen from binding the androgen receptor. Testosterone synthesis inhibitors do this by reducing the androgen levels.

The disrupted developing tissue does not distinguish among these events. As I said, the tissues do not function like radioimmunoassays or binding assays. They just know there is less. What they detect is that there are fewer androgen-activated genes to initiate development. I think they argue that a cumulative risk assessment using a framework should be based at least on common systems or common developing tissues, and that the common mechanism of toxicity is not predicting what we’re seeing in these experiments very well.

Now, on some of these, we are going back and doing dose response studies, but that requires a very different experimental design than we have used here where you need to use a larger number of animals and you need to load up the low dose groups quite a bit. In addition, I think a challenge of some of this is what are the real environmental levels of a phthalate or a phthalate mixture? We know people are exposed to multiple mixtures and it varies so much from day to day and throughout the day. Our model is shown in Figure 25 below. Thank you.

![Figure 25](image)

**Discussion**

Unidentified Audience Member: My question is about the second part of your talk. Have you tried any *in vitro* models where you have combined an AR agonist with an AR antagonist to see if you can interact...
between those two *in vitro?* I know you see that interaction *in vivo*, but have you tried a system that might be responsive?

L. Earl Gray: We do not know. We have not, and I am not sure how *in vitro* systems can deal with chemicals with different mechanisms of toxicity like that. I think that is a real challenge. It is a challenge at first to get the *in vitro* systems to be predictive of individual chemicals and then mixtures. However, to start to include different mechanisms of toxicity is a bigger challenge.

Unidentified Audience Member: Okay.

L. Earl Gray: But, no – the answer is no. I mean, we have done mixture studies *in vitro* with individual estrogens, individual androgens, and anti-androgens, and then Wilson’s laboratory is also looking at real-world mixtures of effluents from a variety of sites and then she’s reconstructed some of these mixtures of estrogens to test them for additivity. *In vitro*, these things work well, and I think for the estrogens, the *in vitro* assays are fairly predictive of what goes on in some of the aquatic species, but then mammals do not respond similarly based on the differences in their metabolism.

Unidentified Audience Member: So then, do you think we understand the biology well enough of the individual pathways so that we can describe it computationally? I am also trying to think about how you would model that and use the *in vitro* data to parameterize the model to predict those interactions.

L. Earl Gray: Some pathways are better defined than others, like the androgen receptor antagonist pathway. On all of them, there are major black boxes in these pathways where we do not know various steps. It is likely that an androgen receptor antagonist, which we know that it blocks the androgen receptor and reduces androgen-dependent gene expression, is tissue-, age- and sex-specific. That makes this problem even more complicated.

Andreas Kortenkamp: Could we generalize to adopt dose additions as a default approach and in this way, get rid of all these discussions about the mechanisms, which usually I find lead nowhere? I mean, I am not saying mechanisms are unimportant, but as a starting point to distinguish which model to use to assess mixture effects; I find this very problematic because it normally blocks progress because in most cases, we don’t know. I wonder what your views are on this question.

L. Earl Gray: I think that dose addition would be a better default model to start with, realizing that there are cases in which it is not going to work. However, I think that it is more likely to work than a response addition model. I think there are many other things we have not looked at in these mixture studies yet. For example, if a chemical affects a testis that is in the fetus early on during gonadal development, and then it affects the testis during sex differentiation, and then the chemical affects the testis during pubertal life, how do those interactions behave in a dose or a response addition manner? I think for some of these things, like the precise mechanisms of toxicity like the phthalates, we do not really know. We know something about the disrupted pathway, but we do not know exactly what is happening first, and for that particular class brings about the question of our confidence of inter-species extrapolation from our rats to humans.
Mixtures have been and continue to be a great challenge. The reality is that human populations are exposed to many things throughout life. One challenge is that exposures deposit in compartments where we cannot measure them easily, in large part because of metabolism and other biological processes. An example of one type of mixture exposure is exemplified by the Tar Creek Superfund site in Oklahoma, which is related to mining activities. At this site, there are many heavy metals, PAHs, and organic compounds. Human exposure associated with the Tar Creek site is very specific to the location and mining activities. However, in the case of disinfection byproducts, which were discussed by Glenn Rice (see above), virtually the entire population is exposed due to the ubiquity of water treatment processes in the United States.

Sexton and Hattis (2007) identified key issues in cumulative risk assessment of mixtures, which are also relevant to mixtures issues in toxicology and epidemiology (Sexton and Hattis 2007). They discussed the importance of 1) determining which combined exposures occur in populations, 2) characterizing the nature of the exposure, and 3) identifying the mechanism and public health consequence of the combined exposure. They also discussed the need to recognize the potential for magnification of chemical toxicity in vulnerable populations, such as children. It is clear that combined exposures remain a challenge for epidemiology studies.

One of the challenges for epidemiology studies that are evaluating exposure to mixtures include finding populations either within the United States or overseas that have a predominant exposure of interest and to study them in detail. Examples include occupational populations such as those exposed to welding fumes or benzene, and overseas where we have looked at arsenic exposure in Bangladesh and the Ganges Delta. In some of these cases, people have been exposed to levels ranging from very low to extraordinarily high. Additional exposures in these populations have been handled as secondary exposures that represent potential “confounders” or “effect modifiers.”

Some of the questions raised by Sexton and Hattis (2007) and in discussions at this workshop are important for epidemiology (Sexton and Hattis 2007). What are the experimental mixtures that are most important? What types of mixtures are they? For example, organic compounds, inorganic compounds, mixtures of organic and inorganic, and urban air pollution can be extremely complex. Furthermore, what are the high-priority mixtures, who are the exposed populations, what is the potential severity of the outcome(s) and what is the likelihood of interactions among components? Vulnerability is also an important factor. Vulnerability is a broad question and can refer to inheritable susceptibility (e.g.,
genetic susceptibility) but also to acquired susceptibility (e.g., co-existing conditions and diseases such as diabetes) that can make someone much more vulnerable to peripheral vascular and cardiovascular disease, growth and developmental effects, and other effects, such as those seen in elderly populations. All of these factors can influence vulnerability to disease following chemical exposures.

One important issue in mixtures for both epidemiology and toxicology is the concept of interactive effects. This will be discussed in our breakout sessions. The concept of interaction is different from a toxicological versus an epidemiological perspective, although there should not be inherent conflicts. It is critical to be clear on terminology and definitions. There are many terms that are used to describe combined effects, such as: independence, additivity, synergism, greater than additive, multiplicative effects, antagonism, or agonism. In terms of mechanisms of action, in the pharmacology world, most drugs and most compounds are antagonists. There are some exceptions like β-2 agonists for treatment of asthma and epinephrine-type compounds for treating shock, but most compounds are inhibitors or antagonists. This phenomenon probably carries over in the environmental world as well.

**Synergistic: Prenatal Tobacco and Current Lead Exposures on ADHD**

- 2001–2004 NHANES
- 8 to 15 years of age (N=2,588)

<table>
<thead>
<tr>
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<tr>
<td>Prenatal tobacco</td>
<td>2.4 (1.5 to 3.7)</td>
</tr>
<tr>
<td>Blood lead</td>
<td>2.3 (1.5 to 3.8)</td>
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<tr>
<td>Tobacco and third tertile lead</td>
<td>8.1 (3.5 to 18.7)</td>
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(Froehlich et al. *Pediatrics* 2009)

In this presentation, examples of epidemiology studies of mixed exposures will be highlighted that focus on environmental exposures, rather than highly exposed occupational populations. In the first study, prenatal tobacco exposure and current lead exposures were measured and compared to attention-deficit hyperactivity disorder (ADHD) scores (Froehlich et al. 2009). Greater than additive effects were observed for moms’ prenatal exposure to tobacco and blood lead (Figure 29). Another paper showed interactive effects of stress and an integrated measure of bone lead on hypertension in the Veterans Administration normative aging cohort of adults of advancing age (Peters et al. 2007). This prospective
study showed that high stress modified the effect of tibia lead and patella lead on risk for developing hypertension. Obviously, there is noise in the association plots we derive from epidemiology studies, but this reflects the variation in all biological systems.

Our Superfund Center led by Robert Wright, Ph.D., a pediatric epidemiologist, focuses on metal mixtures and builds on three existing birth cohorts that will be followed into childhood. The mixed metal exposures include arsenic, manganese, and lead in three different environments. We are looking at gene-environment interactions and site characterization using extensive biological markers of exposure for these three compounds in blood, nails, and hair. The effects of timing and duration of human activities on bioaccessibility of metals in water and soil are being explored.

We have also performed epidemiological studies in Bangladesh and in Mexico City. In Bangladesh, we are assessing the relationship of gestational arsenic exposure to birth outcomes in a population with low-to-moderate exposure levels in mothers who moved into post-remediation areas (Huyck et al. 2007). We were not particularly interested in, nor ethically challenged by, studying only very highly exposed populations and following the moms through gestation and birth. In our study, the mothers were exposed to levels that are not that different from those levels seen in the United States, and we have very good data on maternal exposures. The Bangladesh cohort is the group for whom we are now assessing neurological development in the new Superfund project. In another project, Robert Wright, Howard Hu, Ph.D., and others have studied and established a birth cohort at Tar Creek in Oklahoma, which is on the Superfund list with a population of 4.1 million in an area of about 40 miles (Hu et al. 2007). We are studying many outcomes including child development and cognitive function. Metal mixtures at such sites tend to contain more lead, manganese, and cadmium, and less arsenic. Our hope is to characterize these outcomes and three metals longitudinally. We already have some data on the Mexico and Tar Creek cohorts. In Bangladesh, the cohort of children is just being born, and we have not gathered substantial neurodevelopment data yet. These data will be collected in the next 24 to 40 months.

My student, Birgit Claus-Henn, Ph.D., studied manganese and neurological development in the Mexico City cohort from 1997-2000 (Claus-Henn et al. 2012). The data indicates a bi-phasic dose response relationship between early-life, low-level manganese exposure and infant neurodevelopment. However, because the curve is U-shaped, there was some question of manganese having both important beneficial effects as an essential nutrient in very low levels and then toxicant effects at the higher levels (Figure 30). She also looked at manganese and lead co-exposures in the same group and found that in the highest quintile of manganese exposure, there was a synergistic effect with lead on mental development, and on psychomotor development at a slightly older age (Figure 30).
Mixed Metal Exposures: Manganese and Lead Co-exposure with Neurodevelopment

- 486 children, Mexico
  - 12 months (n = 296)
  - 24 months (n = 475)
- Outcome
  - Mental Development Index (MDI)
  - Psychomotor Development Index (PDI)

Air pollution studies have assessed multiple pollutants combined with particulate and air pollutant measures. Eun-Hee Ha, Ph.D., and Jong-Tae Lee, Ph.D., for example, have collected a large amount of data on air pollution in Korea. In collaboration with Joel Schwartz, Ph.D., a working group was established to apply some of the methods that he used in air pollution studies in the United States and in Europe to the Korean study. The goal was to look at combined and individual pollutants and outcomes, such as overall mortality and children’s development. We found that using some of Joel’s models, a combined index of total particulate matter (PM-10), oxides, nitrogen, SO2, and CO, seemed to better explain the exposure/response relationship with total mortality than any individual air pollutant (Hong et al. 1999). This suggested that the individual pollutants should be considered together in assessing air pollution.

We also looked at interactive effects of air pollutants on risk of stroke mortality in Seoul, South Korea (Hong et al. 2002). We used a single pollutant model using PM-10 according to inter-quartile ranges of PM-10 stratified by the level of gaseous pollutants, and then evaluated the data using the single pollutant model of gases stratified by the inter-quartile range of PM-10. Both approaches resulted in the
same conclusion: PM-10, oxides, and nitrogen correlated more strongly than other constituents as far as predicting stroke mortality, which was increased in middle- and advanced-age adults.

We assessed the relationship between low birth weight and air pollution in Inchon and then Seoul, South Korea, with exposure measured at gestational age 37 to 44 weeks over a prospective two-year period (Ha et al. 2003). We found that the relative risk of low birth weight increased as inter-quartile changes in the criteria pollutants went up in single pollutant models (Figure 31). As expected, there were very strong correlations between some of the criteria pollutants, such as SO2 and total suspended particulates. Again, we concluded that we needed to consider these pollutants collectively, rather than separately, depending on the outcome.

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>First and Third Trimesters Separately</th>
<th>First and Third Trimesters Simultaneously</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO (100 ppb)</td>
<td>RR: 1.08 (1.04–1.12) 95% CI: 0.99–1.09</td>
<td>RR: 1.07 (1.02–1.14) 95% CI: 1.01–1.09</td>
</tr>
<tr>
<td>NO2 (ppb)</td>
<td>RR: 0.97 (0.92–0.98) 95% CI: 0.92–0.98</td>
<td>RR: 1.00 (1.02–1.14) 95% CI: 0.99–1.09</td>
</tr>
<tr>
<td>SO2 (ppb)</td>
<td>RR: 1.06 (1.02–1.10) 95% CI: 1.01–1.10</td>
<td>RR: 1.00 (1.02–1.10) 95% CI: 0.99–1.09</td>
</tr>
<tr>
<td>TSP (μg/m³)</td>
<td>RR: 1.04 (1.00–1.08) 95% CI: 1.03–1.10</td>
<td>RR: 1.01 (1.00–1.08) 95% CI: 0.99–1.09</td>
</tr>
<tr>
<td>CO (ppb)</td>
<td>RR: 0.92 (0.88–0.96) 95% CI: 0.92–0.96</td>
<td>RR: 0.96 (0.87–0.97) 95% CI: 0.91–1.04</td>
</tr>
</tbody>
</table>

Finally, we looked at maternal secondhand smoke exposure. Tobacco smoke has been widely studied, and represents a complex mixture of volatiles and particulates comprising many, many compounds. In mothers and their offspring in South Korea, there were significant differences in urinary 8-OHdG levels, a measure of oxidative damage, between the secondhand smoke-exposed and non-exposed groups (Figure 32). This marker varied by glutathione methyltransferase and one deletion polymorphism status. In this study, a biomarker, 8-OHdG levels, was studied, and not a disease outcome.

One key issue is whether joint exposures are more toxic than individual ones. It is important to define the precise characteristics of such interactions in epidemiology studies. Genetic and acquired susceptibility present a challenging issue. In epidemiology, populations are characterized by multiple exposures, perhaps several outcomes, and multiple genes, which creates a fairly substantial moving target when the goal is to explain the variability in disease occurrence and distribution. Lastly, we need to consider what the environmental conditions are that increase human exposure to these mixtures. This information is important for exposure control and elimination.
As mentioned previously, “interaction” is a term that is defined differently in toxicology and epidemiology. It is not an inherent conflict, but a case of comparing apples to oranges. Accordingly, we have to be very clear about stating the definitions of terms such as “interaction.” This issue will be further discussed in the interdisciplinary breakout sessions at this current workshop.

Another key issue is that epidemiology is not a stand-alone discipline; it is completely dependent on the accuracy of exposure measurements. If exposures are misclassified, environmental epidemiology generates invalid results. Epidemiology is also dependent upon appropriate statistical power and statistical methods.

Additionally, it is valuable to have support from toxicology and biological plausibility. Otherwise, the findings are going to be difficult to interpret even if the study was conducted in a valid manner. All of these considerations are secondary to a foundation of solid study design, meaning standard designs continue to be the hallmark of epidemiology cohort studies, case control studies, and case cohort panel studies.

The need for larger sample sizes is implicit in epidemiological studies. If people are exposed to a range of mixtures and outcomes of interest may vary, larger and larger studies are needed. It is highly unlikely that any single cohort is going to be large enough and designed in such a way to deal with mixed environmental exposures of interest in a country like the United States. There will have to be multiple studies, just as no single clinical trial is going to get us the answer for the best regimen for treatment of breast or prostate cancer. There are always parallel studies going on that have to be done to cross-validate results.

The need for larger sample sizes also becomes a problem when financing these studies. In genetic studies, investigators routinely combine studies, even those that were conducted with different designs; this raises significant pitfalls and limitations in interpreting studies. However, in cases where it is appropriate to combine studies (e.g., meta-analysis), as long as the proper quality control checks are in place, then this option should be considered. In the case of environmental epidemiology, it is hard to combine a study where the purpose, the exposure of interest, and the outcomes of interest are very different in their designs, but there may be situations where it is possible.

In epidemiology, disease and death are often measured outcomes. However, intermediate outcomes such as DNA damage and urinary biomarkers from mothers (as in the relatively small study in Korea mentioned previously) as well as lung function, blood pressure, and hormone levels can be very important. Using quantitative traits and physiologic or biologic markers can significantly improve statistical power and ultimately, might improve prevention. If we understand the early physiological changes that occur on the pathway to disease, other opportunities for intervention may become apparent.
We have just scratched the surface in the epidemiology of mixtures. It has been a concern in epidemiology for a long time and it is likely to get more complicated as we bring in more and more variables to examine in these studies, such as genetic markers, for example. However, there are many opportunities to make advances in this area and to reflect the reality of exposures to populations.

Discussion

Audience Member: Ray Yang, Colorado State University. What exactly is the difference between the definitions of interaction by toxicologists versus epidemiologists? Can you describe it in plain language?

David Christiani: Yes. In epidemiology, it is going to be fairly straightforward. Do the effects of A and B add up or are they greater than additive, as in moving towards multiplicative? I have seen that definition of “interaction” applied in risk assessment. I do not want to speak for toxicologists, but sometimes toxicologists-biologists use “interaction” to mean any interaction between compound A and compound B, not necessarily in relation to the change in magnitude of the outcome of interest. Chemists, for example, have a further diverging definition of “interaction.” For example, sodium chloride interacts with sodium chloride, which is a chemical reaction or some kind of interaction.

Audience Member: Thank you.

Audience Member: Andres Kortenkamp, Brunel University. I would like to comment in exactly the same vein. It seems to me that this will be a great opportunity, this workshop, to open the dialogue between the toxicologists and epidemiologists and like Ray Yang, I feel it has to start with terminology. It is very clear that in mixtures toxicology, “interaction” means something totally different from the definition you described for epidemiology.

In mixtures toxicology, “interaction” is the phenomenon that the observed mixture effect doesn’t fit the expected one, when the expectation is formulated on the basis of some additivity assumption, which is not necessarily a mathematical adding up of the effects. That is what we mean by interaction, and I would like to know what you mean.

The same question relates to when you use the term “additivity.” In your talk, you refer to some effects of epidemiological studies as additive. In mixtures toxicology, this term is also used, but it denotes the situation where we make a prediction of the combination effect on the basis of the toxicity of the individual components, so we have a clear reference case and judge everything that deviates from it as either synergism or antagonism.

David Christiani: Correct.

Andreas Kortenkamp: I don’t know whether you do that as well.

David Christiani: We will try to keep it simple. In this case, again, in epidemiological terms, here is a case of the prenatal tobacco exposure and blood lead. The adjusted odds ratio for prenatal tobacco exposure was 2.4; for lead, it was 2.3. This is not ADHD, which is a case control study, so it is a yes-no. The
combination of the two together is an odds ratio of 8.1, which is greater than the addition of 2.3 and 2.4 — a bit simpler than what you were getting into. You can go further in the lab. For example, antagonism and agonism can be characterized in the lab, and that gives you an opportunity that we do not have in epidemiology. This is actually fairly simple.

As in the example of prenatal tobacco smoke and lead, individual odds ratios are 2.4 and 2.3. In epidemiology, “additive” would be the addition of those two odds ratios (i.e., 5.7). If the combination odds ratio is more than the addition of those two, as in the case described, it is more than additive. If it is in the multiplicative range, it has to be greater than the multiplication of the two odds ratios. It is quite simple. However, in the lab, you have more ability to actually refine biological interactions, which is what you just described, so it really is a matter of keeping our terminology clear and understanding what is being done, not necessarily a matter of changing procedures.

There are times, of course, epidemiologically where there is some demonstration of antagonism (e.g., selenium and arsenic). The treatment for arsenic toxicity acutely in clinical terms is selenium. Most people do not realize, although toxicologists do, the treatment for selenium toxicity, which is rare, is acute arsenic trioxide.

Andreas Kortenkamp: One key point that is relevant to epidemiology is that exposures do not happen at random. Exposures are driven by the purpose of chemicals, which suggests that there is a huge challenge for doing chemical epidemiology because the chemicals are markers for behavior and for conditions that may themselves be related to the effects, rather than being direct markers of the chemical causing the effect.

Therefore, the challenge to environmental epidemiologists is to include in their study design recognition of how the chemical could be a marker for other stressors and then to build into the design measures aimed at addressing that question formally through collecting the appropriate data to correct for those biases. The obvious example is that lead in paint tends to be associated with older houses, which tend to be associated with lower income. Pesticide exposures tend to be associated with houses with insect infestation, which may be markers for other chemical stressors in the house.

David Christiani: Actually, that is a very good point, and it obviously has implications for design.
EMPIRICAL WHOLE MIXTURE APPROACH FOR RISK EVALUATION: COMBINING EXPOSURE AND MIXTURES TOXICITY DATA – CHRIS GENNINGS, Ph.D., VIRGINIA COMMONWEALTH UNIVERSITY

Statistics Overview

Chris Gennings, Ph.D., is an expert in statistical approaches available for predicting the toxicity of chemical mixtures including both component-based approaches and determining sufficient similarity of complex mixtures. She has participated in numerous cross-discipline collaborations to assess the effects of a diverse array of mixtures, and she has served as an expert on multiple advisory panels (e.g., NAS phthalate panel, EPA PAH panel). She is a Professor of Biostatistics and Director of the Research Incubator for the Center of Clinical and Translational Research at Virginia Commonwealth University. Her research interests focus on design and analysis methodologies in toxicology and epidemiology studies of chemical mixtures.

The focus of my presentation was on whole mixture approaches for predicting the toxicity of “unknown” mixtures (i.e. using data from a reference mixture to infer information about another mixture). The component-based approaches mentioned in Glenn Rice’s presentation can be thought of as “bottom-up” approaches. In effect, component-based approaches are built by incorporating data from individual chemicals into additivity models. Whereas, whole mixture approaches are “top-down”, meaning they begin with a complex mixture and determine whether the mixture is associated with adverse effects. Additionally, whole mixture approaches include both complex mixtures and constructed mixtures that are defined based on exposure.

This presentation focused on testing for sufficient similarity, which included highlights from some of our ongoing work. Sufficient similarity was discussed from a data-rich point of view and then also a data-poor perspective, which is more relevant to most real-world situations. Lastly, I discussed considerations for choosing mixtures to study experimentally that could be used in a whole mixture approach.

We are exposed to many, many chemicals. There are approximately 83,000 chemicals that are in production in the United States. It is important to note that regulatory agencies and legislative policies tend to be focused on categories of chemicals, but there is not much emphasis on mixtures based on real exposure scenarios. For example, categories of these chemicals include: pesticides, consumer products, drinking water contaminants, food, and drugs.

In cumulative risk assessment, an emphasis should be placed on chemicals that co-occur and have a common adverse outcome, which was proposed by the 2007 National Academy of Sciences report on phthalates (National Academy of Sciences 2008).

Component-based approaches were discussed in some detail (see presentations by Drs. Glenn Rice and Earl Gray); therefore, the focus of this presentation was on whole mixture approaches. At first glance, you might think that whole mixtures present an impossible problem. But based on some of the discussion at this workshop and an interesting paper by Rogelio Tornero-Velez et al. (2012), it could be that mixtures are not random and are generated by human behavior, such that not all possible combinations of chemicals would be expected to occur together (Tornero-Velez et al. 2012). Therefore, in prioritizing mixtures for study, we can try to find patterns of exposure, and then focus on some of the
“top-down” approaches on those patterns. In this way, there may be a more manageable number of mixtures that would be reflective and typical of human exposures. Such a strategy may supplement the more common components-based approaches.

The advantage of whole mixture approaches is that we do not have to worry about default assumptions of additivity. U.S. EPA guidance for cumulative risk assessment of chemical mixtures describes whole mixture approaches as preferred compared to component-based methods, but mixtures data are not generally available. Mixture-specific toxicity data considered “sufficiently similar” to mixtures in the environment may be used as surrogates. However, the guidance does not provide specific information on how to define sufficiently similar mixtures.

Our approach proposed to define sufficient similarity by using equivalence testing methodology. Equivalence testing is commonly used by the FDA for generic drugs. Here, the null and the alternative hypotheses are opposite of those generally used (i.e., generally, the null hypothesis is that the generic drug does not differ from the name-brand drug vs. that the generic drug does differ from the name-brand drug). Thus, using equivalence testing methods, we allow the data to provide evidence of sufficient similarity.

A preliminary step in the method is to determine what is meant by similarity using a reproducible process that results in a numeric value for the boundary of similarity. In short, we estimate the ‘distance’ between the benchmark dose (BMD) estimate for a reference mixture and a candidate mixture in a data rich and data poor scenario. The method was demonstrated in a data rich case where dose-response data are available for a reference mixture and for a candidate mixture. When the upper confidence limit on the distance between the two BMDs is less than the boundary of the similarity region, the mixtures are claimed to be ‘sufficiently similar.’ In other words, the data support the alternative hypothesis of similarity.

In a data poor scenario, the more typical case, dose-response data are only available for a reference mixture and exposure or occurrence data are available for candidate mixtures. With simplifying assumptions, a similar strategy is followed to test for sufficient similarity.

In both cases, we construct a “similar mixtures risk indicator” which is analogous to the hazard index. It is a comparison of exposure data with toxicity information from sufficiently similar mixtures. We illustrate the approach using mixtures of pyrethroids from floor wipe samples in day care centers.

Details of the approach were presented at the NIEHS Mixtures Workshop. However, our paper is still under peer review and we have elected to only summarize the work as described above.
This breakout session provided opportunities for researchers to discuss current challenges in mixtures within their disciplines. Participants were divided into five breakout groups according to their specialty (biology/toxicology, epidemiology, statistics, exposure science, risk assessment). They were asked to develop a comprehensive, discipline-specific list of knowledge gaps in mixtures research. The discussion within these breakout groups was based on the Request for Information (RFI) (active March-April, 2011; http://ntp.niehs.nih.gov/go/rfimix), and the following questions were provided to each group:

1. What are the underlying scientific knowledge gaps for assessing the effects of mixtures on human health?
2. What are the scientific issues encountered in performing risk assessments of mixtures that can be addressed by new research?
3. What types of scientific data (e.g., mechanistic, epidemiological, etc.) are needed to address these underlying knowledge gaps?
4. What are the new technologies and innovative approaches that could be leveraged to address these underlying knowledge gaps?

Participants were asked to prioritize the issues based on importance. Each chairperson later provided a 15-minute presentation describing the output from the discussion within their groups. In addition, Cynthia Rider (DAPT, DNTP) provided the results gathered previously from the RFI (March-April, 2011) (see page 79). The presentations were followed by open discussion with all attendees. These talks and discussions are presented below.
Most of the discussion within the epidemiology group focused on identifying the underlying scientific knowledge gaps for assessing the effects of mixtures on human health. This group chose not to address the second question because it was outside of the area of expertise of the group. Along with knowledge gaps, this group focused on conceptual issues in mixtures research.

**Knowledge Gaps**

<table>
<thead>
<tr>
<th>Group discussion results:</th>
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<tbody>
<tr>
<td>• Proper exposure assessment/measurement</td>
</tr>
<tr>
<td>o Knowledge of chemical co-occurrence</td>
</tr>
<tr>
<td>o Windows of susceptibility</td>
</tr>
<tr>
<td>o Vulnerable populations</td>
</tr>
<tr>
<td>o Misclassification</td>
</tr>
<tr>
<td>o Product information (what is in formulation X?)</td>
</tr>
<tr>
<td>o Validated questionnaire data</td>
</tr>
<tr>
<td>• Translation between toxicology and epidemiology (poor bridge)</td>
</tr>
<tr>
<td>o Mechanisms, exposure groupings</td>
</tr>
<tr>
<td>o Animal versus human doses</td>
</tr>
<tr>
<td>• How do you prioritize mixtures?</td>
</tr>
<tr>
<td>o “Lamp post” versus biologically-relevant mixtures</td>
</tr>
<tr>
<td>• If we don’t know dose-response with single chemicals, what would additivity look like</td>
</tr>
<tr>
<td>• Number of exposures overwhelming</td>
</tr>
<tr>
<td>o Need to be related via mechanism, potency, structural groups, outcome</td>
</tr>
<tr>
<td>▪ Can exposures be summed?</td>
</tr>
<tr>
<td>o Often this information does not exist (e.g., PFCs)</td>
</tr>
<tr>
<td>o What’s the approach?</td>
</tr>
<tr>
<td>▪ E.g., high throughput, binding assay</td>
</tr>
</tbody>
</table>

The data gaps were presented by priority order above. The first data gap identified was the need for proper exposure assessment (i.e., measurement of exposure). One issue under that heading includes the knowledge of chemical co-occurrence. For example, there are an infinite number of possible chemical combinations, but people are more likely to be exposed to certain combinations, and other combinations are likely to be extremely rare. Next, “windows of susceptibility” is a critical issue when performing exposure assessments in the epidemiological study of mixtures. Other issues to consider include susceptible and vulnerable populations, and the possibility for misclassification of exposure status. For example, if an exposure is measured at a particular moment-in-time, but the timing is not relevant to the endpoint of interest, it can lead to incorrect interpretation of results (e.g., measuring exposure post-natally for an in utero effect). Further, if the potential exposure is due to a commercial product, information on that product is needed to fully characterize the exposure. This is often hindered by lack of access to proprietary information. Finally, the issue of validating of questionnaires to assess...
exposure was discussed. Questionnaires are often used to assess exposure in epidemiology studies. Therefore, it is very important that when asking about exposures, the questions are relevant to those exposures. For example, nutritionists have really advanced the field by designing questionnaires that can determine the dietary components related to a person’s food intake.

A second knowledge gap that was identified was the need for translation between toxicology and epidemiology. The bridge between these two disciplines requires strengthening to leverage knowledge from both fields. This meeting has furthered discussions on this topic. Further collaboration between epidemiology and toxicology is needed to contribute toward understanding biological plausibility of linkages identified in epidemiological studies. Additionally, mechanistic data from in vitro and in vivo experiments can be used to elucidate mechanisms and modes of action, as well as disease pathways. This information can be used in grouping exposures (i.e., which chemicals should be the focus of epidemiological research based on biological activity). Lastly, because animal and human doses can be very different, it is important to develop accurate models for extrapolating between them.

The third knowledge gap identified was how can mixtures be prioritized? During the morning plenaries, the concept of “looking under the lamppost” was discussed. Essentially, this is the practice of only monitoring chemicals with known toxicity and/or only testing the toxicity of chemicals with known exposure. In the lamppost scheme, which is the more justifiable approach: should the focus of research be on mixtures that are more biologically relevant or more relevant to measured co-exposures? Alternatively, can more unbiased approaches be developed and/or implemented (e.g., exposome approach for characterizing exposure; use of high throughput screening to assess thousands of chemicals for biological activity).

Another gap we identified is a lack of dose-response data for individual chemicals. Because most models of mixture toxicity require input from individual chemicals, the lack of data can preclude our ability to assess additivity.

Certainly, the number of potential co-exposures is overwhelming. In considering large numbers of exposures, there is a need for grouping chemicals. This can be accomplished by using mechanistic criteria, potency, structural features, or by grouping according to a targeted endpoint. This information is often not available, but is required for accurately grouping chemicals. Potential approaches to attaining this information could be high throughput screening or binding assays. At this time, there are not clear methods for simply summing exposures, but this could be an area to develop.

Conceptual Issues

<table>
<thead>
<tr>
<th>Group discussion results:</th>
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</thead>
<tbody>
<tr>
<td>• What is the goal of the research?</td>
</tr>
<tr>
<td>◦ Mechanistic, policy, etiologic</td>
</tr>
<tr>
<td>• Scientific versus opportunistic (funding)</td>
</tr>
<tr>
<td>• Unmeasured contaminants and changing formulations (PBDEs)</td>
</tr>
<tr>
<td>• Multiple comparisons</td>
</tr>
</tbody>
</table>
• Epidemiology can be confounded and or biased; less likely in experimental designs, and needs to be considered

Some of the conceptual issues included addressing the appropriate scientific question when developing a study. For example, it may be acceptable to evaluate the effects of a whole mixture (air pollution), without considering the component parts. If the question pertains to temporality or seasonality of health effects associated with air pollution, then a researcher could measure air pollution as a whole and would not need to determine the specific components. If, instead, the study is for the purpose of remediation or linking specific biological effects to a toxicant, it might be necessary to identify the specific components. Therefore, the approach depends on the goal of the research (e.g., identification of a specific mechanism or a policy question to reduce exposure).

Another conceptual issue involves research drivers, such as the availability of funding sources and whether studies are motivated by opportunistic circumstances or scientific considerations.

In epidemiology studies, there are an abundance of unmeasured contaminants. Additionally, in epidemiology studies assessing the health effects from exposure to commercial formulations or personal care products, the ingredients can change over time due to environmental conditions and degradation processes or they can be altered by industry in response to innovation, replacement of ingredients, and changing consumer needs. For example, the polybrominated compounds replaced other flame retardants in the late 1970s and were subsequently found to be toxic and persistent. This type of evolving formulation of flame retardants, flavor enhancers, personal care products, etc. can lead to a “moving target” in the field of mixtures research.

Epidemiology is challenged by the need to make multiple comparisons. In epidemiology studies, it is also important to account for potential confounders and limit sources of bias.

**Types of Scientific Data Required**

<table>
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<th>Group discussion results:</th>
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<tbody>
<tr>
<td>• Large scale surveys to identify contaminant-contaminant associations</td>
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<tr>
<td>• Toxicology data on choosing chemical groupings</td>
</tr>
<tr>
<td>• Better data on disease mechanisms (to help select mixtures)</td>
</tr>
<tr>
<td>• Database on endpoint-specific potency factors</td>
</tr>
<tr>
<td>• Validation of intermediate endpoints for disease risk</td>
</tr>
<tr>
<td>o Exposure AND outcome</td>
</tr>
<tr>
<td>o Because of power, high throughput screen concordance</td>
</tr>
<tr>
<td>o Both biomarkers and functional assays</td>
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</table>

In terms of the types of scientific data required, several ideas were discussed. For example, availability of large-scale surveys/databases would be useful (e.g., NHANES data to identify contaminant-contaminant associations). Next, toxicology data is needed to aid in grouping chemicals. Better data on
disease mechanisms is needed to facilitate selection of mixtures for study. One specific idea that was discussed was development of a database on endpoint-specific potency factors that would be useful in prioritizing mixtures for investigation.

Validation of intermediate endpoints for disease risk is important. Increased information is needed to link intermediate endpoints to disease outcomes. The use of intermediate endpoints has the potential to provide greater statistical power and larger sample size for epidemiological studies. Intermediate endpoints include both biomarkers and functional assays. More data in the area of intermediate endpoints would facilitate greater translation between epidemiology and toxicology. This area requires research attention.

Technologies and Innovative Approaches

**Group discussion results:**
- Intermediate assays (like estrogen receptor)
- EWAS
- Problems with timing of exposure, persistence of toxicant
  - Probably not feasible at this time
  - Pooling data, harmonization of testing
- Exposure technologies and approaches
  - Validating exposures
  - Opportunities with mobile devices
  - Exposure assessment that reduces participant burden

One of the technologies and approaches that would fill the data gaps discussed above includes more effort on intermediate assays. Also discussed were environment-wide association studies (EWAS), but there are limitations with the EWAS concept, and it may need further development before it can be applied to mixtures research. Another approach would be to pool data from multiple independent projects. Additionally, it would be useful to increase the harmonization of testing practices and develop methods for combining data across disciplines. Finally, exposure assessment is critical to epidemiology. This includes validation, the use of mobile devices to measure exposure, and reducing participant burden by increasing measurement efficiency in exposure assessment.

More communication and collaboration between disciplines is required. The current workshop is an excellent example of moving toward that goal.

**Discussants:** Rosemary Castorina (University of California, Berkley), David Christiani (Harvard School of Public Health), Julie Daniels (University of North Carolina, Chapel Hill), Lyndsey Darrow (Emory University), Caroline Dilworth (NIEHS DERT), Kimberly Gray (NIEHS DERT), Russ Hauser (Harvard School of Public Health), Irva Hertz-Picciotto (University of California, Davis), Todd Jusko (NIEHS DIR, rapporteur), Kembra Howdeshell (NIEHS DNTP), Freya Kamel (NIEHS DIR), Susan Korrick (Harvard Medical School),
Andreas Kortenkamp (University of London), Scott Masten (NIEHS DNTP), Paige Tolbert (Emory University), Tom Webster (Boston University), Beth Whelan (NIOSH, chairperson).
**Group Discussion Results**

**Underlying Scientific Knowledge Gaps in Hazard Characterization**

- Lack of toxicity data for individual compounds
- Advancing/defining methods for clustering/classification/grouping
  - Example: sufficient similarity (substantial equivalents)
- HTS: Establishing linkage between different biological levels for bioassay data
  - Example: linking *in vitro* endpoints to *in vivo* endpoints
- Describing uncertainty – knowing you have interrogated enough biological dimension
- Interventions and risk reduction

The risk assessment group began their discussion using a figure (Figure 33) developed by Mike DeVito, Ph.D. This figure illustrates the principles of mixtures evaluation. There are three different approaches. One approach is to evaluate the mixture of concern using existing data on that mixture. In this approach, the mixture is treated the same as an individual chemical for conducting the risk assessment. If the mixture data is not available, an alternative would be to use data from a similar mixture in order to conduct a risk assessment. However, this introduces the question of how to determine what defines a similar mixture. One recommended approach was to assess the substantial equivalence, which is used in determining the safety of novel foods (i.e., reference mixture demonstrates the same characteristics and composition as the mixture of interest). In the sufficient similarity approach, the mixture is still treated as an individual chemical for risk assessment purposes. If data for a sufficiently similar mixture is not available, then a risk assessor can utilize the third, and most commonly used, component-based approach. In this approach, data on individual chemicals exists and are used in risk assessment. The hazard index is an example of the component-based approach. There are many issues in using component-based approaches, but one of the most important data gaps associated with its use is the lack of data on individual chemicals. Another issue inherent in the component-based approach is the need to classify chemicals, or cluster them together, which requires criteria to determine how to decide which individual chemicals to include in the risk assessment.
One way to generate data on individual chemicals is through high throughput screening. However, it is not clear how risk assessors should utilize \textit{in vitro} data. HTS has the potential to generate large quantities of data. In order to make use of these data in risk assessments, \textit{in vitro} data must be linked to \textit{in vivo} end points. Methods for extrapolating among different platforms must be developed.

There is inherent uncertainty in risk assessment. One data gap is the need to determine how much biological ground should be covered to reduce uncertainty. For example, how much data is needed to have confidence in chemical-to-chemical and route-to-route extrapolation for a mixtures risk assessment.

Finally, the end goal for public health is to reduce the risk associated with exposure to mixtures through intervention strategies and by promoting risk communication.

\textbf{Underlying Scientific Knowledge Gaps in Exposure Assessment}

<table>
<thead>
<tr>
<th>Group Discussion Results</th>
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<tbody>
<tr>
<td>• Identification of mixture of interest/Better define exposure data to identify mixtures</td>
</tr>
<tr>
<td>• Defined mixtures: mixtures we know components of</td>
</tr>
<tr>
<td>• Identifying mixtures we don’t know about (real world mixtures)</td>
</tr>
<tr>
<td>• Lack of data on mixtures at different routes of exposures</td>
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</table>

Several scientific knowledge gaps in exposure assessment were also discussed by the risk assessment group. First, a priority for risk assessors is to identify the mixture(s) of concern, due to the many possibilities and permutations of environmental chemicals. There is a need to focus risk assessments on mixtures that represent real-world exposures. For example, a cumulative risk assessment has been performed on organophosphates as a group, but people are rarely exposed to organophosphates alone.

Risk assessors should consider utilizing databases to identify which chemicals are likely to co-occur in the human population. It is important to recognize that this may vary from community-to-community and from person-to-person. There is a need to confirm that risk assessments are being conducted on the most relevant mixtures.

Another very important consideration is in extrapolating across different routes of exposure. For example, experiments in animals use intravenous or intraperitoneal dosing, but these do not necessarily represent relevant routes of exposure for humans. More work is needed to validate this type of extrapolation.
Issues Encountered in Performing Risk Assessment of Mixtures

Group Discussion Results

• Issues of uncertainty encountered in doing a RA can inform underlying scientific knowledge gaps
  • Iterative process where RA improves
• Life stage toxicity testing—differences in susceptibility
• Relating in vitro HTS assays to apical endpoints – need more case studies
• Developing methods to predict interactions that are not dose additive

Uncertainty is included in the calculation of risk such that it should protect even the most susceptible populations (e.g., children). However, one goal in risk assessment is to decrease the uncertainty by increasing our understanding of the relationship between different variables, such as animal-to-human and route-to-route extrapolations. Although uncertainty is a general concern in risk assessment, it will require sophisticated solutions when considering the complexity of mixtures risk assessment.

Life stage toxicity represents another complication to performing risk assessment. Most of the time, data only exists for adults, and data for children or other susceptible populations is lacking.

Another consideration is the utilization of high throughput screening and how to link data from in vitro to in vivo studies. Case studies that move from in vitro to in vivo should be developed in order to validate extrapolation. This will increase confidence in using data from HTS or other in vitro studies.

When conducting risk assessments using whole mixtures approaches, the interactions among component chemicals are accounted for in toxicity testing with the whole mixture of interest or a reference mixture (e.g., in testing diesel exhaust for toxicity, the interactions among PAHs and metals do not require characterization). However, if a component-based approach is utilized, then interactions must be accounted for in order to accurately characterize risk.

Because dose additivity is often used as the default approach in risk assessment, it is critical to define it very clearly. Additionally, more work is needed to determine when dose additivity is appropriate and when another model might provide a better estimate of joint toxicity.
Types of Scientific Data Required

<table>
<thead>
<tr>
<th>Group Discussion Results</th>
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<tbody>
<tr>
<td>• <em>In vitro</em>, whole animal, and epidemiological data (new and better use of existing)</td>
</tr>
<tr>
<td>• Common chronic diseases across population as anchor for mechanistic data</td>
</tr>
<tr>
<td>• Expanding technology for detecting real time exposures</td>
</tr>
<tr>
<td>• Technologies and innovative approaches</td>
</tr>
<tr>
<td>• Multidisciplinary teams required</td>
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</tbody>
</table>

In terms of the types of scientific data required, the integration of *in vitro*, whole animal, and epidemiological data will be important in advancing mixtures risk assessments. There is an opportunity to connect the currently existing epidemiology data on chronic diseases and toxicology exploring the mechanisms of these diseases. This is an area that has a lot of data gaps and is ripe for research. Collaboration across disciplines is necessary to develop new technologies and innovative approaches in the study of mixtures.
The statistics group discussed many of the same themes outlined by the previous breakout groups. The statistics group used a different approach from the breakout groups described previously. Four challenges in mixtures research were selected and discussion revolved around how statisticians could contribute to the planning and conduct of studies to respond to those four issues.

**Exposure Characterization and Uncertainty**

<table>
<thead>
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<th>Group Discussion Results</th>
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<tbody>
<tr>
<td>• Statisticians should work with exposure scientists to characterize distribution of exposures to mixtures in populations</td>
</tr>
<tr>
<td>• Important to have raw data exposures on exposures rather than summary data</td>
</tr>
<tr>
<td>• Statisticians need to help define:</td>
</tr>
<tr>
<td>• Uncertainties (multivariate) surrounding exposure</td>
</tr>
<tr>
<td>• Susceptible sub-populations</td>
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<tr>
<td>• Temporal, spatial exposures</td>
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</table>

The first area discussed was exposure characterization. It is important that the mixtures that are studied should be related to mixtures to which people are exposed. Statisticians need to work with exposure scientists to characterize the distribution of exposures to mixtures in populations. In order to characterize distributions, statisticians should have access to raw data, rather than summary data. “Distribution” is a key term because there are several sources of variation within exposed populations (e.g., person-to-person, temporal, and spatial variation). These distributions are multivariate (i.e., they encompass the consideration of more than one statistical variable at a time) and require statisticians to evaluate several variables simultaneously. To add to the complexity, correlations between these variables need to be determined. Another consideration is the variation in responses to low dose versus high dose exposures, which may lead to dynamic mixtures or effects.

Statisticians need to know what the exposure distribution is across populations with respect to these variations, and also need to define the uncertainties in these distributions. Statisticians are also concerned with data from susceptible populations and need to determine how this information plays a role in the health effects associated with mixtures. For example, it is not sufficient to only determine average exposure within a population as there may be susceptible populations that demonstrate significant health effects. Exposure information can be used along with distribution data to prioritize mixtures for study and to characterize the probability of health effects associated with those exposures. These types of data could drive regulatory decisions on acceptable limits.
**Data Mining: NHANES Biomonitoring Data**

<table>
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<th>Group Discussion Results</th>
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<tbody>
<tr>
<td>• Valuable, underutilized resource to characterize population exposures</td>
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<tr>
<td>• Provides background levels of multivariate exposure</td>
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<tr>
<td>• Captures nutritional information, some environmental background information, and biomonitoring</td>
</tr>
<tr>
<td>• Family histories, non-chemical stressors</td>
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<tr>
<td>• Biomonitoring information can be used to prioritize mixtures for study</td>
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</table>

Biomonitoring data is collected through multiple databases. NHANES was given as a prime example of a valuable resource to characterize population exposures. These biomonitoring databases are used to build libraries of exposures in various populations. The NHANES provides data on multivariate exposures and more effort should be expended to mine this and other similar resources.

In addition, NHANES has data on a number of non-chemical stressors such as family histories and nutritional information. Researchers can also study the population and temporal distributions of exposures. Data from NHANES and other biomonitoring programs should be used to prioritize mixtures to be studied.
Another area of discussion focused on the need to develop methods for characterizing the toxicity of complex classes of chemicals. Because the potential combinations in a large class are infinite, methods for predicting the toxicity of the mixture of interest based on sufficient similarity to a reference mixture are needed. A reference library would be comprised of a set number of reference mixtures. A reference mixture refers to a well-characterized sample, both chemically and biologically. The discussion revolved around how to create a reference library of mixtures that would provide adequate data to gain information about the toxicity of unknown mixtures. For example, polycyclic aromatic hydrocarbons (PAHs) are a class where a reference library approach has been recommended by a scientific advisory board to the EPA (U.S. EPA 2012).

If you consider the chemical space as including all possible combinations of PAHs, the first questions in building a reference library are how many reference mixtures are needed and how will you select the reference mixtures. In effect, it is necessary to decide the make-up of the PAH reference mixtures. Consideration of both the specific PAHs to include in each reference mixture and their ratios within the reference mixtures is essential. Additionally, it is important to think about whether the driver in
selection of reference mixtures should be environmental relevance or coverage of the widest possible chemical space. For example, should the selection of reference mixtures be based on combined exposures that are most likely to occur in human populations (e.g., cigarette smoke, Gulf Oil Spill) or alternatively, should selection be independent of exposure considerations and instead involve mixtures that represent the widest variations possible?

Once a reference library has been established, the next step would be to develop statistical methods to interpolate from the reference mixture to the experimental sample. This process requires the development of criteria to determine sufficient similarity. The criteria will include both chemical and biological comparisons between the reference and experimental mixtures. Ultimately, scientific judgment and assumptions will be involved in the process.

Special design considerations will be required to limit the number of tests, while providing the most coverage. For example, fractional factorial designs have been implemented in mixtures toxicology for many years in order to select representative mixtures for study, while using the minimal number of tests. These representative mixtures are tested and results can then be extrapolated from these representative mixtures to the many possible combinations of the untested mixtures. In the case of PAHs, highly fractionated designs can be used to estimate the toxicities across the entire range of PAHs.

Another method that can be applied to comparing reference and experimental mixtures is a sequential adaptive program that is based on the dose-response relationships of the reference mixtures. In effect, mixtures that display very steep dose-response relationships will require more data points in order to characterize the shape of the curve. Once the dose-response relationship of each reference mixture has been characterized, the data from the reference mixture will be used to estimate the experimental mixture dose-response curve and determine which doses of the experimental mixture to test. This is an iterative process and testing can be completed when sufficient data is generated to support the assumptions.
### Unknown Mixtures – No Reference Data

**Group Discussion Results**

- **Problem:** How do you design a program and come up with scientifically credible clean up?
  - Also need to consider cost
  - Some ideas:
    - Default: dose additive model
    - Start testing surrogate synthetic mixtures
    - Sample enough to understand components of mixtures
    - Artificially created by-products
  - Look at genomic signature (e.g., genomics data available in CEBS)
    - Use it as an effect marker to predict what type of toxicity is expected from the unknown mixture (not a validated method – needs more work)
    - Genomic data could serve as starting point, but has to be validated with in vivo approach
  - Multivariate *in vivo* approach

The last scenario discussed involved unknown mixtures that require clean-up. For example, this may include development of a strategy for remediation of the unknown mixture at a Superfund site or determination of a safe level of exposure. In cases such as this, a component-based method can be utilized. The first step would be to determine the major components of the unknown mixture using analytical chemistry approaches. Next, a component-based mixture toxicity model such as dose addition could be used to estimate the toxicity of the mixture based on the constituents.

Another approach would be to develop a synthetic mixture that would resemble the unknown mixture from a hazardous waste site. Various concentrations of the synthetic mixture could be used to determine a reference dose-response curve. This data could then be used to assess the validity of a dose addition model by comparing the observed mixture toxicity to the toxicity predicted based on the individual components.

Genomic approaches were suggested as another potential method for determining the effects of an unknown mixture. There has been an on-going effort to collect genomic data on many different classes of compounds. If a genomic signature of the unknown mixture could be generated, it could be compared with signatures from chemical classes contained in a genomics reference library. This process could provide information about possible toxicity pathways associated with the unknown mixture and could be used as a starting point for future mixtures toxicology studies. Currently, the data is more qualitative than quantitative, but it is a promising approach for mixtures toxicology.
Discussants: John Bailer (Miami University), Abee Boyles (NIEHS DNTP), Gregg Dinse (NIEHS DIR), Paul Feder (Battelle, chairperson), Chris Gennings (Virginia Commonwealth University), Dale Hattis Clark University), Richard Hertzberg (Biomathematics Consulting), and Amy Herring (University of North Carolina, Chapel Hill), Grace Kissling (NIEHS DIR), Elizabeth Mauell (NIEHS DNTP), Shyamal Peddada (NIEHS DIR), Cynthia Rider (NIEHS DNTP), Keith Shockley (NIEHS DIR), Woody Setzer (EPA NCCT), Sheetal Thakur (NIEHS DERT, rapporteur).
Underlying Scientific Knowledge Gaps

Group Discussion Results

- Need to understand the utility and limitations of in vitro (including HTS) – verification needed with in vivo systems
- Need for modeling across different physiological systems (e.g., immuno, neuro) and species
- Need to understand how internally circulating chemicals (e.g., hormones, nutrients) may interact with man-made chemicals (e.g., drugs)
- Need for integration of systems biology with computer modeling/simulations
- Mixture testing strategies should be focused on developing predictive models of mixture toxicity
- Need to understand how collection/concentration of samples affects the biological response to the mixture
- Need to calculate accurate individual exposure data (i.e., may vary with gender, activity, age, race)

This group began a discussion of underlying scientific knowledge gaps in biological/toxicological studies of mixtures. One very important issue identified was the need to translate effects from *in vitro* and *in vivo* experiments. *In vitro* platforms offer a great deal of promise for the study of mixtures, but validation in *in vivo* systems is required in order to provide biologically meaningful data. Another area discussed was the need for modeling across various physiological systems and species. Physiologically-based pharmacokinetic models are needed to extrapolate from species-to-species and route-to-route. The next point discussed was the consideration of the mechanistic interactions between internally circulating chemicals (e.g., hormones, immune modulators) and external chemicals (e.g., drugs, pesticides). This requires an understanding of the “normal” milieu and how it is disrupted through chemical exposure.

There was discussion of the movement in toxicology away from characterizing the effects of individual chemicals and towards consideration of interconnected networks or systems. This network/system perspective takes perturbation of the target system as the starting place and radiates outward to identify and characterize the role of different stressors in that perturbation. In order to accomplish this goal, computer modeling/simulations will be needed to integrate across biological systems. The data generated from these methods will allow for identification of potential mixtures of concern.

Another data gap discussed by this group included the need for biological studies to support the development and validation of predictive models of mixture toxicity. The dynamic nature of mixtures
increases the complexity of study design. There are important considerations when using complex environmental samples in toxicity testing. For example, the effects of sample collection and concentration of complex environmental samples should be considered in designing biological studies. Additionally, toxicologists should be aware of the inherent differences between lab studies and human populations. For example, toxicologists should consider real-world human exposures when designing mixtures for use in laboratory studies (i.e., using mixing ratios observed in human populations).

**Issues Encountered in Performing Risk Assessment of Mixtures**

<table>
<thead>
<tr>
<th>Group Discussion Results</th>
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<tbody>
<tr>
<td>• Appropriate experimental designs for addressing mixture-related questions</td>
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<tr>
<td>• Need to know the underlying dose-response curves of the components of the mixtures</td>
</tr>
<tr>
<td>• Dose-response extrapolation relating to humans and experimental models</td>
</tr>
<tr>
<td>• <em>In vitro</em> to <em>in vivo</em> validation required prior to application of <em>in vitro</em> data to risk assessment</td>
</tr>
<tr>
<td>• What is the impact of the biological extrapolation from the selected model (current models may not capture biological relevance)?</td>
</tr>
<tr>
<td>• Need to study mixture and toxicological thresholds</td>
</tr>
<tr>
<td>• Need to calculate accurate individual exposure data (i.e., may vary with gender, activity, age, race)</td>
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</table>

In terms of risk assessment-related issues, experiments conducted by toxicologists should have appropriate experimental design to address mixture-related questions (e.g., power, appropriate statistical tests). Increased communication between risk assessors and toxicologists is needed to determine the types of mixtures data that would be the most useful for risk assessment purposes. Toxicologists play a key role in generating dose-response information that can be utilized in cumulative risk assessments. More dose-response data from single chemical components as well as whole mixtures is needed to inform risk assessments. Methods for extrapolating doses across species (e.g., determining chemical levels within tissues, PBPK modeling) are needed to effectively use that data.

There has been increased interest in using *in vitro* data for risk assessments. Before these data can be used, more work is needed to link *in vitro* data to *in vivo* disease endpoints. Selection of appropriate experimental models is necessary. For example, the effect observed in the model should be relevant to human disease pathways.

Another area that requires attention is the calculation of mixture and toxicological thresholds of effect. It is critical to define the point at which individual chemicals and mixtures transition from no effect to effect (e.g., No Observed Adverse Effect Levels, benchmark doses). This area requires a greater focus on the lower end of the dose-response curve, as opposed to high dose mixtures studies, which are often conducted to characterize joint action (e.g., identify interactions).
Types of Scientific Data Required

Group Discussion Results

- Define exposure scenarios and collection of exposure data
- Use lower organisms (e.g., zebrafish) to bridge conserved pathways
- Data on chemical and mixture “signatures”
- Chemometrics/toxicometrics (component analysis versus whole mixtures)
- Life cycle analysis of the chemical mixture (e.g., nanomaterials in plastic composites)
- Integrating mechanism of action into epidemiological studies

The group then discussed the types of scientific data required to move the field of mixtures research forward. The first type of information that is needed is a description of the exposure scenario and a more complete collection of exposure data from epidemiological studies. Collection of better exposure data would help inform the design of animal studies to further explore causal relationships between exposures and disease. Toxicologists should consider using lower/smaller organisms (e.g., zebrafish, *C. elegans*), which could provide important information on pathways that are conserved between species. This would also further the goal of decreasing the numbers of mammalian animals used in research, and lower order organisms provide an alternative to the use of mammalian species. The recognition of “signatures” in high-density platforms (e.g., –omics, HTS) can be utilized to develop testable hypotheses and select mixtures for study.

The concepts of “chemometrics” and “toxicometrics” were discussed. The chemometric approach involves measurement of chemical parameters for use in predicting chemical behavior. Toxicometrics takes into account measurement of toxicity parameters for predicting mixture effects. These approaches can be used in either component or whole mixture evaluation.

Another topic discussed included the need for increased data on the lifecycle of chemical mixtures. Environmental mixtures have a dynamic nature and they change over time. For example, nanomaterials, which begin as mixed entities, can be used in plastic composites that break down over time to create new mixtures that could display different toxicities than the starting materials.

The final data need discussed was integration of mechanism of action data into epidemiological studies. This does not necessarily require the generation of new data, but a modified application of existing toxicological data. Although this need exists in single chemical assessments, the added complexity in studies of mixtures emphasizes the need to determine biological relevance.
Many of the technologies and innovative approaches discussed mirrored topics covered in response to the previous questions on data gaps and needs. New types of analysis that include the latest modeling software will aid in the study of systems biology. For example, clustering and pathway analysis programs can be used to identify patterns of toxicological interest from robust data sets. As described above, there is a need to apply mechanism of action data in an epidemiological context.

It was noted that ecologists have experience in evaluating complex exposure scenarios. For example, ecological risk assessments involve incorporation of both chemical and nonchemical stressors for a variety of key species in the ecosystem of interest. Techniques and tools used in ecological assessments should be considered in mixtures studies. It is important that toxicologists utilize the “lessons learned” from ecologists.

Lower organisms (e.g., zebrafish and *C. elegans*) hold promise in the development of innovative approaches. The multiple benefits include: low cost, rapid results, and ease of manipulation. For example, morpholino, knock-out, and humanized models can be created. They offer the whole organism benefits (e.g., metabolism and system interactions) with less time invested than in mammalian systems. Some lower organisms can be visualized non-invasively allowing for phenotypic evaluation throughout the life cycle. However, it was noted that investigation should focus on conserved pathways between lower organisms and humans, as well as selection of appropriate models for the targets of interest.

The importance of focusing research on the low-dose effect region in mixtures studies was discussed. New approaches for studying in the low dose region include more sensitive analytical techniques to measure concentrations in biological samples, refinement of modeling approaches to assess points of
departure, and development of intermediate biomarkers of toxicity. Future endeavors may address methods for incorporating non-monotonic dose-response relationships into predictive models of mixture toxicity.

New technologies to better assess exposure are needed. Development of wearable monitors that can non-invasively measure multiple chemicals (and their validation) offers promise for the future of exposure biology. This will require an interdisciplinary effort between not only engineers and biologists, but statisticians, molecular biologists, etc.

There was discussion that there has been a concentration of mixtures research on certain biological systems (e.g., reproductive/endocrine). However, future mixtures studies should explore other systems that are related to common human diseases (e.g., cardiovascular, neurological, immunological and metabolic, etc.). Awareness of the mixture effects on important disease states may help in identification of environmental contributors to disease and may eventually reduce healthcare burden. This would require collaboration with healthcare economists to conduct economic analysis of the real costs of mixed exposures.

**Discussants:** Mamta Behl (NIEHS DNTP, rapporteur), Chad Blystone (NIEHS DNTP), Christopher Borgert (Applied Pharmacology and Toxicology), Danielle Carlin (NIEHS DERT), Deborah Cory-Slechta (University of Rochester), Stephania Cormier (Louisiana State University), Kevin Crofton (EPA NHEERL), Paul Foster (NIEHS DNTP), Julia Gohlke (University of Alabama, Birmingham), L. Earl Gray (EPA NHEERL), Jerry Heindel (NIEHS DERT), Marike Kolossa-Gehring (Umweltbundesamt/Federal Environment Agency), Andreas Kortenkamp (University of London), Susan Schantz (University of Illinois, Urbana-Champaign), Jane Ellen Simmons (EPA NHEERL), and Raymond Yang (Colorado State University, chairperson)
Underlying Scientific Knowledge Gaps

**Group discussion results:**

- **Top-Down vs. Bottom Up**
  - Top = Outcome; Bottom = Chemical

- **Temporal/Spatial Variability**
  - Exposure as a function of life stage
  - Historic vs. chronic vs. acute

- **Non chemical stressors**
  - Lifestyle: diet, circadian disruption, etc.

- **Prioritize what is studied based on exposure**
  - Variability, prevalence (qualitative), dose

- **Biological/Individual Diversity**
  - Life stage
  - Disease state
  - Genetics
  - Voluntary/pharmacological exposures

- **Behavior and sources as exposure determinants**

This group began by identifying knowledge gaps in mixed exposure science. For exposure assessment to be conducted successfully, two different approaches should be considered: top-down and bottom-up. There was discussion of whether they should be integrated or simply improved. Top-down approaches focus on an outcome, such as a disease or a health endpoint, whereas bottom-up approaches involve taking the chemical(s) as the starting point for exposure assessment.

Another important data gap was the need for temporal and spatial information. Although this is a theme throughout the workshop, it is important to remember that exposure is a function of life stage. For example, *in utero* exposures will differ significantly from adult exposures. It is also important to distinguish exposures in terms of historic, chronic, or acute exposures. Historic exposures could include exposure to legacy chemicals, which have been banned but continue to be present in the environment. Chronic exposure could include daily contact with a chemical (e.g., use of personal care products), as opposed to acute exposures that may be more like those found in occupational settings. More effort is required to better characterize exposure and use available data (e.g., predict chronic effects from acute measurements).

The group agreed that it is necessary to consider the contributions of non-chemical stressors in health effects associated with exposure to chemical mixtures. It is important to determine the types of non-chemical stressors that have a significant impact on response to environmental chemicals. The nonchemical stressors that were mentioned involved lifestyle choices (e.g., diet, circadian rhythm, stress).

The group also thought it was important to prioritize mixtures for study based on exposure. There are many different aspects of exposure that should be taken into consideration. These include:
the effects of variability in a population and how it affects exposures; prevalence of a chemical or mixture within the study population; and dose of chemicals (i.e., focus on chemicals that display the highest concentrations).

The exposure is also going to be driven by individual biological diversity, which includes life stage as discussed above. Other contributors to biological diversity include the disease state of a person, genetics, and personal behavior (e.g., use of products and pharmaceuticals).

The last data gap identified was the need for more information on how behavior and sources influence exposure. For example, more work is needed to model how certain behaviors, such as an infant crawling, can contribute to potential exposures.

**Types of Scientific Data Required**

<table>
<thead>
<tr>
<th>Group discussion results:</th>
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<tbody>
<tr>
<td>• Components in mixtures people are exposed to</td>
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<tr>
<td>o Qualitative identification of chemicals</td>
</tr>
<tr>
<td>o Point-of-care analytical tools</td>
</tr>
<tr>
<td>• How people interact with mixtures</td>
</tr>
<tr>
<td>• How the above translate into disease</td>
</tr>
<tr>
<td>• Demographics</td>
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<tr>
<td>o Sex, age, susceptibility, etc.</td>
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The types of scientific data that are required to fill the identified data gaps were discussed. Exposure scientists need to examine components and real-world mixtures to which people are exposed. More rapid and inexpensive methods for detecting exposures are needed. These may include qualitative screening of environmental samples to identify components that may be part of a chemical class, and the use of point-of-care analytical tools (e.g., portable measurement devices) for biological sampling.

Other types of data required to characterize exposure to mixtures include information on the exposed population (e.g., sex, age, health status, and potential susceptibilities) and exposure patterns. Data is needed to assess how all of these exposure factors translate into potential disease.
Needs in Performing Risk Assessment of Mixtures

Group discussion results:

- Need for informatics approach
  - Patterns of exposure
  - “Behaviome”
  - Database, ontologies, standards [QA/QC]
  - Data maintenance – sustainability
    - Public/private partnerships
  - Relational approach
    - Account for individual variability
  - Validation versus development of approaches
- Biomarker panels
  - Endogenous versus exogenous responses
- Screening approaches
  - Source/media and exposure pathways – forensics
    - Signature chemical (e.g., formaldehyde)
  - Chemical and biological activity
- Communication between disciplines
  - Translation to public health

The first need discussed was the use of informatics approaches to identify patterns of exposure. The term “behaviome” was proposed to describe the use of behavior to characterize exposure. The incorporation of portable devices such as the cell phones described in Paul Price’s presentation could be used to track patterns of exposure based on everyday behavior (e.g., monitoring activity, biological parameters, and chemical exposures).

The group discussed the need to develop more publicly accessible databases containing exposure information. Some databases have already been established and contain information on exposures, co-exposures, and non-chemical stressors. To ensure the availability of high quality data to the research community, the group recommended the use of quantum information and quantum control (QIQC) methods. One stated challenge was that databases are often created as part of studies in the extramural community. These databases are an excellent resource during the lifetime of the project. However, they might not be maintained when the project ends. It was suggested that public-private partnerships could be created to sustain and maintain these useful databases. Furthermore, there should be an effort to compare across individual databases in order to validate exposure measurements.

Another need discussed was the development of biomarker panels that could include endogenous versus exogenous responses. For example, non-invasive sampling results could be compared to results from blood, urine, or tissue sampling.

Several screening approaches are available, but this group focused on a forensics approach, which uses information on source, media, and exposure pathways to determine unknown chemicals in
environmental samples. Signature chemicals can be used as identifiers of mixture components. For example, if the signature chemical formaldehyde is present in an air sample, it may indicate that other chemicals are also present. Another screening approach to identify chemicals of interest is to assess biological activity (e.g., estrogenic activity).

Throughout this workshop, the need for interdisciplinary cooperation has been a key theme. It is important to have communication and translation between the different disciplines in order to link exposure data to toxicology and epidemiology data and ultimately improve public health.

Technologies and Innovative Approaches

**Group Discussion Results**

- Multivariate Approach
- Agnostic Approach
  - Discovery-driven
- Sharing of Epidemiology Data Sets
- Public/Private Data Maintenance Partnership
- Bioinformatics is a must!

The technologies and innovative approaches that were mentioned fell into two categories: multivariate and agnostic. The multivariate approach involves assessing multiple exposure variables without collapsing data into broad categories (e.g., averaging across time). The agnostic approach is discovery-driven, without a predisposed or pre-established hypothesis. The group wanted to emphasize the importance of sharing data sets and developing public databases. Bioinformatics and appropriate statistical analyses will play a role in all of these proposed technologies.

Last Thoughts

**Group Discussion Results**

- Foster interactive dialogue & collaboration among all disciplines
- Integrative measures/approaches
- Take advantage of technological progress
A few “last thoughts” were presented. All disciplines need to foster dialogue for collaboration. There is also a need to use integrative measures and approaches. For example, this may include using blood samples to assess exposure and biomarkers of effect. Lastly, it is critical that the scientific community take advantage of technological progress in order to move the field of mixtures research forward (e.g., advances in bioinformatics and exposure-monitoring devices).

**Discussants:** Scott Auerbach (NIEHS DNTP), David Balshaw (NIEHS DERT), Dana Boyd Barr (Emory University), Herb Buxton (U.S.G.S), Antonia Calafat (CDC, chairperson), Brian Curwin (NIOSH), June Dunnick (NIEHS DNTP), Richard Kwok (NIEHS DIR), David Lawrence (Wadsworth Center), Joanna Matheson (CPSC), Minerva Mercado-Feliciano (NIEHS DNTP, rapporteur), Sri Naddadur (NIEHS DERT), Chirag Patel (Stanford University), Paul Price (Dow Chemical Company), James Rusling (University of Connecticut), Rogelio Tornero-Velez (EPA NERL)
NIHES sent out a Request for Information (RFI) prior to this conference to get a broad perspective on the important knowledge gaps in mixtures research. The presentation of the results from the RFI was reserved for the end of the breakout session discussions, in order to allow the breakout groups to discuss mixtures without influence from the RFI results. The history of the RFI is also a history of the workshop. When discussions about mixtures began at the National Toxicology Program (NTP), there was interest in developing a dedicated mixtures research program. It was necessary to narrow the field to tractable projects that would be a good fit for the NTP testing program.

These internal discussions focused on the development of a testing program aimed at defining the limitations of dose additivity. Next, the NTP mixtures group consulted with some of our regulatory partners, and they had much broader ideas of what the focus of an NTP mixtures program should be. They had questions about how to use epidemiological data in risk assessment, how to look at different routes of exposure, whether to add up total tumors instead of focusing on one tumor type at a time, how to use HTS in cumulative risk and in assessing sufficient similarity of complex mixtures, and more.

Instead of returning to internal discussions, a decision was made to seek an even broader perspective and develop an RFI to ask the public about mixtures. In developing the RFI, NTP worked with DIR and DERT, which was already leading a trans-NIEHS effort to develop a mixtures program. The title of the RFI was “Needs and Approaches for Assessing the Human Health Impacts of Exposure to Mixtures.” It was open March through April 2011 and was advertised through notices in the Federal Register and NIH Guide, as well as through emailing various NTP and DERT listservs. We posed the same questions that were posed to the breakout groups.

The response breakdown was: 27 responses total, 19 from academia, four from government, two from consulting, one from the non-profit sector, and one from industry. The approximate breakdown in terms of respondent specialties was: 13 toxicologists, eight exposure scientists, three epidemiologists, two risk assessors, and one statistician.

There were some major projects that were referenced, which was very helpful, as many times there is not an awareness of the activities of other groups. For example, the NIOSH Mixed Exposures Research Agenda was brought up, the Toxicity Risk Assessment in the 21st Century (Tox21) cumulative risk sub-team provided a response, and the Multi-Pollutant Science and Risk Analysis Workshop that took place early in 2011 was mentioned.

The responses were divided into key concepts. Next, the most highly cited key concepts were identified (Figure 34). By far, the most highly cited key concept was interactions (i.e., how unexpected interactions between chemicals such as synergy, antagonism, and potentiation can be addressed). The second most common topic was the need for better exposure characterization. The third key concept was defined as predictive mixture modeling approaches and included comments about the application of these predictive modeling approaches as well as questions about different types of approaches (e.g., dose
addition, response addition, PBBK modeling, and sufficient similarity). The fourth most cited theme involved temporal considerations.

A second tier of referenced issues included HTS and omics data and their use for identifying mechanisms of action and interactions, networks and systems biology, pharmacokinetic and pharmacodynamic considerations associated with mixtures, and moving from *in vitro* to *in vivo*. HTS and omics were discussed in terms of potential uses, but also included cautionary statements and the need to link *in vitro* results to *in vivo* data. Other topics included biomarkers and sensors for measuring exposure, cross-discipline communication, and low-dose issues. Some examples of topics in the theme of low-dose issues included extrapolation from animal studies to human exposures and interactions and mechanisms of action that might differ at low doses as compared to the high doses that are often tested.

Some of the particular stressors that were referenced in the RFI included both chemical and non-chemical stressors, which have been mentioned a lot at this workshop. Nonchemical stressors included things such as noise, vibrations, and the backdrop of hormones and psychosocial stress. Chemical stressors mentioned included air pollution, metals, PAHs, and solvents.

Technical challenges that were cited included improving analytical chemistry methods for characterizing mixture constituents and developing inexpensive lightweight sensors, which Paul Price discussed in his presentation. Another challenge described was the difficulty in getting support for basic research in mixtures. Although these problems will not be solved today, the more they are discussed, the better. More dose response data for individual chemicals, developing public databases that contain both single
chemical response data as well as interaction data that can be used by all, and developing databases also with meaningful data on network and pathway disruption of chemicals are called for.

When we break it down into keywords, we can see how respondents to the RFI brought up each of these key issues in their submissions. This matrix was useful in developing the topics for focus for Day 2 of this workshop. There was an attempt to capture a breadth of key topics identified through the RFI in the descriptions of the Day 2 focal topics.

**Topics for Day 2**

![Figure 125](image)

**Topic 1: Modeling Mixture Toxicity: The Constraints of Extrapolation**

For this topic (Figure 35), the issues surrounding mixture modeling were captured, including pharmacokinetic and pharmacodynamic (PKPD) considerations, some of the low dose issues, the need for individual dose response data, and potential statistical methods. In one of the scenarios, polycyclic aromatic hydrocarbons (PAHs) were mentioned specifically.
In the second topic (Figure 36), exposure characterization was captured and the range of topics included biomarkers, biosensors, susceptible populations, and how to prioritize chemicals for testing.
Topic 3: Epidemiology: Reconciling Epidemiological and Toxicological Approaches to Mixtures

The third topic was developed both from the RFI data and in discussions with the NIEHS Epidemiology Branch (Figure 37). Themes in this topic include cross-discipline communication, investigation of non-chemical stressors, difficulty in incorporating multiple stressors into epidemiology studies, prioritization methods, and recommended statistical methods.
In Topic 4 (Figure 38), the concepts of HTS and omics were combined with interactions because of the infinite number of possible chemical combinations; high-density approaches will be needed. Use of systems biology network approaches and consideration of moving through \textit{in vitro} to \textit{in vivo}, and QSAR are also covered under this topic.
Lastly, Topic 5 (Figure 39) provides a starting place to discuss temporal considerations in studying mixtures. Pharmacokinetic and pharmacodynamic considerations, biomarkers, and susceptible populations represent some of the key concepts included in this topic.
Mike DeVito (NIEHS DNTP): In listening to the presentations today, many issues discussed were very specific to individual fields. Tomorrow, the focus will be on similarities between different groups. One of the themes discussed today that cut across disciplines was the recognition that researchers will not be able to “test our way out of this” by dosing animals. In other words, there are too many potential mixtures to test and alternative approaches are needed. All of the groups mentioned high throughput technologies and/or alternative species because of the realization of the overwhelming number of single chemicals and possible mixtures. However, there was also concern about the linkage of those approaches to apical endpoints.

Another interesting theme that emerged from multiple presentations was the need for more individual exposure data that can be searched by researchers. Many groups discussed the need for databases that can be helpful in prioritizing: either exposure databases or toxicology databases. Additionally, there is a need to build bridges between exposure and toxicology to prioritize the type of mixtures and endpoints of concern.

There was also a theme of top-down versus bottom up approaches. In other words, it would be helpful to start from components of individual exposures and explore effects associated with those exposures, and then start from effects observed in different communities and identify associated exposures. Both approaches can contribute to the overarching goal of improving human health in populations by understanding environmental contributions to disease.

Lastly, a point that came out of the discussions today was that some kind of sufficient similarity methods are needed to begin to classify exposures and help with prioritization.

Linda Birnbaum (NIEHS): I am the director of NIEHS and NTP. I wanted to reflect on a couple of things that I heard. First of all, I want to reiterate what someone mentioned earlier: the questions asked are dependent on the end goal. It is very important to recognize that there is not necessarily one approach; the prescribed approach will depend on what is being asked.

Another thing is that multidisciplinary teams are absolutely essential. Complex mixtures are the reality. We can continue testing single compounds until forever, but that will not tell us about the reality of our human exposure. Also, mixtures are more than just the environmental chemicals and the drugs and naturally occurring compounds. They include the nutrients in your food and many other things.

Susceptibility is based on more than just differential exposure or differential timing of exposures; the inherent differences in susceptibility can play a role in the response to exposures. It was mentioned that we might not be ready to look at environment-wide association studies (EWAS). However, I do not think we have a choice. We must start looking more broadly. NHANES is a wonderful resource and allows us to ask some very specific questions. There are researchers currently developing mixtures for testing that resemble patterns observed in NHANES. However, there are many chemicals that are not measured through NHANES.
In other words, there is a tendency to measure chemicals that are known or of interest or for which analytical methods are available, but that does not provide a complete exposure picture. This is not a criticism of the current approaches, but it does highlight future possibilities.

Another point that is related to earlier comments by Ray Yang is the need to look at multiple mixtures and multiple physiological endpoints. From a public health point of view, it is important to ask: if we see an effect of a mixture on one health endpoint, do we really need to study other endpoints? This is a philosophical question about whether the multiplicity of effects associated with chemicals requires complete characterization or whether the identification of a single adverse effect is enough to warrant public health action.

This brings us to the issue of dose-response. I noted the use of the phrase “animal versus human dose” and would like to take this opportunity to express my frustration with the ongoing discussion about “high dose” animal studies. If an appropriate dose metric is used, which is usually some measure of internal dose, than you can compare across species without having to worry about converting the dose using complicated calculations. In order to actually perform a risk assessment, the administered dose to the animal must be related to the actual dose in the animal, because it is completely inappropriate to compare animals and humans on the basis of the exposure dose alone. Again, this highlights the importance of biomonitoring efforts and the need to get internal doses from animal studies for comparison.

Another point that I would like to make is that we should spend more time thinking about what we can learn from the ecological approaches, because ecologists are usually dealing with very complex mixtures and have been developing methods for dealing with them.

Those are just a couple of things I wanted to reflect upon based on the presentations from the breakout sessions. Thank you and I look forward to Day 2 of the workshop.

Kathleen Gilbert (University of Arkansas): I am really happy that we saw on the last slide that the concept of interactions actually came out on the top. I was missing it all day because I don’t think we are at the stage where we know single compound toxicology, and there is a lot of data about single compound experiments and toxic effects. Now we move into mixtures, and we know already we cannot measure all the constituents in mixtures.

But I think what we might want to do is to refine the mixture and see how the mixture affects the toxic response of the single compound, which is present in that mixture. Then, a mechanism-based grouping of compounds can be done. For example, if it is known that a mixture increases activity of cyp1A1, then I know all the compounds in that mixture that induce this one enzyme have to be adjusted with the factor as well. I also think we should correct the value. When we correct the mixture based on how it enhances or reduces the toxic effect of some compounds, we can access some of the older data and improve the risk assessment process.

Audience Member: Yes, I wanted to reinforce one of the discussion points that came out of the epidemiology breakout in relation to epidemiology and toxicology and how we can each help and reinforce each other in terms of the design and the questions that we are asking.
One of the questions that we, as epidemiologists, really need help with is what to do after we have gone out and measured a bunch of chemicals, whether they are PBDEs, phthalates, etc. These are classes of compounds where we may end up with 5, 10, or 15 parent chemicals and/or their metabolites. We need to get closer to understanding both the disease endpoints and the mechanisms by which these compounds get us to those disease end points. We need to develop assays that are mechanistically relevant and have toxicological testing that would then allow us to figure out how to combine the information on all of those individual compounds into a meaningful metric that might relate to anything ranging from estrogenic activity to mitochondrial disruption to thyroid hormones to whatever the functional mechanisms are that are really relevant in human pathophysiology.

I think the thing that I often see absent when I look at the toxicology literature is enough information on chemicals within a class that are evaluated in the same assay testing for the same mechanism so that there would be a whole panel of results. I think the toxic equivalency (TEQ) approach is a great idea, and we can apply it to many different kinds of toxicities and that could really help us in epidemiology.

Linda Birnbaum: I would like to suggest that we ask Marike Kolossa-Gehring, one of our participants, to discuss the state of in vitro and high throughput testing in Germany because the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) Program in the EU has practically banned animal studies, which have been replaced largely with in vitro assays. I think we, in the United States, could learn from the European experience in discussing how in vitro studies and alternative methodologies have worked thus far.

Marike Kolossa-Gehring: Under the new European chemical legislation, REACH, there was a shift from requiring in vivo data to in vitro data for the large majority of chemicals to be tested.

Before this European legislation, 28-day toxicity tests were required for all chemicals, including chemicals that were produced in minor amounts or for which no critical effects such as carcinogenicity or reproductive toxicity were known. Today, only in vitro data is required. The first point is that these in vitro data are difficult to interpret.

The second point is that the available in vitro methods are not very well characterized and in many cases, they have only been evaluated in a single lab. The biological relevance of these tests has not been properly investigated. For these reasons, in Germany, most toxicologists think that we need a paradigm shift back to more in vivo testing. If we do not know something about the biological relevance of a test, it is difficult to use for risk assessment, which is the ultimate purpose.

Mike DeVito: I will push back on that point. Say we have 10,000 chemicals. How many animals are you going to use in a year? When we think about the potential combinations of chemicals, the number gets even bigger. I think that eventually we are going to have to move to the HTS and in vitro approaches. I don’t think they are currently ready for prime time. I don’t think we are comfortable that we have covered all the biological space that needs to be covered.

I also think we have issues with in vitro to in vivo extrapolation as far as dose goes, but I don’t think dosing more animals is going to get us to a point where we are going to be able to deal with the large number of chemicals and the large number of possible exposures. There are limitations to these
approaches, but there are as many limitations to some of the animal approaches as there are to the in vitro. At some point, what was clear from the group presentations is that we need to build the appropriate linkages between these in vitro assays and apical endpoints.

Furthermore, if a particular in vitro assay does not link to in vivo, you need to rethink that in vitro assay. That is actually going to be where some of the challenges have been; some of the in vitro assays haven’t been as successful as we would like. That is my personal opinion and does not represent the views of NIEHS or others.

Raymond Yang (University of Colorado): Michael, the point is well taken and that’s the reason I think in our group, our number one issue was building in vitro to in vivo linkages. It is a data gap between in vivo-in vitro correlation or validation, not just from in vitro to in vivo animal studies, but all the way to human relevance. That is issue number one. Issue number two is the testing strategy. If you have 10,000 chemicals, you don’t do random testing. It would be far better if you have some sort of a goal and goal-oriented testing to fill important data gaps.

Mike DeVito: Agreed. I think the people who are actually doing the HTS work have thought about these issues. However, the issue of HTS has snowballed into whether we can do full-blown risk assessment with only in vitro data, and I think we are not at that point yet.

Moiz Mumtaz (ATSDR): The issue of how we can use in vitro data was discussed a lot in the risk assessment group. Perhaps assays that target certain points along a pathway could be used to give us a better idea of which chemicals disrupt that pathway. This would be a more targeted use of in vitro assays, instead of simply testing with every available assay and generating a lot of data. The point is that there might be something more specific that can be done with in vitro assays that is more goal-oriented.

Linda Teuschler (U.S. EPA): How much mixture testing is going on in the high throughput programs. How much whole mixture and component-based testing is involved?

Mike DeVito: To put my response in context: I work in the National Toxicology Program, where I participate in the Tox21 effort, and I am one of the co-chairs of the Targeted Testing Workgroup. Tox21 is a multi-agency partnership that is trying to develop high throughput testing methodologies. It includes EPA, NTP, NIEHS, the National Chemical Genomics Center (NCGC), and more recently, FDA.

A number of commercial formulations have been tested. The first phase of Tox21 involved assessing 1,408 chemicals. Tox21 is now entering the second phase, which will include about 10,000 chemicals. There are some mixtures being tested, but the mixtures included in Phase II of Tox21 represent an initial step. It is important to note that Phase I Tox21 testing really brought to light some of the challenges of high throughput testing for toxicology. For example, high throughput testing for toxicology is quite different than high throughput testing for pharmacological agents, for which these technologies were originally developed.

In using high throughput testing to screen mass numbers of structures for pharmacological activity, chemistry and dose-response are not of concern. A million chemicals are going to be screened to look to see which ones are active at 1 µM, and the ones that are active enter the next phase of testing. In other
words, a high rate of false negatives is acceptable, and the goal is to find a small number of strong positives. Concerns about chemistry (e.g., whether the dosed concentration is the concentration that reaches the target, or whether the individual test chemical is sticking to the plate) do not enter the picture until after the initial screening phase.

This contrasts sharply with the situation in toxicology, where there is a need to understand chemistry and dose-response. In toxicology, most environmental chemicals are on the weak end of the spectrum and we want to use high throughput screening as a first pass to pick up any possible positive signal. In other words, a high rate of false positives would be preferred to a high rate of false negatives. Additionally, it is important to know that the chemical that was put in the well is the chemical responsible for the signal (i.e., chemical stability is an issue). Finally, new methods of analysis are needed for high throughput toxicology data. Imagine doing 1,408 fifteen-point dose-response analyses. This cannot be accomplished by a chemical or by chemical approach, but requires automated analysis methods.

The challenges discussed above hold for testing mixtures in high throughput screening, as well. Currently, there are some targeted mixtures studies (i.e., “pilot studies”) to figure out what additional issues will arise from including multiple chemicals. Because this is a first attempt, there will be mistakes, but this will give us an opportunity to learn how to move forward.

One example from the pilot mixtures studies involves cytotoxicants. Based on data from Phase I, cytotoxicants were binned into 24 different categories. Based on the slopes of the dose-response curves and time course for cytotoxicity, chemicals that were found to be cytotoxic in Phase I could be grouped into 24 different sub-groups. A series of mixtures was designed using those cytotoxic chemicals. There were a total of 62 chemicals. There will be fifteen-point dose-response curves for each of the individual chemicals as well as each of the test mixtures. There are approximately 40 mixtures of cytotoxicants representing different combinations and ratios. The data from those mixtures will be collected as the plates are run through the different assays included in the Phase II suite of tests. At this time, I do not know how we will apply dose addition models automatically to that data to analyze it at a rate consistent with the rate at which we collect it. The pilot studies that are on-going will help to identify the issues. One of the major goals of the pilot studies is to determine whether or not the dose addition models that are available, that have worked in previous in vivo and in vitro studies, will work in a high throughput context for a large number of chemicals.

Our longer-term goal is to develop whole plates of mixtures for analysis using the high throughput approach. Each plate contains 1,408 wells, and each well has a fifteen-point dose response curve. We can make a lot of mixtures. For example, 100 different mixtures based on NHANES exposure patterns can be included and 100 mixtures of PAHs, and there would still be room on the plate for 1,208 mixtures. Moving forward, we would like to design mixtures for inclusion that are environmentally relevant and provide data to answer the most important questions possible.

Gail Prins (University of Illinois, Chicago): I would like to hear more about it. I had the pleasure of sitting at a workshop and Francis Collins was there. He announced a new initiative that they are funding in collaboration with Defense Advanced Research Projects Agency (DARPA) and FDA to build chips for high
throughput screening for drug toxicity. These could easily be applied to any toxic chemicals or toxic chemical mixtures. The idea is to have these biological chips of 3D cultures of different cell types in the body, and you could screen very rapidly to determine whether your drug, chemical, or mixture of interest might have adverse effects on any number of different systems.

Perhaps this could be applicable to people at this workshop; there will be funding opportunities out over the course of the next five years as they develop this program. It is a very interesting concept that could be applicable to the in vitro-in vivo bridge that's been discussed at this workshop.

Mike DeVito: The Tox21 community is aware of that approach, and we plan to learn from the successes that come out of that program. Drugs are very useful because they involve an intentional exposure with known doses and monitored toxicities. A couple of hundred “failed drugs” (i.e., drugs that did not meet safety standards in clinical trials) have been included in Phase II of the Tox21 testing program, and they will be used as truth sensors. Meaning, if they have clear toxicities and we know what the doses are in humans, then we can compare the known effects in humans to results from the high throughput screening battery.

Audience Member: I understand the validity of high throughput screening assays, but I have a lot of concerns about it. For one, you're using a cell line, I assume, instead of primary cells. Cell lines start throwing out pieces of chromosomes all over the place. When you get something that is a negative result for that one cell line, it does not mean it is not a toxicological issue for some other system.

Mike DeVito: That is correct.

Audience Member: Is there a way to deal with those issues, because HTS is very important and I think we should look at it. It is just a question of how it should be done.

Mike DeVito: Currently, we are in the second phase, and we are adding assays and technologies. The process is not done. We have not covered the complete biological space that we would like to occupy. For example, in a standard NTP bioassay, we examine 41 tissues. There are not even close to 41 cell types, let alone 41 tissues, included in the HTS battery. There are definitely some limitations.

Right now, we envision HTS as a prioritization and screening methodology. It is not a risk assessment effort. We appreciate some of the limitations of HTS, and what we really need is people to think about how to get around them.

We don't think the Tox21 members will be able to answer everything. We have tried to engage with the public, academia, and the private sector to figure out which assays would be useful and what approaches are available.

Rogelio Tornero-Velez (U.S. EPA): One of the things that struck me this afternoon in hearing all of the reports was that every group mentioned exposure in various ways and for me the lesson was that we cannot ignore that. As complicated as the toxicology is, we have to think of the exposure as well, which is every bit as important.
Mike DeVito: Agreed. That is what I heard in every group as well; the exposure is what we really need information on.

Scott Auerbach (University of Massachusetts Amherst): One of the things that comes to mind in this issue of interpreting in vitro data is that scientific cartography is needed. In other words, we need to use in vitro and genomics data, in which we look at multiple different biological contexts, along with high-dimensional data with chemicals that we actually know a tremendous amount about in terms of in vivo effects, so we can fill in the details of the biological space.

Then, when we have a new chemical or mixture, we can look at this multi-dimensional data and be able to put it into multivariate space and compare it to well-characterized chemicals. Right now, we are using a “pathway” view of biology that over-simplifies the biological networks.

If we more carefully map the molecular space as it relates to chemical responses, we will be able to put these new technologies (e.g., HTS) into context and be more comfortable with actually saying something about the responses measured through –omics and HTS approaches.

Mike DeVito: I’ve heard consistently the concern about linkage of the in vitro to in vivo, and I think there is a real consensus about this point.
Day 2 consisted of presentations that described tools or approaches that are relatively new and could contribute to integrating disciplines and advancing the field of mixtures research.

**INTRODUCTION TO DAY 2 – LINDA BIRNBAUM, Ph.D., DABT, ATS, DIRECTOR OF NIEHS**

Linda Birnbaum, Ph.D., has been involved in mixtures research since the 1980s and believes it is a priority area of research. She expressed her enthusiasm for the various types and level of expertise of the attendees. The cross-disciplinary and inter-disciplinary approach is the only path toward progress in studying more relevant exposures. We live in a world that involves exposure to chemical mixtures, not exposure to a single chemical at a time.

Real-world exposures, in fact, are even more complex because they include both chemical and nonchemical stressors. For example, consider some of the recent environmental disasters that have occurred, such as the Gulf Oil spill, massive flooding along the East Coast and other areas (the Hudson Valley and upstate New York and Vermont), and the earthquake in Japan that caused the Fukushima nuclear disaster. The resultant exposures are not only to various environmental chemicals, but also to infectious agents, mold, and more.

Regulators also realize that they have to manage risk associated with real-world mixed exposures. For example, EPA is working on developing a relative potency factor approach for PAHs, which builds upon the well-known dioxin-like chemical potency factor approach. EPA is also using a cumulative risk assessment approach for evaluating mixtures of similar-acting pesticides including organophosphates, methylcarbamates, and pyrethroids. Additionally, we should consider that chemicals rarely have one mode of action, and they also may have different modes of actions in different tissues. In 2008, the National Academies of Science (NAS) recommended a cumulative risk assessment approach for phthalates, which focused on grouping chemicals according to a common target (i.e., androgen-sensitive endpoints), not strictly a similar mechanism of action, which is often difficult to identify.

There has been a great deal of activity in the area of mixtures this year, and our workshop is just one of a number of conferences on this topic. In February 2011, EPA held a multi-pollutant risk analysis workshop related to the National Ambient Air Quality Standards (NAAQS) Review. In March 2011, SOT held three sessions dealing with mixtures work at their annual meeting: a continuing education course, a workshop discussing the incorporation of non-chemical stressors into cumulative risk assessments, and a symposium on mixtures and reproductive toxicity. In July, NIEHS and other federal partners sponsored an NAS Emerging Science Committee Workshop entitled “Mixtures and Cumulative Risk and Looking at
New Approaches Using the Latest Science and Thinking about Pathways.” Lastly, there will be an International Toxicology of Mixtures Conference in October 2011 in Arlington, Virginia, sponsored by Elsevier.

The unique aspect of the NIEHS Mixtures Workshop is its multidisciplinary breakout sessions focusing on key topics in mixtures research. NIEHS has a long history of funding research in the area of mixtures. The first NIEHS dedicated mixtures grants were funded in 1998 as a response to the Request for Applications (RFA) titled “Chemical Mixtures in Environmental Health,” led by Claudia Thompson, Ph.D. Another avenue for mixtures research has been the Superfund Research Program (SRP), which continues to support research related to Superfund sites. Several of our 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 are shown in Figure 40. As you can see, these studies assess a variety of mixtures, some of which are complex. In fact, these mixtures studied by our 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.

NIEHS’ intramural research efforts have included investigation of the Deepwater Horizon Oil Spill. NIEHS staff responded immediately by providing emergency response and worker training for the cleanup. Two months after the oil spill, NIH Director Francis Collins, Ph.D., announced before Congress that he was going to give NIEHS $10 million to study the long-term health effects in people who had been exposed to the Deepwater Horizon Oil Spill. Additionally, we recruited additional funds to facilitate these efforts. NIEHS partnered with academic institutions and government agencies to conduct a study, for at least five years, but with the goal of a 20-year study ($36 million) in mind. This study is evaluating the health effects, including biochemical and physiological effects from exposure to chemical and nonchemical stressors. This study will create a platform for biomedical specimen collection that will be available for many other researchers, who can collaborate with Dale Sandler, Ph.D. (NIEHS Study Principal Investigator) on related projects.

The effects from oil spills are of great interest, as there have been over 40 oil spills over the last 20 years. However, the long-term health effects of oil spills are largely unknown because there has been inadequate follow-up in previous oil spill studies, which have only followed-up for up to 2 years. For example, in the two-year study of a spill off the coast of Spain, the Prestige oil spill, those workers
continued to exhibit respiratory effects, but also had persistent chromosomal changes. That study has provided us with some insight into the long-term health effects from this spill, which can be assessed in the current NIEHS study. Furthermore, the NIEHS study is unique in that it will follow exposed individuals for up to 5 years and potentially beyond.

NIEHS, along with eight other NIH institutes, is also funding an extramural program focusing on research related to the oil spill through a U19 grant mechanism led by 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 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.

The DNTP has a long history of engaging in mixtures research. Nearly 20 years before Linda Birnbaum became director of NIEHS, she was an investigator in the DNTP and evaluated the additive, synergistic, and antagonistic behavior of dioxins and PCBs with regards to development of cleft palate in animal models. Much of that led to the development of the toxic equivalency factor (TEF) approach, which was tested very elegantly by DNTP starting in the mid-1990s. The reports were first published in 2004 and 2005 showing that the TEF approach not only worked for endpoints like enzyme induction and various short-term effects, but also worked very well for carcinogenesis.

The DNTP also has a program focusing on complex mixtures, such as the whole mixtures testing of herbals and flame-retardants. The flame-retardant commercial formulation FF1 was the firemaster product involved in the 1974 Michigan polychlorinated biphenyl (PCB) disaster, the effects of which continue to be evaluated by Michelle Marcus, Ph.D., and co-workers. Other examples of mixtures explored by DNTP include marine diesel fuels and welding fumes. Regarding defined mixtures, Ray Yang, a former DNTP investigator (currently at Colorado State University), led an effort in the 1980s through the early 1990s assessing a 25-chemical mixture that contained relative proportions of contaminants measured in groundwater samples. Also, June Dunnick, Ph.D., led studies of AIDS drugs in combination therapies.

Another area of NIEHS interest is in the development and use of new tools and computational approaches for mixtures toxicology. This effort was stimulated by the DNTP roadmap led by Chris Portier, Ph.D., in 2004, and was strengthened by the NAS report on “Toxicity Testing in the 21st Century.” Much of that report discussed high throughput screening and various “omics” approaches. Tools such as these can be used to increase our understanding of gene-environment interactions. For example, at this workshop, Chirag Patel, Ph.D., discussed environment-wide association studies (EWAS) (see page 109), which could merge with genome-wide association studies (GWAS). These types of tools can also be used in exposure science and development of the exposome.
The NAS report provided a roadmap for a conceptual shift towards rapid automated assays for activation of toxicity pathways (Figure 41). These were defined as cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects. It is important to note that toxicity pathways are the same as disease pathways, and these concepts should be merged. Key goals are to understand cellular changes throughout the pathway, develop appropriate pathway biomarkers, and relate pathway changes to disease outcomes.

The NAS report stimulated the development of the Tox21 collaboration, which is a partnership between NIH (NIEHS/DNTP, NHGRI, and NCGC), EPA, and FDA. Part of the Tox21 effort will also involve the new NIH National Center for Advancing Translational Sciences. The goal of this effort is to develop a high throughput in vitro assay system capable of screening many thousands of chemicals for toxicity. This effort was motivated by the large number of chemicals in use today. The majority of these chemicals have had inadequate testing, due to limited resources and inconsistent testing requirements. The Tox21 testing platform can be used to test the vast number of chemicals in the environment, as well as potential mixtures including environmental contaminants combined with pharmaceuticals, nutrients, or naturally occurring chemicals. One advantage of testing thousands of chemicals in a battery of in vitro assays is the ability to observe patterns of effect. This is beginning to be done using the first wave of chemicals, which were selected based on the availability of toxicity data in animals and humans. By comparing results from the in vitro assays with the toxicity data, we can begin to associate certain phenotypes with the patterns of in vitro responses.

Another promising area is in “omics” technologies and accompanying data analysis approaches, which should continue to be utilized in an effort to make sense of the many chemicals to which we are exposed. In addition to genomics, proteomics and metabolomics, there are a number of emerging “omics” technologies, such as
lipomics, exposomics, etc. These “omics” technologies will require parallel advances in bioinformatics in order to effectively utilize the resulting large datasets. User-friendly and searchable databases will also need to be developed.

The NIH roadmap project on genes, environment, and health (Figure 42) 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 pleasure and 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 dietary intake, physical activity, and psycho-social stress. Some of the 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 cross-sectional. There is a need for ongoing longitudinal prospective studies to collect samples at different time points 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 an exemplary ongoing California Biomonitoring Program. The NIEHS Children’s Environmental Health Centers, co-funded with EPA, have several children’s cohorts for which subjects are followed from before 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 (Figure 43), as described by Steve Rappaport, Ph.D., and Martyn Smith, Ph.D., provides a characterization of total exposure and metabolomic analysis (Rappaport and Smith 2010). This perspective takes advantage of new approaches, including chemical analysis and metabolomic tools. The exposome holds promise for contributing to our understanding of the relationship between total exposure and disease and the development of biomarkers.

The field of mixtures research is moving forward. Previously, there was a focus in both toxicology and risk assessment of one chemical and one source. This has evolved to include chemical classes. However, in the future, we need to be evaluating all routes, all sources, and all chemicals. In addition, we should consider the interaction of environmental chemicals with internal biomolecules (including the microbiome), nutrition, pharmaceuticals, and mediators of non-chemical stress. An interdisciplinary approach will be required to move the field forward and to address these pressing issues.
Paige Tolbert, Ph.D., is a leader in the field of environmental epidemiology and currently serves as the Co-Director for the EPA-funded Southeastern Center for Air Pollution and Epidemiology, which is a multi-institutional, cross-disciplinary center dedicated to characterizing complex ambient air pollution mixtures and exploring the effect of these mixtures on public health. She is Professor and Chair of the Department of Environmental Health at the Rollins School of Public Health at Emory University and holds a joint appointment in the Department of Epidemiology.

This presentation will provide the epidemiologic perspective on a path forward in developing strategies to advance multi-pollutant epidemiologic research in the context of air pollution. Air pollution presents a classic case of complex mixtures. There are multiple sources and multiple transformation processes that result in very dynamic mixtures with multiple potential descriptors. Even components of the mixtures are mixtures themselves, e.g., particulate matter (PM) 2.5 (particulate matter less than 2.5 microns in diameter) and organic carbon compounds are generic descriptors for complex mixtures.

Figure 44 is a photo of a view in China showing an example of extreme air pollution. Figure 45 presents a view of Atlanta, which is more similar to other cities in the United States. We are comparing the relationship of health outcomes on days that look like the left side of the photo to days that look like the right side of the same photo.

The U.S. EPA recently named Emory/Georgia Tech Southeastern Center for Air Pollution and Epidemiology (SCAPE) as one of the four new Clean Air Research Centers, which builds on a history of NIEHS-supported studies. The focus of the center is on mixtures. During the five–year funding period, progress will be made on the kinds of issues being discussed at this workshop.

The center has several different projects. A measurement project is assessing reactive oxygen species (ROS) and other oxidants and using those markers for both measured and modeled input into several on-going health studies. For example, one study focuses on health effects in commuters and includes intensive measurements of vehicle exposures. Two other health studies include longitudinal studies with birth cohorts that will be examining relationships of air pollution, birth outcomes, and asthma development and exacerbation. Finally, a multi-city study is assessing air quality and acute health outcomes in a number of cities across a broad region of the United States.

All of these studies have hundreds of analytes available for epidemiologic analysis. We are using the Air Quality Core of SCAPE to develop a number of fused inputs that will be a combination of the modeled
data and monitoring data. We are also using satellite data, a very broad variety of air quality characterization methods, and a Biostatistics Core to extend epidemiological methods. We are also borrowing from other fields such as genetics and applying other types of statistical approaches that have been developed for high-dimensionality data (Figure 46).

SCAPE is approaching mixture characterization from several perspectives: 1) an evidence-based perspective with a focus on vehicular emissions and roadway impacts to determine the major role of traffic on health endpoints; 2) a biologically-based perspective, in which we are evaluating ROS and other oxidants as a group with potential biological activity relevant to oxidative stress; 3) an environmental management perspective using state-of-the-art methods for source apportionment to better understand roles of groups of agents that are co-emitted from specific sources and their transformation products; and 4) an empirical perspective in which we are using database approaches to sort species and group them according to their associations with health endpoints of interest to the center.

We are using a number of different approaches in the center to address questions regarding the air pollution mixtures. The goals in multi-pollutant research will be discussed in the context of air pollution, but these goals are generalizable to other contexts, such as water pollution and other mixed exposures.

A broad range of goals for multi-pollutant research have been expressed by epidemiologic investigators (Figure 47). Goals range from estimating the total health effects associated with exposure to multiple pollutants (i.e., the joint effect of the combination), as described by Francesca Dominici (Dominici et al. 2010), to modeling complex air pollution mixture effects more explicitly to gain better insight into the features of an air pollution mixture that are most toxic, as described by Sverre Vedal and Joel Kaufman (2011). Goals cover a broad range. An example at one end is the work of Francesca Dominici (2010) who has been estimating the total health effects associated with exposure to multiple pollutants (i.e., the
joint effect of the combination) (Dominici et al. 2010). An example at the other end is work by Perry Vedal and Joel Kaufman (2011) describing modeling complex air pollution mixture effects more explicitly to gain better insight into the features of an air pollution mixture that are most toxic (Vedal and Kaufman 2011). This second goal is more “deconstructionist,” taking apart the joint effects to try to understand what the specific component effects are.

To advance the field, the conceptual issues underlying the mixtures research paradigm must be clear. The statistical issues flow from clarification of the conceptual issues. Once the conceptual side is clear, implementation is the next hurdle. These issues associated with performing multi-pollutant research will be presented briefly. A more in-depth discussion of these conceptual issues, including co-variation, interaction, joint effects, and disentangling the effects, is the topic of a manuscript by my colleague, Mitch Klein (in preparation).

Covariation

Many pollutants co-vary temporally and spatially because they are co-emitted or because of common atmospheric processes, and it is because of this that multi-pollutant models historically have been fit to air pollution data. Typically, when the multi-pollutant models have been used, it has been to estimate the effects of a single pollutant controlling for the effects of other pollutants.

As an example, our Atlanta studies compared a single-pollutant and multi-pollutant model for respiratory emergency department (ED) visits (Figure 48). On the left panel are the single pollutant model results for some of the classic air pollutants. The results show that there are significant associations for ozone, NO$_2$, carbon monoxide, and PM10 with ED visits. The question is whether any of these associations are real or are the artifactual result of co-variation of the pollutants with each other. Furthermore, when we run a multi-pollutant model and address that question of confounding across the pollutants, we observe that ozone and PM10 remain significant, while NO$_2$ and CO go to the null. These results suggest that NO$_2$ and CO were confounded by their correlation with ozone and PM10.
In applying these kinds of models, problems can arise with overlaid correlated data leading to a decrease in the power to detect associations. In general, this is not a major problem in these kinds of studies. A more important problem, in terms of interpreting these models, is differing levels of measuring error across pollutants that are being compared directly. This can be a problem even in single pollutant models, but in multi-pollutant models with direct comparisons across the pollutants, differing levels of measurement error can lead to the possibility of misleading results. Another related issue is that one pollutant, or a combination of pollutants, can act as a surrogate for a more biologically important pollutant that happens to be either poorly measured or not measured at all.

Again, using data from Atlanta, we examined cardiovascular disease ED visits (Figure 49). We compared single pollutant results (left panel) with multi-pollutant results (right panel). When a multi-pollutant model of the three input variables that were significant in the single pollutant models was used, NO₂ and total carbon go to the null and CO has an increased association. However, CO is probably operating as a surrogate for traffic emissions, including total carbon, which is potentially less well-measured than CO in this example, and other unmeasured from traffic. This example demonstrates complications associated with applying these multi-pollutant models.
A second approach to dealing with co-variation is source apportionment models in which we group co-emissions from a common source. This can either be a step forward or backward. It is helpful for environmental management purposes, and for identifying sources that can be controlled and have an impact on health. It helps with dimension reduction in our models and leads to less correlated input variables, but it adds a layer of uncertainty into the modeling process that is often difficult to quantify. Any toxic agent could be present in multiple sources, and if one source is associated with a particular outcome of interest, we will want to know how and why that source has that association.

An example of this approach is shown in the graph below (Figure 50), also using data from Atlanta. We initially found an association between cardiovascular disease ED visits and PM2.5, which was slightly positive, but did not quite achieve significance. To evaluate this health effect, we used two different receptor modeling approaches to perform source apportionment analyses: the Prioritization Matrix Factor (PMF) and the Chemical Mass Balance (CMB)-based approaches. Relative risk for cardiovascular ED visits was then estimated for each of the identified sources. Results show a signal coming from diesel, gas, and biomass using each of the source apportionment methods. These results show that it is helpful to include source apportionment analyses, and also provide a potential mechanism for prioritizing specific source mixtures for further study.
Interaction

Interactions among pollutants represent another important consideration. Knowledge of single pollutant effects, even with multi-pollutant modeling, may not be sufficient to predict the effects of the combination. Epidemiologists generally define “interactions” as statistical interactions and effect measure modifications. Therefore, the definition of interactions is model-dependent and may reflect biological or chemical interactions. Based on some of the discussions during the Day 1 Epidemiology breakout session, it is clear that there are some differences in how the toxicologic and epidemiologic communities define “interactions.” It is possible that the concept of “dose additivity” in toxicology could be consistent with the epidemiological approach for modeling an interaction.

It is important to keep in mind that the interactions do not necessarily need to be between simultaneous exposures. They can be non-contemporaneous; for instance, when there is evidence that ozone may potentiate the effects of particulate matter.

A primary challenge in assessing interactions is that we are splicing the data into smaller pieces, and we ultimately have major power constraints in being able to identify interactions. The potential numbers of combinations can become unmanageable moving from two-way and three-way combinations up to “n”-way. Generally, going beyond three-way combinations has not been productive, but regardless, there is a multiple comparisons problem that emerges from the number of potential combinations. It is very important to develop approaches for prioritizing the interactions that are assessed.

This prioritization process should involve consideration of actual co-occurrence of mixture combinations and biological plausibility. It is an area that presents an opportunity for a lot of interaction between epidemiologists, toxicologists, systems biologists, and others. Getting input from the high throughput screening is going to be very helpful in predicting interactions in cases that involve hundreds of potential
analytes. The idea of developing a reference library with interaction data, which was discussed in Day 1 of this workshop, could be very helpful in the prioritization process.

Lastly, there are a number of biostatistical methods that can be brought to bear on the high-dimensionality problem that can be used for dimension reduction and model stabilization. There are a number of techniques that are being applied now in this context, such as LASSO and ridge regression, and additional techniques are under development.

**Joint Effects**

Another important goal in environmental epidemiology is in determining the total effect of the mixture (i.e., issues associated with joint effects). Joint effects can be approached from a number of different directions. One approach is a “bottom-up” approach that uses multi-pollutant models where a number of different components are fit to the model, the parameter estimates for those components are summed, and then the sum is exponentiated to get the relative risk for the simultaneous increase of one unit of each pollutant. This approach offers one way to provide a description of the risk across multiple pollutants. Interaction terms can be included in these kinds of models, but it is very important to choose appropriate units because the units determine the weights given to the individual components and their impact on total risk. This approach can become unmanageable if there are hundreds of potential analytes in the complex mixture.

At the other end of the spectrum, another approach uses the “one atmosphere” paradigm to estimate risk associated with unit increments of the mixture. However, this approach does not provide information on which component is driving the association with the mixture overall and how the risk varies by composition of the mixture.

**Disentangling Effects**

The final issue to be discussed is one of the underlying purposes of multi-pollutant modeling, which is to disentangle the effects and to deconstruct from the joint effect which components, or groups of components (i.e., sub-mixtures) are driving observed associations with the total mixture.

The critical question is how to group components. It is important to note that there are some constraints on chemical grouping based on how components are measured (e.g., some air pollutants are routinely measured as a group, such as PM2.5 and ultra-fine particulates). Choices available for grouping chemicals include source apportionment (as already discussed), mode of action, or other mechanistically relevant properties. For example, ROS is one group definition that we are using in SCAPE. We are using methods including empirically based techniques, principal components analysis, random forest and supervised and unsupervised clustering, which are emerging as useful tools. The supervision can involve prior knowledge of the health outcome.

**Conclusion**

These kinds of approaches are ultimately providing the means for more closely reflecting the true complexity of the epidemiologic picture involved in real world exposures. As stated previously, clarity regarding the concepts in multi-pollutant epidemiology is critical. Additionally, thoughtful approaches
are required to minimize the vulnerability of multi-pollutant studies to common issues associated with mixtures research, such as concerns about data mining and multiple comparisons as well as differential measurement errors.
MIXTURE EFFECTS AND EPIDEMIOLOGY: THE NEED FOR UNBIASED APPROACHES TO EXPOSURE ASSESSMENT – ANDREAS KORTENKAMP, Ph.D., UNIVERSITY OF LONDON

Andreas Kortenkamp, Ph.D., is a renowned expert in mixtures research and is a lead author on the “State of the Art Report on Mixture Toxicity” (2009). He is a Professor in Human Toxicology at Brunel University in London. His research focuses on exploring the effects of multi-component mixtures of endocrine-active substances. His work assesses whether the effects of mixtures of chemicals can be predicted quantitatively on the basis of information about their individual toxicity.

There has been enormous progress in the mixtures area with a couple of key findings. First of all, we now know that mixture effects are predictable when the toxicity of components is known. Secondly, there have been many studies exploring a dose range at the low effect levels of the individual components. The general conclusion from this seems to be that dose addition is an extremely powerful tool for approximating mixture effects.

The term “approximating” is appropriate because predictions from dose addition are not always entirely correct, but they do provide a good approximation. The striking feature is that there are very few examples in the literature where the competing model of response addition (also known as independent action) actually provides as accurate an approximation as dose addition. Yesterday there was a presentation that showed that response addition consistently underestimates the joint effect of mixtures (see L.E. Gray section of this document), and that is true for many other mixtures as well.

Taking these findings into consideration, there are several important questions that arise. First, are the acceptable daily intakes (ADI) or tolerable daily intakes (TDI) used in the traditional single chemical risk assessment approach actually sufficiently protective? Secondly, are exposures defined? In effect, do we have a complete picture of exposures that occur to human populations? Lastly, what should be done about the large number of chemicals that are untested and for which we have no toxicity information whatsoever?

The overarching question is: when is a mixture safe? The answers to that question depend on whether we are using a model of dose addition or response addition to predict mixture toxicity. The formula for dose addition is fairly simple mathematically (Figure 51), and the terms (Intake1 over Tolerable Daily Intake1) are familiar to everyone who does single chemical risk assessment. When this quotient approaches one for an individual chemical, there is concern for potential negative health effects in humans.

For dose addition, an additional assumption is made that the sum risk of all the individual chemical components of the mixture has to be less than one (< 1) in order to be considered protective of human health. Using that information, it is possible to determine when the joint effect of the mixture is similar to the effect probability associated with an individual chemical.
tolerable daily intake. That is the case when every component is present at its individual Tolerable Daily Intake (TDI) divided by “n,” where “n” is the number of components in the mixture. It then becomes clear that the question reduces to how many mixture components are present that contribute to the effect under consideration. This is a critical question.

When the concept of independent action is used to predict the toxicity of a mixture, we actually arrive at a very similar answer to that described above with dose addition, only by a different path (Figure 52). According to the formula for independent action, the predicted joint effect is zero when all of the chemicals in the mixture have a zero effect. It is important to note that the zero effect level must be literally mathematically zero. For example, if 100 chemicals all display a 1% effect, the joint predictive effect is already 63% and if they display a 0.1% effect, the joint predictive effect is close to 10%. Therefore, under the model of independent action, in order to be protective of human health, the ADIs should have zero effect levels. Only in that case is the joint predicted effect with independent action actually zero. However, is there certainty that the ADIs are always equal to a zero effect? The risk assessment dogma assumes that they are always equal to a zero effect, but considering all of the steps involved in calculating ADIs, this is doubtful. It is likely that for some number of chemicals the ADI is not equal to zero effect.

The issue of non-zero ADI values mentioned above is one complication in using the independent action model to predict mixture effects. Another potential issue can be found in the data limitations that are frequently present when performing cumulative risk assessments. We have conducted an exercise where the hazard index approach was used to assess risk associated with anti-androgens. In order to include chemicals in this risk assessment, they had to meet certain demands. For example, there was a requirement for availability of both in vivo evidence of anti-androgenicity and exposure information. Surprisingly, these two straightforward criteria already drastically reduced the number of chemicals that could be considered for a cumulative risk assessment for anti-androgens. The chemicals that had available data and were considered in the risk assessment included: six phthalates, six pesticides, brominated flame retardants, DDE, bisphenol A, and parabens (Kortenkamp and Faust 2010). On the basis of that analysis, it became obvious that seven chemicals explained more than 90% of an expected combination effect (Kortenkamp and Faust 2010). Parallels can be drawn between this exercise and the maximum cumulative ratio methods described by Paul Price (see page 22). However, it is important to remember that the cumulative risk assessment described above has been carried out in the context of the severe restrictions in available data.

The example just described dealt with “known knowns,” but in the study of real-world mixtures there are many unknowns. It would be interesting to see what the results of the risk assessment would have been with a wider range of chemicals for which biological activity and exposure data may or may not be
available. This inclusion of a wider set of chemicals sets the scene for epidemiological work, which has similar data restrictions.

Lopez-Cervantes et al. (2004) conducted a meta-analysis to ask, in a case control study format, if organochlorines were associated with breast cancer risk (Figure 53) (Lopez-Cervantes et al. 2004). They concluded that there was no association, but they only evaluated \( p,p' \)-DDE. The reason for limiting the evaluation to only \( p,p' \)-DDE is not clear. It appears that the selection of \( p,p' \)-DDE for analysis was not driven by a hypothesis about the biology of breast cancer, but by the availability of analytical chemistry techniques. In effect, \( p,p' \)-DDE is very easy to analyze. Analytical methods for other interesting chemicals in this context were simply not available. Therefore, the authors concluded that the results should be regarded as strong evidence any relationship between \( p,p' \)-DDE and breast cancer risk should be discounted (Lopez-Cervantes et al. 2004). However, this conclusion provides an incomplete answer because it focuses specifically on one compound. The same type of example can be found in a paper by Bhatia et al. (Bhatia et al. 2005) regarding organochlorines (i.e., DDT and DDE) and their ability to cause hypospadias and cryptorchidism. This study did not support an association of DDT or DDE with hypospadias or cryptorchidism.

This type of epidemiological analysis inspired Steve Rappaport to write the following in a paper, which appeared in *Toxicology Letters* (Rappaport et al. 2012):

“However, with few exceptions, the identities of major environmental toxicants and their role in causing chronic diseases have not been addressed. Given the poor state of knowledge about health-impairing environmental exposures, epidemiologists pursue narrow hypotheses that largely skirt disease etiology [sic] in favor of known environmental risk factors, even when the attributable risks are small. Although such hypothesis-driven studies confirm some environmental sources of disease, they offer only fragments to our understanding of the major causes of mechanisms of chronic diseases.”

That is the background against which Rappaport and others advocate what is called the “Exposome: an Unbiased Approach to Exposure Assessment.”

In terms of assessing mixtures in epidemiology, there are two potential ways of dealing with the issue of which chemicals to focus upon. First, in designing cohort studies, it is just as important to study the health impact of combinations of chemicals, as it is to work from chemical to effect, such as in the current practice of epidemiological studies. In case control study designs, typically the impact of chemical exposures on diseases is assessed to determine whether chemical exposures are indeed risk factors for certain disease processes. However, there must also be an effort to work backward from the disease to the chemicals.
An example (Figure 69, left panel) can be found in a study that was discussed in Dr. Christiani’s talk at this current workshop (see page 41) (Froehlich et al. 2009). In this study, joint effects between prenatal tobacco exposure and lead were assessed. The joint effect (highlighted by red arrow) is larger than any of the individual risks attributable to the either of the two components.

This is analogous to experimental approaches in mixture toxicology where they examined liver foci induced by different carcinogens (Figure 54, right panel) (Futakuchi et al. 1996). Each of the individual chemicals produces a small effect, and the joint effect of the mixture is bigger than the individual effects of each of the components. On the basis of this difference between individual effects and mixture effects, the authors concluded that the mixture elicited synergistic effects. However, this does not necessarily indicate synergism. In order to determine whether synergy was present, more data would be required. Perhaps the term “synergism” is used in the original meaning of the Greek word, which is working together. Indeed, in both examples it is clear that the chemicals do work together (i.e., are not acting independently). The data provided does not allow for discrimination between additivity or synergy.

It is only rarely possible to isolate the individual contribution to risk of individual exposures as in this example by Froehlich et al. with smoking and lead (Froehlich et al. 2009). It is essential to know the individual exposure contribution to the risk before the combination effects can be understood. In most cases, especially with multi-component exposures, this information is simply not available and will not be available.

From a multi-component mixture perspective, it may be more feasible to look at the case-controlled led study design where investigators work from a disease to the potential chemicals that may contribute to the disease. Therefore, researchers can measure multiple chemicals, and this has been practiced in various case-control studies. However, the question arises: which chemicals should be measured and what is the driving hypothesis? For example, in the breast cancer context, it would make sense to measure chemicals with known estrogenic activity.

Suppose a large number of chemicals have been measured in the case-controlled study design. The question of how to handle these data arises. The simplest approach would be to add all of these individual measured concentrations (e.g., $M_1 + M_2 + M_3 + M_4$). However, the major flaw in this approach is that that the potency of individual chemicals is not taken into account, so this is not a valid method for aggregation. (Implicitly, the method of summing up concentrations or doses assumes that all mixture components have equal potency, which is very unrealistic.)
In principle, a better way of aggregating would be to introduce weighting factors (e.g., \( W_1M_1 + W_2M_2 + W_3M_3 + W_4M_4 \)) to account for the individual chemical potencies. The weighting factors could be tailored to each individual measure, which is mathematically equivalent to the dose addition formula or the hazard index equation.

This approach could be a step in the right direction to introduce the mixture approach used in toxicology into epidemiology by somehow aggregating information about prevalence, exposure estimates, measured level, and information that is available about potency. However, in the case of epidemiology, the method for estimating mixture effects is not clear. In the hazard index formula (Figure 55), the numerator contains exposure levels and an acceptable level is defined. These ratios calculated for individual chemicals are then added to get the hazard index for the mixture. Acceptable levels for single chemicals could be defined as the NOAEL divided by uncertainty factors. In order to get an accurate estimate, the total number of chemicals must be known, but it is possible to begin by incorporating those chemicals for which information is available. One approach for translating this approach to epidemiology would be to correlate the sum of hazard quotients with health outcomes in the case control study format.

This represents a data-rich approach that requires a lot of input data. However, exposure levels are needed, and these can be calculated from measured concentrations in tissues. We also require good toxicokinetic models. For example, for phthalate levels, a toxicokinetic model exists and data is available on the concentrations in human urine.

Another issue in epidemiological studies is the derivation of potency estimates. They would have to come from in vivo animal studies that have relevant endpoints. Phthalates are a good example of this because the reproductive toxicity endpoints measured in animal models are relevant to humans. The advantage of this approach is it is also easily compatible with the regulatory risk assessment, such that values calculated for risk assessment purposes such as reference doses could be used in epidemiology. The disadvantage is the paucity of data. Currently, this approach is only possible with a very small number of chemicals.

The alternative approach for deriving potency factors would be to use concentrations measured in human samples to derive potency factors from in vitro assays (Figure 56). There is much data available for measured tissue concentrations. In order to connect dose-metrics between tissue concentrations and in vitro effects, the acceptable levels (i.e., ADIs) would have to be effective concentrations in in vitro assays. Again, information about the total number of chemicals and the components in a mixture would be needed, and the potency estimates would have to come from
suitable *in vitro* assays. An example would be *in vitro* assays to evaluate androgen receptor antagonists and estrogen receptor agonists. The advantage is that this approach is easy to apply. The disadvantage is that the extrapolation from the *in vitro* assay to the disease outcome in humans must be validated, which is a challenge for many *in vitro* assays.

An example that used this approach is a study from Ibarluzea et al. (2004) where they looked at breast cancer risks (Figure 57) (Ibarluzea Jm et al. 2004). They measured 16 individual organochlorines in adipose tissue and observed no differences between cases and controls. Then, they measured the total estrogenicity of the organochlorines in adipose tissue using an *in vitro* assay, especially in lean women, and they observed that for the highest quartile there was a substantial odds ratio for breast cancer (shown in bold). The analogy for this type of epidemiological approach would be whole mixture testing approaches in experimental toxicology where e.g., the effects of the entirety of substances in sewage effluents are measured, as if this complex mixture were one single compound. Next, the estrogenic mixture present in adipose tissue would need to be deconstructed in order to identify the components responsible for eliciting the estrogenic signal in this *in vitro* screening assay. This is not a trivial undertaking.

In order to make progress, collaboration between various disciplines is required. Returning to the theme of an unbiased approach to exposure assessment, it is important to ask whether the full spectrum of estrogenic agents in human adipose tissues is known. This is not likely to be the case. Therefore, a concerted effort will have to be made to extract mixtures from tissue samples, determine the components within the mixtures, investigate the components for biological activity with relevant *in vitro* assays, and characterize which components/fractions show activity. This is a major collaborative research effort. This approach offers a path forward for better epidemiology and toxicology studies and also better risk estimates. Lastly, this would enable the unbiased investigation of a wider range of environmental contaminants in tissues.

**Discussion**

Audience Member: I think that it is really nice to have a very fresh perspective on some of these questions and this really highlights the need for toxicologists and epidemiologists to work together and get some sort of common ground in terms of the terminology and concepts and what we are all trying to achieve. This perspective will be helpful in trying to iron out some of the issues that you have described to have additional discussions among toxicologists and epidemiologists. For example, it sounded to me like you were really suggesting that the case control methodology would be preferable to other epidemiologic approaches.

Andreas Kortenkamp: These were just thoughts that flowed out of my primitive mind.
Audience Member: I would say your primitive mind provides a lot of good, new ideas. It will also be helpful, I think, to discuss some of the issues involved. There are a number of concerns with case control studies in terms of understanding the longitudinal exposure history and the possibility for the disease itself to affect those exposure levels that you are measuring. I think you are right, that there are a number of good potential ways to get at this. We really have to fully understand the toxicokinetics of each of the components that you are measuring.

For instance, the whole exposome approach is a very exciting, interesting area that, with collaboration and input from all the different communities including epidemiologists, could eventually have a lot to offer. But there are these enormous practical issues to get past.

Andreas Kortenkamp: Yes. I agree with you. I think this is not done just in a Wednesday afternoon. I think it will take a concerted effort of five years maybe at the very least. But perhaps then we are there. I really believe we need unbiased approaches to exposure assessment.

Audience Member: I have a quick question, if you could clarify something. I don't know a lot about the exposome approach, but it seems like there is this tension between the “let’s look at everything” approach that people are really exposed to in an unbiased way, and having some kind of hypothesis-based approach to choosing the chemicals that you focus on in any given study. I wonder how you resolve that tension.

Andreas Kortenkamp: I am attracted by Paul Price’s ideas. As usual, in this area, aquatic toxicology and ecotoxicology are ahead of human toxicology. There are, in Europe at least, hundreds of chemicals that can be found in surface waters, but if you then approach this with the question of, how many of them are really potent enough to kill daphnia? You quickly find that only a dozen of them really count. The other chemicals are there, but either the potency is too low or the level at which they are present is too low. They do not contribute. This is what I call the “Dirty Dozen” hypothesis of mixture toxicology.

Aquatic toxicology has pointed in this way. All of the available evidence currently points in the direction that it also may be true for human exposure scenarios, although, I am very uncomfortable with the fact that this analysis is biased. We need unbiased approaches to confirm or refute that, but I think in principle, this is an extremely powerful filter to bring order into this complexity, the great complexity associated with thousands of chemicals.

We have to test this hypothesis and if it is also true for human exposure scenarios, we then have a very powerful tool to focus on the handful of chemicals that really matter. Then we can do better epidemiology. I think that is the way forward. We cannot do epidemiology with mixtures with hundreds of chemicals. I cannot see that.
Chirag Patel, Ph.D., is a postdoctoral researcher at Stanford University in the field of translational bioinformatics. He has been developing methods to conduct environment-wide association studies (EWAS) and conducting analysis of the expansive amount of “omics” data available in the public domain to answer complex questions on human diseases and mixtures. Prior to attending Stanford University, Patel worked as a software engineer at Life Technologies writing algorithms for DNA sequence detection devices.

The field of translational bioinformatics uses data-driven informatics methods to extract information relevant to disease and health. This presentation will focus on the Environment Wide Association Study (EWAS), which utilizes computational tools to create hypotheses about disease and health. This topic relates to concepts mentioned by Andreas Kortenkamp in his talk about using unbiased methods to generate hypotheses.

Common diseases are a function of both genes and the environment, and the contribution from the environment can be quite significant. In this presentation, heart disease and type II diabetes will be used as examples for which estimates of the environmental contribution to the disease development range from approximately 30–70%, excluding any potential gene/environment interactions. These are diseases that are of primary importance in the United States and worldwide, and they are estimated to represent 2%–13% of the death burden worldwide according to World Health Organization (WHO).

Although common diseases are a function of both genes and the environment, there is greater research emphasis on the genetic contributions to disease. This emphasis can be visualized through a simple exercise (Figure 58). A review of the literature in MEDLINE related to WHO-prioritized diseases (e.g., cardiovascular disease, type II diabetes, kidney disease, chronic obstructive pulmonary disorder) for association studies assessing genetics or the environment resulted in a clear finding that since the 1990s, the emphasis has been on genetics and not on the environment.

A potential explanation for this is related to the intense efforts associated with the human genome project, which has led to platforms such as Genome-Wide Association Studies (GWAS). Data-driven methods such as GWAS can be used to create hypotheses about genetics and disease. For example, in 2001, the first genomes of a family of individual donors were sequenced. Just three years later, measurement modalities such as the Affymetrix and Illumina microarrays allowed for the assessment of over 100,000 to a million genetic factors against disease. These data-driven methods facilitate the assessment of large case-control studies (currently in the range of 100,000 cases and...
controls) to evaluate genetic variants. This work started in 2008 and is ongoing, with over 250 GWAS have been performed to date (Hindorff et al. 2009).

In considering the possible approaches for accounting for the environmental contribution to disease, perhaps the exposome project could follow in the footsteps of the human genome project and allow for Environment-Wide Association Studies (EWAS). This possibility was outlined in Rappaport and Smith (2010), which touches on many of the topics in this workshop. Many questions surrounding the exposome require attention, including:

- What methods (e.g., metabolomics, genomics, etc.) should be used to evaluate the exposome?
- What is actually being measured (e.g., signaling molecules, metabolites, etc.)?
- Do these measurements represent direct biomarkers?
- Most importantly, how do we use these measurements in relation to health? In effect, how can these data be analyzed to provide a more comprehensive view of the environmental impact on human disease?

As stated in Rappaport and Smith (2010), “A more comprehensive view of environmental exposure is needed to discover the major causes of diseases,” such as the WHO priority diseases mentioned earlier (Rappaport and Smith 2010).

The primary hypothesis of the work presented here is that genome-based methods can be applied to the environment. It is our assertion that comprehensive connection of environmental factors to disease is predictable using high throughput analysis methods, which are now common in genome-based investigations.

This presentation will address EWAS and GWAS, and some of the methodologies used. Examples from publications will be presented. Based on the Day 1 breakout session, a “linkage disequilibrium map” of the exposome will be presented. As was pointed out previously, epidemiology can be limited by certain constraints. For example, epidemiological studies often deal with candidate environmental factors and ignore multiple hypotheses. There is a great deal of selective reporting of these hypotheses. An example of this is presented in Figure 59. Essentially, people in the study population are categorized as diseased and non-diseased, and exposed and non-exposed. When only one “E” (i.e., environmental factor) is considered, other potential hypotheses are ignored. It is the lack of a comprehensive view that has led to fragmented literature, and this phenomenon is hotly debated in the medical literature. However, genome-wide tools have the potential to overcome this issue.
First, consider GWAS that, in this example (Figure 75) looks at cases and controls of all the genetic variants that are measureable, including common genetic variants. Instead of testing one or a handful of candidate gene hypotheses, 100,000–1 million hypotheses are tested at once. The entire genome is being queried for differences and frequency of these genetic variants between people who have disease and people who do not.

Instead of the candidate gene hypothesis (e.g., is gene X associated with type II diabetes?), the agnostic question is asked: what genetic loci are associated to disease simultaneously? It is this approach that is needed in order to gather more information about the environment and its association to disease, using a tool like GWAS. In other words, an EWAS (see Figure 60, right panel) approach is needed. In an EWAS, the X-axis could be different categories of exposures or mixtures rather than the order of different chromosomes (as used in GWAS). The Y-axis shows the strength of association for a particular environmental factor, and many environmental factors can be queried at once in order to compare cases and controls for a particular factor. Rather than asking a hypothesis-driven question, the question is: what specific environmental factors are associated with disease (e.g., diabetes) and from there, hypotheses can be generated.

The reasons for performing this type of study are numerous. First, EWAS and GWAS will enable comprehensive and transparent assessment of the factors contributing to disease. Everything is tested at once, and there is transparency to our observers of the elements that have been tested (i.e., they are listed along the X-axis). Second, these approaches offer standards. In GWAS, the old and now widely accepted statistical significance level is the genome-wide significance, indicating that a genetic association is probably real rather than being a false positive (i.e., $5 \times 10^{-8}$ generally indicates statistical significance). In effect, an accepted threshold of significance (such as a Bonferroni corrected level of significance) must be passed in order to publish a manuscript. This mechanism for controlling for multiple hypotheses in EWAS must be established. Additionally, the comprehensive nature of the assessment allows for new hypotheses and novel findings that can eventually be validated in larger cohorts and molecular studies.
Instead of asking whether a particular environmental factor is associated with disease, the question being asked should be which environmental factors could possibly be associated with disease. In order to illustrate this approach, an NHANES dataset will be used (Figure 61). It represents our “E-chip” for this purpose. Laboratory measures (e.g., clinical measures of glucose to assess type II diabetes) as well as biomarkers and exposure markers will be used. It should be emphasized that NHANES is a one-of-a-kind dataset that is representative of the U.S. population. The “environmental chromosomes” are binned by environmental categories already collected by NHANES and other exposure surveys. Independent surveys collected in 1999 and onwards will be used to conduct the EWAS. Every exposure will be analyzed for an association with the diseases of interest. For example, nutrients (carotenoid nutrients, vitamin A, etc.), PCPs, perchlorates, and more will be queried for associations with different diseases.

The methodology, like GWAS, is quite simple (Figure 62). For each of the four individual cohorts (1999-2000, 2001-2002, 2003-2004, 2005-2006), all exposures will be queried for associations with disease. Well-established analytic methods are used to test the association between a particular environmental factor and disease, such as linear or logistic regression. Further, potential confounding risk factors, such as age, sex, ethnicity, etc. will be included as adjustments to the regression models.
For each cohort surveyed, the result will be a significance value or a \( p \) value of the association between that disease and each particular environmental factor, which can be expressed in a table format. Because many queries are being performed, some are likely to represent false discoveries. Therefore, another genomics methodology will be applied to compute the false discovery rate (FDR; developed by Benjamini and Hochberg (1995) and also at Stanford by Rob Tibshirani, Bradley Efron, and John Storey (Benjamini and Hochberg 1995; Dabney and Storey 2010; Storey and Tibshirani 2003). This methodology will provide an estimate of the number of false discoveries that could occur beyond that particular \( p \) value. For example (Figure 78), if the FDR for a \( p \) value of .01 happens to be 10%, then 10% of results could be expected to be false at that particular \( p \) value (Storey 2002; Storey and Tibshirani 2003). The FDR is calculated by first permuting (i.e., rearranging) the disease labels, then assessing the FDR, which is the number of permuted results added.
to your $p$ value divided by the total number of results (Tusher et al. 2001). This is a standard method of computing the FDR in genomics studies. For a given threshold, the question posed is whether the calculated FDR is exceeded in two or more cohorts. If it is, then the result is tentatively validated. Additionally, tentative validation involves checking the signs of the factors to confirm the effect size to ensure that everything is in line across cohorts.

The first example of application of this data-driven approach is from a study that focuses on type II diabetes (Figure 64) (Patel et al. 2010). As mentioned previously, environmental factors (a.k.a. “E chromosomes”) are shown on the X-axis. All subjects with fasting blood glucose levels over 125 mg/dL (i.e., the American Diabetes Association threshold level to diagnose diabetes) from each cohort are represented by symbols (see legend in Figure 64). All estimates are adjusted by BMI, SES, ethnicity, age, and sex. The calculated odds ratios correspond to a change in one standard deviation of the log exposure value, allowing for comparison across the Y-axis. The Y-axis denotes the strength of the association or the $p$ value and is the negative log 10 of the $p$ value; the red line indicates an FDR of 10%, and the open symbols are validated (i.e., had a low FDR in more than one survey).

Each of the different symbols denotes a different NHANES survey, with the four surveys represented (1999-2000, 2001-2002, 2003-2004, and 2005-2006). Five different factors were identified as having significant odds ratios: two forms of beta carotene (precursor to vitamin A) had an odds ratio below one; gamma tocopherol (which was surprising because it is a form of vitamin E) had an odds ratio above one; and two pollutants (e.g., PCB170 and heptachlor epoxide) had odds ratios above one. Using this unbiased approach, two additional environmental factors were identified that could be associated with the disease (i.e., gamma-tocopherol and heptachlor epoxide), in addition to other factors that have already been associated with the disease, such as vitamin C, D, and PCBs.
A second example of this approach can be found in a study assessing which lipids are primary risk factors for coronary heart disease and targets for intervention (Figure 65). It is well known that serum lipids are influenced by both non-genetic and genetic factors. A 1% increase in LDL cholesterol level correlates to approximately a 1% increased risk for coronary heart disease; whereas, for a 1% increase in HDL cholesterol level, there is a 2% decreased risk. There is a nominal increased risk with increased triglyceride levels.

Again using NHANES data for the EWAS on LDL cholesterol, we found validated associations with organochlorine pesticides, some hydrocarbons, cotinine, as well as vitamin E and other markers for nutrition. Values were adjusted for body mass index, ethnicity, age, etc. When effect sizes were assessed for these particular factors, small to moderate effect sizes for HDL cholesterol were found. Some nutritional factors are presented on the left side of the graph (Figure 66). As in the first example, gamma tocopherol was associated with lower levels of HDL cholesterol, showing an association with adverse lipid profiles in two cohorts with \( p \) values less than .01 with an FDR less than 10% and an effect size of -1 mg/dL, which equates to roughly 1% increased risk for coronary heart disease.

The carotenes display a consistent association across three different surveys. The combined estimates represented by open symbols are fairly consistent with the individual cohorts, thereby adding some strength to these associations. The greater sample size was used to adjust for more factors, such as waist circumference and fasting blood glucose. The effect sizes for these environmental factors were on the same order as genetic effect sizes.
A major challenge is determining the validity of these estimates, which can be made difficult by the possibility of reverse causality or confounding factors (Figure 67). Reverse causality occurs when the disease actually leads to the measured exposure. The data from NHANES is cross-sectional, and the sequence of the exposure and the disease are unknown. An example of a potential case of reverse causality can be found in gamma tocopherol, which was associated with both diabetes and low HDL levels. It is possible that supplements taken by people who know about their cardiovascular disease status result in the observed association between gamma tocopherol and decreased HDL. Alternatively, confounding factors can be either known or unknown. For example, some nutrient factors were associated with high HDL levels in our study. It is possible that behaviors, such as statin use, could be confounding the observed association between beta-carotene and high HDL levels.

To address reverse causality and confounding factors, it is necessary to validate findings in these hypotheses-creating exercises. Currently, methods are being developed to conduct EWAS longitudinally. In effect, baseline measures are generated (e.g., gamma tocopherol) and the development of the phenotype of interest will be monitored over time. This approach offers a potential solution to the challenge of reverse causality biases. Another approach that is under development and offers promise for countering reverse causality bias is the inclusion of incidence cases and controls in the association study.

It is also necessary to develop approaches for minimizing confounding bias (Figure 82). A systematic approach involves using large dimension data from databases such as NHANES. The large database allows for the systematic addition of known confounders to models. Effect size changes are monitored to observe the degree of change associated with the additional factor. For example, disease status could be a source of bias. Self-reported diabetes or cardiovascular disease can be added to the association models to measure the effect size change; the use of a certain statin could be added to the models, etc. This exercise was performed using NHANES data because it is a high-dimension dataset which captures disease state and
drug use jointly. Results from this exercise assessing some biases from the known possible risk factors are shown in Figure 68. For each of the validated factors shown on the X-axis for HDL cholesterol (17 total, including heptachlor epoxide on the right and cotinine on the left), the increase or decrease in effect size was assessed after adding the particular factors to these models. The decrease of the factors can be seen in the lower third of the plot and the increase in the upper side of the plot.

![Figure 68](image)

For example, when accounting for recent self-reported alcohol use, the effect of cotinine on HDL levels increased up to 20%. On the other hand, when accounting for self-reported cardiovascular disease, the effect of cotinine decreased up to 15%. Furthermore, cardiovascular disease status attenuated the estimates for many of the other factors (e.g., hydrocarbons and carotenoids).

Based on some of the talks and breakout session discussions on Day 1 of the workshop, the question of whether an exposome approach could be used to elucidate combinations of exposures was formulated. This exercise involves assessing whether there is linkage disequilibrium of the exposome when studying mixtures (Figure 69). In a GWAS context, linkage disequilibrium is the correlation of alleles at two or more loci, or “non-random” association between alleles. The phenomenon of linkage disequilibrium is used to identify the actual causal genetic factor, or “causal locus.” To describe this more clearly, under linkage equilibrium, we would expect alleles inherited from one’s mother and father to occur at random, and thus, zero correlation between the genetic frequency between two or more loci. With linkage disequilibrium, inheritance of alleles occurs non-randomly, such that the presence of an allele on one locus is correlated with the occurrence of an allele on another locus on the genome. This mechanism is used in GWAS and other genetic studies to determine “causal loci.” In effect, an identified single nucleotide polymorphism (SNP) associated with type II diabetes could be associated with other correlated markers that would need to be examined for links to the disease.
The possibility of using linkage disequilibrium to find correlated exposures in the exposome should be explored. To do this, methods for deciphering identified linkage disequilibrium in terms of mixtures would need to be developed. An example from Sladek et al. (2007) (Figure 84) demonstrates the identification of the causal gene, SLC30A8, using the correlation matrix of variants (Sladek et al. 2007). Currently, work is being done to compute linkage disequilibrium plots for NHANES exposures. Linkage disequilibrium between some environmental factors has been observed, some of which are novel (data not shown). This type of analysis can promote discussion about how prevalent and relevant mixtures can be identified.

In summary, EWAS is a generalizable, comprehensive, and transparent study of the environment. The exposome will allow for the unbiased creation of hypotheses regarding environmental contributors to the development of disease. Through prototype EWAS, novel associations have been found between environmental factors and type II diabetes and HDL cholesterol. The associations are comparable to those observed in genetics. If these associations can be validated in larger cohorts, the effects observed could be significant. These environmental factors cannot be discounted as confounding variables of genetic investigations anymore, and these EWAS are examples of potential large-scale “exposomic” or “environment-wide” studies. Lastly, it is important to keep in mind sources of bias (e.g., confounding and reverse causality) and follow-up initial observations with longitudinal studies, including both molecular and population level assessment.

Discussion

Audience Member: Yes, biology sometimes complicates things. One of the complications that you could expect to arise some of the time is that some of the effects of some of the environmental agents can saturate at high doses. For example, the effect of reported cigarette smoking on birth weights tends to saturate at high doses. Have you explored ways to incorporate saturation-type phenomenon in your hypotheses?
Chirag Patel: That is a great question. I think in general, we are looking at different ways of modeling some of these effects. I think once we define how these are distributed in the population and so forth, we can actually build better ways of doing these association studies. So you are right, we need to take into account the distribution of some of these factors and how we are doing the associations.

Julia Gohlke from University of Alabama: I really enjoyed your presentation. I like how you are using genomics and genetics techniques to look at the environment, but of course, the one thing that is different is that genetics are very stable in general, as opposed to the environment, which is incredibly dynamic.

Chirag Patel: Yes, exactly.

Julia Gohlke: The genomics literature has not really had to deal with this extreme variability. I wonder if you have any ideas of how you are going to deal with that. That is the obvious thing that is completely different when you look at GWAS versus an EWAS approach.

Chirag Patel: I agree, and we are lucky because we are measuring a lot of these things that are persistent, but I think there have been efforts in the transcriptomics literature to look at longitudinal changes in gene expression. I think we can borrow some of those tools that are well known right now to look at how things are changing over time, but I think at the end of the day, we need to have these longitudinal measures to begin with to develop these methods. One place to start would be the expression quantitative trait loci (eQTL; i.e., genomic loci that regulate expression levels of mRNAs or proteins) and the transcriptomics literature to look at longitudinal changes.
Participants were placed in multidisciplinary groups such that there were representative experts from each discipline within every group. Each group was provided with a specific topic (see brief descriptions of topics below) and several scenarios to facilitate discussion of approaches for integrating research across disciplines, research strategies to address the topic, and potential challenges in conducting research on this topic. Groups were given a “Priority Matrix” (Figure 70) to help frame their discussions.

**Note:** The scenarios associated with each topic were provided ONLY to stimulate discussion. Alternatively, breakout groups could have proposed other scenarios for discussion that were relevant to the topic.

**Group 1 Topic – Modeling Mixture Toxicity: Constraints of Extrapolation**
What are recommended research approaches to overcome the key challenges to the application of predictive models of mixture toxicity (e.g., dose addition, sufficient similarity of complex mixtures, or alternative models)?

**Group 2 Topic – Exposure Assessment: Making Sense of Biomonitoring Data**
How can we use exposure data (e.g., the Exposure Biology Program, NHANES, and the exposome) in designing toxicology and epidemiology studies to better understand the contribution of environmental exposures to human disease?

**Group 3 Topic – Epidemiology: Reconciling Epidemiological and Toxicological Approaches to Mixtures**
What are suggested research strategies that incorporate lessons from both epidemiology and toxicology to increase our understanding of the role of environmentally-relevant mixtures in human disease?

**Group 4 Topic – Chemical Interactions: Predicting the Unpredictable**
What types of *in vitro* to *in vivo* approaches are needed to screen chemicals for potential interactions and how can they best be applied to provide biologically-meaningful information?
Group 5 Topic – Mixtures across Time
What types of research approaches are required to elucidate principles of both mixture exposures and associated health effects across time?
Group 1 Topic Description:

Two basic approaches for predicting the toxicity of mixtures that are promising for widespread application are: models that use single chemical dose-response data to calculate mixture toxicity (i.e., dose addition, response addition, and integrated addition) and models that compare the toxicity of a known mixture to estimate the toxicity of a related mixture (sufficient similarity). Dose Addition (DA), Response Addition (RA), and Integrated Addition (IA) are mathematical models that have been used to assess the toxicity of mixtures when the mixtures in question are relatively simple and individual dose-response data of the constituents is available. Whereas, approaches based on Sufficient Similarity (SS) have been suggested as a path forward in cases dealing with complex mixtures that are often ill-defined and when constituent dose-response data is not available. In this breakout session, participants will focus on questions that challenge the application of these approaches and discuss how they should be incorporated into biologically-based models (e.g., PBPK), or other relevant predictive models of mixture toxicity. General questions, example scenarios, and specific questions are provided below to help guide discussion.

General Questions:

• What key questions need to be addressed to move forward on this topic?
• What scientific approaches could be used to address these questions?
• What are the scientific or technology barriers and anticipated limitations to implementation of these approaches?
• How does each key question/proposed approach rank according to time? Use the priority matrix provided (see instructions above).

Group 1 Example Scenarios:

Scenario 1 – Modeling complex, low dose mixtures

Leachate from a particularly contaminated site has reached groundwater that supplies a small population with their drinking water. Chemical analysis of the groundwater identified over 100 chemicals that target a common signaling pathway. Although many of the chemicals have different primary targets, all of these chemicals have the capacity to disrupt the signaling pathway of interest. All chemicals are present well below the levels that would elicit significant toxicity individually. Is it possible to test the hypothesis that the 100 chemicals will contribute to dose additive toxicity and if so, what is the best approach to test this hypothesis?

Scenario 2 – Sufficient similarity of complex mixtures

The Gulf Oil Spill and subsequent remediation efforts (e.g., application of chemical dispersants) resulted in a complex and dynamic mixture. What is the best way to move forward on using a sufficient similarity approach to estimate the toxicity of this type of mixture with the example of polycyclic aromatic hydrocarbons (PAHs)? Consider the following specific issues: 1) what characteristics of the complex mixtures should be used to determine sufficient similarity of the unknown mixtures to the reference mixture(s) (e.g., concentration of the most potent component, ratios of top 10 components); 2) how many defined mixtures do you need as “references” to have
confidence in the sufficient similarity of the unknown mixture; and 3) what is the best approach for designing reference mixtures (e.g., design reference mixtures based on human exposures versus statistical approaches for designing mixtures that differ in systematically in components and ratios of components)?

**Scenario 3 – Target tissue approach to applying mixture models**

A population of factory workers is experiencing unusually high incidence rates of liver-related diseases and you suspect that some combination of exposures is responsible. You identify several chemical exposures that are known to target the liver, but through different biochemical pathways. What experimental support would you need to determine whether these chemicals are contributing to liver toxicity in an independent (i.e., response additive), dose additive, or interactive (synergistic or antagonistic) manner?

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**Group discussion results:**

**High Impact/Short Timeframe**

- Testing interaction threshold
  - High Throughput Screening (HTS) approach
  - Many mixtures, many endpoints
  - Fixed ratio ray design studies
- MCR hypothesis (maximum cumulative ratio)
  - Are toxic mixtures dominated by 1 or 2 components?
  - HTS of many mixtures using many endpoints
- Build database identifying interactions
  - Analyze mechanisms related to interactions
  - Look to drug-drug interactions for beginning
- Behavior-based exposure modeling for cumulative exposure
- Statistics methods development
  - Clustering techniques
  - Prioritization methods
  - Multivariate dimension reduction
  - Principle components
- Sufficient similarity
  - Chemical analysis comparison
  - Toxicity data (endpoints, sequence), *in vitro*
  - Herbals, petroleum mixtures, stored farm products
  - Systematic library of PAHs in different scenarios (e.g., high exposure)

During the group meeting, the first several minutes were spent defining what was meant by the term “modeling.” Due to the highly inter-disciplinary nature of the group, there were four or five definitions of what a model should be, ranging from simple dose addition to toxicokinetic/toxicodynamic models. Other topics discussed included: interpretation of high throughput data in epidemiologic modeling, as well as empirical, statistically based multivariate models. There was no consensus as to the most appropriate model on which to focus and many types of models were discussed.
The first short time-frame project discussed by the group involved identification of interaction thresholds. The interaction threshold is defined here as the point of departure of the observed mixture toxicity from an appropriate model (additivity) of the no-interaction scenario. The group recommended encouraging high throughput approaches for screening to prioritize mixtures for assessment. This project involves examination of many mixtures and many endpoints using ray design studies, either \textit{in vitro} or \textit{in vivo}, to determine whether or not the interaction threshold lies at a dose that is higher than the toxicity threshold. If the interaction threshold is above the toxicity threshold, than maintaining environmental pollutants below their toxicity thresholds should be protective of public health.

The maximum cumulative ratio that Paul Price presented on Day 1 of the workshop was another project that should be investigated. This would involve gathering information to determine situations where the mixture is dominated by just a few components. Again, use of high throughput screening is recommended to narrow the field of potential mixtures for study. The group identified that there is a need for development of statistical tools to evaluate the high throughput data.

The third project was building a database with what is known about interactions, especially regarding interactive mechanisms of toxicological importance. Once a catalogue of these processes is available, then predictions can be made of the chemical combinations likely to elicit those interactions. Next, exposure data can be used to determine whether these combinations occur in the real world. In building an interaction database, drug-drug information could offer a useful starting place to identify toxicological pathways that are likely to result in interactions.

The next project would be to use modeling to assess combined exposures and determine human behaviors that result in exposure to a number of different components. The group did not provide specific details, but provided an example work by Valerie Zartarian (U.S. EPA), which involved tracking or following an individual throughout the entire day to determine exposures (Zartarian et al. 2012). Studies such as this have been conducted with children exposed to copper from playground dust. The group proposed projects focused on statistical methods development to narrow the infinite world of mixtures to more manageable projects. This may include novel methods, or hybrids of existing models. These types of models are of high interest in the field of bioinformatics.

Next, the group discussed projects addressing sufficient similarity, which was discussed in detail by Glenn Rice and Chris Gennings on Day 1 of this workshop. The group suggested developing more case studies. More examples of the application of sufficient similarity methods are needed in order to identify methodology challenges, data needs, and conceptual problems. The group proposed focusing on certain test mixtures. For example, herbals are complex mixtures currently being studied at NIEHS because they are widely used supplements and there is little toxicology data available. Sufficient similarity approaches would aid in manufacturer-to-manufacturer comparisons. Petroleum mixtures provide another example of the need for sufficient similarity studies; EPA and some state agencies have evaluated petroleum fractions as surrogates for various components. A final example is using sufficient similarity approaches to determine when stored pesticide, herbicide, and fertilizer combinations become altered and result in cardiovascular toxicity following exposure. Another project would be to develop a systematic library of reference mixtures. The example that was discussed was PAHs in different exposure scenarios. The concept was to identify a PAH mixture, or set of mixtures, to which
people are most likely to be exposed (e.g., diesel exhaust, cigarette smoke). These reference mixtures would be well characterized and used for comparison to various unknown environmental samples to estimate the predicted toxicity of the PAH fraction.

### High Impact/Long Timeframe

**Group discussion results:**

- **In vitro** (HTS battery, -omics) ↔ animal ↔ epidemiology
  - Defined mixtures (occupational?)
    - Same or different pathway
  - Mirror 4-lab study with another mixture
  - Sufficient similarity of research mixture to epidemiological exposure
- **Multiple-route mixtures cumulative effect**
  - **In vivo**
  - Modeling
- Systems biology models/prediction
  - PBPK/PD – biochemical reaction network modeling

The *in vitro*/*in vivo* linkage needs to be established. Projects should be developed that incorporate *in vitro* (high throughput, medium throughput), model animal (both mammalian and alternative species), and epidemiology studies. Researchers in these various disciplines should engage in dialogue early in project development. For example, this will allow epidemiologists to have input into high throughput screening studies to ensure that relevant endpoints are assessed. One area of mixtures research that could provide cross-study opportunities for collaboration is defined mixtures. In this context, defined mixtures include combined exposures of known constituents. Some examples of defined mixtures include occupational exposures, defined mixtures identified through NHANES, and pesticide combinations. A second proposal was to mirror the four-lab study that Glenn Rice discussed on Day 1 of the workshop. This study synthesized a mixture of disinfection byproducts based upon known water treatment processes. This synthetic mixture was characterized to determine the similarity between the synthesized mixture and environmental samples, which is an example of the sufficient similarity approach. The biological similarity of the mixtures was also compared using *in vitro* approaches.

Another project worth pursuing is one focused on the effects of exposure via multiple routes. A major concern was whether sufficient examples are available that represent multiple routes of exposures to combinations of chemicals. Furthermore, are there examples where we would predict that route might influence the interaction between chemicals? The group discussed the possibility of toxicokinetic and toxicodynamic interactions among chemicals. This project would require a modeling component to estimate systemic and tissue levels of the various chemicals. These predicted concentrations could be compared to results from *in vivo* exposures in order to determine the types and magnitudes of interactions.
The final project that was discussed was the use of a systems biology approach to address questions in mixtures research. Systems biology offers promise of combining kinetic and dynamic processes to examine the interconnected networks of biological reactions. Elucidating these biological networks will allow for the development of multivariate statistical tools to relate complex systems with relevant endpoints.

Finally, the group provided some general thoughts about the path forward. The group viewed the proposed topics as separate projects to be prioritized by the NIEHS and divided into short-term and long-term components. Some of these shorter-term projects might be proof-of-concept or demonstration projects. The sufficient similarity project could utilize on-going research to develop a blueprint for demonstrating appropriate design and evaluation for this type of study. This will provide an opportunity to identify statistical issues that arise and develop tools to address them. The group indicated that there is a substantial need for the development of mathematical and statistical methods in mixtures research. This goes beyond typical data collection and analysis, and involves innovative model development by statisticians and mathematicians in collaboration with toxicologists and epidemiologists. In addition, communication of the results is necessary for maximizing the public health impact of data. Furthermore, the group recommended that grant proposals and journal articles addressing mixtures research require specific mixture study sections and experts in order to properly review these proposals and manuscripts. These review panels should include a strong biomathematical/statistical component.

**Discussants:** John Bailer (Miami University), Lyndsey Darrow (Emory University), Mike DeVito (NIEHS DNTP), Gregg Dinse, (NIEHS DIR), Chris Gennings (Virginia Commonwealth University), L. Earl Gray (EPA NHEERL), Bethany Hannas (EPA NHEERL, rapporteur), Richard Hertzberg (Biomathematics Consulting, chairperson), Marike Kolossa Gehring (Umweltbundesamt/Federal Environment Agency), Anna Lowit (EPA OPP), Paul Price (Dow Chemical Company), Glenn Rice (EPA NCEA), Cynthia Rider (NIEHS DNTP), James Rusling (University of Connecticut), Bill Suk (NIEHS DERT), Raymond Yang (Colorado State University)
Group 2 Topic Description:

The ability to identify and characterize exposures is rapidly advancing as new tools for characterizing exposure are developed and methods for chemical analysis improve and become more sensitive. Vast stores of data on potential co-exposures are being collected from programs such as the Exposure Biology Program, NHANES, and the evolving exposome project. However, translating exposure and health status data into a meaningful basis for designing relevant toxicological and epidemiological studies has been less clear-cut. How can we use these data to understand the contribution of environmental exposures to human disease? In this group, participants will focus on developing suggested paths forward in this area. General questions, example scenarios, and specific questions are provided below to help guide discussion.

General questions:

- What key questions need to be addressed to move forward on this topic?
- What scientific approaches could be used to address these questions?
- What are the scientific or technology barriers and anticipated limitations to implementation of these approaches?
- How does each key question/proposed approach rank according to time? Use the priority matrix provided (see instructions above).

Scenario 1 – Looking for unknowns

Humans are constantly exposed to mixtures of chemicals. Through biomonitoring programs, we are beginning to get picture of the chemicals to which people are most frequently exposed. However, the chemicals included in screening panels are often chosen because of their known effects. Conversely, sensor arrays and functional screens can provide information on exposures to multiple chemicals with less specificity. How can we best use exposure metrics to identify the chemicals that are likely to cause a human health problem without prior knowledge of what these chemicals might be?

Scenario 2 – Highly exposed populations

Exposures to chemical and nonchemical stressors tend to concentrate in certain populations. For example, a heavy concentration of manufacturing and highway pollution concentrated in geographical regions with a high percentage of people of lower socioeconomic status and decreased access to healthcare. How can we identify patterns of exposure in the most highly exposed populations, including both the most frequently occurring co-exposures and the maximum total exposures? Would epidemiological and toxicological studies focused on these populations provide a meaningful “worst case” scenario?

Scenario 3 – Getting a more complete exposure picture

Following the lessons of DDT and other persistent organic pollutants, current-use chemicals tend to be short-lived and therefore, it is difficult to accurately assess exposure in a population. Determination of accurate exposure profiles are further complicated by consideration of multiple short-lived chemicals with differing metabolic profiles. However, due to cost and sample limitations, it is often difficult to move beyond “snapshot-in-time” exposure assessments. What are some practical approaches for getting more accurate assessments of multiple, short-lived chemical exposures? Consider both chronic and historic exposures and exposures from multiple routes.
Group discussion results:

**Overall Goals**

- Develop methods to generate and analyze high-density exposure data.
  - Technology
  - Data
  - Analytical methods
- Develop methods for relating measures of short half-life compounds to exposure.
- Develop better models to bridge fate and transport to distribution of individual-level exposure.
- Exposure database development (building on Expoast™)

This group focused for much of the discussion on new technologies and developing methods for generating and analyzing high-density exposure data. They also discussed the data collection and analytical methods that are required. The second overall goal that they identified was the development of methods to relate measures of short half-life compounds to real-world (sometimes chronic) exposure. The third goal was to identify better models to bridge the fate and transport of chemicals to the distribution of individual level exposures. Lastly, the group discussed the goal of improving and expanding exposure databases (e.g., Expocast™).

Group discussion results:

**High Impact/Short Timeframe**

- Analyze existing high-density exposure datasets with currently available technology.
- Validate current exposure assessment technologies, including questionnaires.
  - Repository of questions (e.g., Phenxtoolkit.org)
- National, random environmental sampling for better environmental exposure characterization. (e.g., EPA wipe sample)
- Evaluate usefulness of commercially available product use/marketing databases.
- Behavior/Exposure characterization using new technologies (cell phone/internet use).
- Evaluate kinetics via high throughput *in vitro* approaches.
- Identify partners to lower biomonitoring technology costs (DOD, first responders, DHS).

For high impact and short timeframe, analysis of existing high-density exposure datasets using currently available technology would be informative. A good example was the EWAS approach presented by Chirag Patel (Patel et al. 2010). Another project would involve validation and standardization of current exposure assessment technologies, including questionnaires in epidemiology studies. For instance, whereas food frequency questionnaires tend to be well validated and use standardized forms, exposure questionnaires do not have the same degree of validation. One example to consider is the PhenX Toolkit (e.g., Phenxtoolkit.org) repository of questions.
More national random environmental sampling programs (e.g., EPA’s efforts to assess childhood exposure to pesticides, lead, and indoor dust) will provide better characterization of environmental exposure. EPA’s program involves collecting random wipe samples throughout the United States in locations of concern (e.g., schools, residential homes, and playgrounds).

Another project to consider is the use of commercially available product information and marketing databases. These have been used in conjunction with remote sensing and GIS databases to develop spatially-distributed exposure matrices based on type of source. However, these databases have not been validated, and they have not been examined from a longitudinal perspective.

The use of behavior patterns to characterize exposure using new technologies was discussed. These technologies could include Internet and cell phone use, as well as spatial information from individuals describing their day-to-day behavior. Paul Price discussed these, and other, technologies in his Day 1 presentation.

One short-timeframe goal would be the use of high throughput in vitro approaches to gain an understanding of the toxicity of mixtures to translate into exposure and epidemiology studies.

Finally, partners could be identified to lower biomonitoring technology costs. These partners could include groups or programs (e.g., DOD, first responders, DHS) that are already collecting demographic or diagnostic information and could facilitate biomonitoring or exposure characterization by researchers.

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<th>Group discussion results:</th>
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<tr>
<td><strong>High Impact/Long Timeframe</strong></td>
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<tr>
<td>• Develop short half-life compound detection technology.</td>
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<td>o Novel matrices (e.g., teeth)</td>
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<tr>
<td>• Environmental sample analysis database (expanding on NHANES).</td>
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<tr>
<td>• Developing better models that link source with behavior to predict exposure.</td>
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One high impact/long timeframe goal is to develop short-half-life compound detection technologies, particularly novel technologies that assess different matrices. One example is teeth, and the group suggested that several other matrices are currently being explored and could potentially be used to detect exposure.

A second long-term approach would be to create an environmental sample analysis database that would expand on NHANES. NHANES currently has some environmental data, but it should be expanded to provide a more comprehensive view of environmental exposures.

Finally, the group discussed the development of better tools to link source with behavior to predict exposure. For example, behavior at different life stages could be modeled to estimate exposure to sensitive subpopulations.
Discussants: David Balshaw (NIEHS DERT), Rosemary Castorina (University of California, Berkley), Brian Curwin (NIOSH), Julie Daniels (University of North Carolina, Chapel Hill), Paul Foster (NIEHS DNTP), Julia Gohlke (University of Alabama, Birmingham, chairperson), Kim Gray (NIEHS DERT), Grace Kissling (NIEHS DIR), Susan Korrick (Harvard Medical School), Richard Kwok (NIEHS DIR), Scott Masten (NIEHS DNTP), Minerva Mercado-Feliciano (NIEHS DNTP), Moiz Mumtaz (CDC ATSDR), Chirag Patel (Stanford University), Woody Setzer (EPA NCCT), Rogelio Tornero-Velez (EPA NERL)
Group 3 Topic Description:

Epidemiology intrinsically involves consideration of complex exposure scenarios that include both chemical and non-chemical stressors. Against this backdrop, the probability of an association between select chemicals or groups of chemicals and disease outcomes is evaluated (i.e., chaos → order). The toxicological approach to mixtures is the direct opposite. Chemical mixtures are designed and carefully controlled to provide information on the joint action of concurrent exposures to multiple chemicals and the information gathered is then extrapolated outward to real-world exposure scenarios (i.e., order → chaos). In this group, participants will focus on research strategies that incorporate lessons from both disciplines to increase our understanding of the role of environmentally-relevant mixtures in human disease.

General questions:

• What key questions need to be addressed to move forward on this topic?
• What scientific approaches could be used to address these questions?
• What are the scientific or technology barriers and anticipated limitations to implementation of these approaches?
• How does each key question/proposed approach rank according to time? Use the priority matrix provided (see instructions above).

Scenario 1 – Dose addition in epidemiology

A mixtures study to assess the toxicity of 18 common workplace chemicals that target the same tissue was conducted in an animal model. The findings indicated that these chemicals acted in a dose additive manner to contribute to the toxicity of the mixture. An epidemiology study has found a positive association between one out of the 18 measured chemicals, which were all present in the population at relatively low concentrations, and development of disease in the target tissue. Are there preferred methods for grouping the 18 chemicals in the epidemiology study to find an association between the disease and the total mixture exposure?

Scenario 2 – Biomarkers approach

Methods have been developed for using a biomonitoring approach to measure the total burden of mixtures of chemicals that share a common target (e.g., using biomarkers of exposure, effect, or response). What are the advantages and disadvantages of this approach? How can we better integrate these biomarkers across epidemiological, toxicological, and clinical sciences?

Scenario 3 – Statistics and epidemiology

Decreased power to determine significant associations is often cited as a major problem in assessing joint effects or effect modification of multiple chemicals in epidemiological studies. Are there study designs or statistical methods that could increase the power for multiple chemical assessments? Another major problem in epidemiological studies where co-exposure is common is an inability to separate out effects of individual chemicals. Are there approaches (e.g., in vivo studies, novel statistical approaches, etc.) that could be used to discern the contributions of the individual components to the observed outcome?
Approaches suggested by the group:

The overall theme discussed by the group was the need for incorporating ideas from toxicology in epidemiology and vice versa. The cross-disciplinary discussion was greatly appreciated by the members of the group.

Group discussion results:

**High Impact/Short Timeframe**

- Collaboration between toxicologists (biologists) and epidemiologists
  - Grouping, potency, from start to finish
- Epidemiology informing toxicology!
  - Experimental testing of interactions (e.g., stress x toxicant) / relevant mixtures for animal models
- Re-analysis of data with potency factor re-weighting or for joint effects
- Resources for:
  - Interactions that have been observed (non-published sources)
  - Potency factors
- Epidemiologists should be involved in Tox21
- People trained to interface between tox and epi (biostat, exposure scientists as well)
  - Knowledge of biology, physiology, mechanisms
  - Organized around a single problem perhaps
- Study section for mixtures
  - Special emphasis panel
- Conferences & workshops
- Could there be a specific grant mechanism for scientists to retrain in other fields (other than clinicians)?
  - K, F-32
- More data on potency factors
  - Would like to use, but data not there
  - Toxicologists would like to know which chemicals we should have potency factors for
- Better data on exposure/outcome intermediates
  - Act as exposure AND outcome
- Ensure chemical analyses overlap in NHANES to provide data on co-occurrence

The group began their discussion with the high impact/short timeframe projects. The main emphasis of these projects was on collaboration between toxicologists, biologists, epidemiologists, exposure scientists, and statisticians. One opportunity for collaboration between these disciplines is to develop a proof-of-concept project involving a select group of chemicals from the project development stage through interpretation of results. The grouping of chemicals could be according to common mechanisms or modes of action. The collaborators would develop potency factors for these compounds based on
toxicological information. The chemicals would then be combined using dose addition models. Finally, this process would be applied to an epidemiology study.

The group also discussed using epidemiological findings to guide the development of toxicology projects. For example, there is often epidemiological data suggesting interactions between chemicals. In other words, the joint exposure results in significantly greater effects than either of the two individual exposures. It would be interesting to assess those combinations in toxicological tests to confirm whether the observed interactions are greater than predicted based on models of dose additivity. Toxicologists could also explore potential mechanisms of the observed interactions.

Another short-timeframe project would be to conduct new types of analyses of existing epidemiological data. For example, potency factors could be incorporated to calculate the total concentration of measured chemicals. This could provide a more refined measure of exposure. The group suggested that many datasets might be available for this type of post-hoc analysis.

The group suggested the creation of data repositories to collect and organize existing data on chemical interactions and potency factors. The creation of mixtures-related databases has been discussed several times throughout this workshop and should be a priority.

Another theme that has been mentioned throughout this workshop is the need for cross-disciplinary interactions. For example, epidemiologists should be involved in programs like Tox21, which aim to provide rapid, human-relevant data on a large number of chemicals. Furthermore, in order to solve complex problems in mixtures research, future generations of scientists should have cross-disciplinary training.

Finally, the group indicated that a special emphasis panel to review grants on mixtures should be created, which was mentioned previously. Additionally, more mixtures-related conferences and workshops would be helpful. Ideas included mixtures sessions at International Society for Environmental Epidemiology (ICEE) and at the Society of Toxicology (SOT), or at independent meetings such as this workshop. It was also proposed that this workshop should reoccur in the future. Lastly, the group suggested that opportunities should be created to cross-train scientists.

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<td>• Ensure chemical analyses overlap in NHANES to provide data on co-occurrence</td>
</tr>
<tr>
<td>• Development of quantitative methods for measuring toxicants in complex mixtures</td>
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<tr>
<td>• Understanding mechanisms of disease</td>
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</table>
Turning to the high-impact/longer timeframe, the group discussed generation of more data on potency factors. There are many epidemiologists in this group who were very interested in applying relative potency factors in their research, but the data may not exist for many of the outcomes of interest. In turn, epidemiologists could provide a prioritized inventory of chemicals for which potency factors are needed from toxicology studies.

Another project involves improved data collection on exposure-to-outcome intermediates. These intermediates are biomarkers that indicate exposure to a chemical mixture. For example, a serum biomarker could be used to help determine components of the mixture that contributed to the exposure.

The group discussed the importance of NHANES data as an excellent resource. They noted the non-overlapping exposure measurements in NHANES present a challenge for mixtures research. Not all chemicals are measured in each sample; exposure comparisons across samples are difficult. Increasing the number chemicals assessed in each sample would be valuable.

Another long-timeframe project is the refinement and development of quantitative methods for measuring toxicants in complex mixtures. It is important to identify the individual constituents associated with the biological activity of complex mixtures. This is an enormous challenge.

One basic concept that requires further study in both toxicology and epidemiology is the understanding of mechanisms of disease. Although, there are some diseases for which mechanisms have been identified (e.g., estrogen-responsive breast cancer), there are many others that are poorly understood. Beyond elucidating mechanisms of disease, perhaps the greater challenge is to connect exposure to mixtures to the etiology of specific diseases.

The final long-term project discussed was the use of high throughput screening (HTS) for identifying potential toxicants and interactions. The group suggested that HTS could be used initially for placing compounds in mode-of-action based groups for dose addition modeling purposes. Additionally, HTS could be used to identify new mechanisms of action of chemicals. Therefore, HTS approaches have a great deal of promise.

The group mentioned a project that was not as high impact as the others. This project consisted of using empirical models to improve the incorporation of uncertainty into mixture analyses. The group
suggested that any mixtures studies that could contribute to increasing the knowledge base would be preferable to continuing the focus on single chemicals.

**Last thoughts**

The group provided a few additional thoughts that represented important points that came up throughout their discussion: identifying exposure outcome assays that can be used by both toxicologists and epidemiologists; the potential for understanding genetic susceptibility in a mixtures context - consideration of both genetic susceptibility and mixtures presents a very complicated challenge; importance of identifying “cocktails” of relevant exposures (e.g., from the occupational literature) and developing reference mixtures for toxicological assessment.

**Discussants:** Mike Babich (NIEHS DNTP), Antonia Calafat (CDC), Caroline Dilworth (NIEHS DERT), Dale Hattis (Clark University), Irva Hertz-Piccioto (University of California, Davis), Kembra Howdeshell (NIEHS DNTP), Todd Jusko (NIEHS DIR, rapporteur), Freya Kamel (NIEHS DIR), Andreas Kortenkamp (University of London), Jennifer McPartland (Environmental Defense Fund), Marike Kolossa (Umweltbundesamt/Federal Environment Agency), Susan Schantz (University of Illinois, Urbana-Champaign), Paige Tolbert (Emory University), Nigel Walker (NIEHS DNTP), Tom Webster (Boston University, chairperson), Beth Whelan (NIOSH)
Group 4 Topic Description:

One key roadblock to understanding the effects of complex mixtures has been the insurmountable task of evaluating the toxicity of what is seemingly an infinite number of potential chemical combinations. The evolving fields of computational chemistry and systems biology coupled with high throughput/high-dimensional screening tools offer the potential to characterize chemical interactions with high efficiency. However, in order to move this area of research forward a number of issues must be addressed. The most important issues include: 1) what constitutes a biologically meaningful chemical interaction (i.e., an interaction that translates to an in vivo context) in an in vitro or in silico system, and 2) what approach(es)/tool(s) (e.g., SAR, genomics, cell biology, etc.) are likely to be most effective at addressing the latter question? In this breakout session, participants will address the types of in vitro assays that can potentially be used to predict in vivo chemical interactions and discuss how they can best be applied to provide useful information. General questions, example scenarios, and specific questions are provided below to help guide discussion.

General questions:

• What key questions need to be addressed to move forward on this topic?
• What scientific approaches could be used to address these questions?
• What are the scientific or technology barriers and anticipated limitations to implementation of these approaches?
• How does each key question/proposed approach rank according to time? Use the priority matrix provided (see instructions above).

Scenario 1 – Understanding interactions

Although interactions are perceived as a significant challenge in the effort to predict the toxicity of mixtures, they are relatively infrequent and often subtle. Before embarking on efforts to identify interactions, it is important to agree on what constitutes a significant interaction. For example, what kind of increase above the “no interaction” case represents a significant synergistic interaction (i.e., is a statistically significant deviation enough or does it require a level that is biologically significant)? For the sake of identifying interactions, is dose addition an appropriate default for the “no interaction” scenario, because it provides a more conservative estimate of additive toxicity in the overwhelming majority of cases?

Scenario 2 – Translation of interactions from in vitro to in vivo

Assuming that a high throughput method is developed that is able to systematically identify chemical interactions, how can we move from in vitro identification of interactions to in vivo characterization of these interactions? What kinds of targeted testing approaches should be employed to provide data that will help develop fundamental principles of chemical interactions?

Scenario 3 – Gene-environment interactions

Chemical-chemical interactions are the focus of the majority of work in the interaction arena. However, it is an accepted fact that both genetics and environmental (e.g., chemical, nutrition, life-stage, lifestyle) factors can affect the same pathways and can play a role in the etiology of certain diseases. What are some research strategies and considerations for identifying important gene-environment interactions?
**Approaches suggested by the group:**

This group did not adhere to the high-low prioritization dichotomy, but considered that each of the projects was of high priority and exist along a continuum. The group used the term “high impact” to denote projects that should be completed before the projects classified as “low impact,” which will become high impact as more information is gathered over time.

**Group discussion results:**

<table>
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<tr>
<th>High Impact/Short Timeframe</th>
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<tbody>
<tr>
<td>• Understanding human exposures</td>
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<tr>
<td>• Improving <em>in vitro</em> assays (metabolism)</td>
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<tr>
<td>• Sufficient similarity techniques in mixtures</td>
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<tr>
<td>o Shrinking the universe of mixtures</td>
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<tr>
<td>o Incorporation of genomic databases to predict whole mixture toxicity for dose-addition modeling</td>
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<tr>
<td>o Start with disease model and work backwards to predict which sets of mixtures may be more relevant</td>
</tr>
<tr>
<td>• Integrate across databases to understand interactions</td>
</tr>
<tr>
<td>o Genomics, physiological models, TK, epidemiological, etc.</td>
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The group had one overarching theme: information about exposures is critical to the discussion of chemical interactions. It is imperative to know to what humans are being exposed, which chemicals co-occur, and which chemicals are of concern because of likely interactions. The group discussed the need to improve *in vitro* assays, especially their ability to predict biological effect, in their use to identify interactions.

A second theme discussed was the use of sufficient similarity techniques in mixtures research. Much of the discussion involved approaches for reducing the universe of mixtures that might be of importance to study.

The third theme discussed by the group was using genomic databases to predict whole mixture toxicity and for use in dose addition modeling. They suggested starting with disease models that have been characterized through single chemical study and working backwards to predict which mixtures may be more relevant to the disease. This was indicated as a potential method for narrowing the field of mixtures.

The group reiterated the need to integrate across databases (from *in vitro* to epidemiology) to understand chemical interactions. The overarching goal is to increase our ability to predict mixture behavior. Therefore, it is important to have confidence in the predictive power of each level of study from *in vitro* assays to epidemiology studies. This will facilitate prioritization of mixtures that are important to study and perhaps, the outcomes that would be of importance as well.
Group discussion results:

High Impact/Long Timeframe

- Understanding human exposures
- Incorporating better non-chemical stressor parameters as “component” of mixture
  - E.g., stress, lifestyle, diet, age, development
- Understanding of common downstream mechanisms of disease
  - Interaction of biological systems – immuno, neuro, repro
- Long latency diseases (chronic) – markers
  - E.g., neurodegenerative

To reiterate, the group believed that understanding human exposure was of the utmost importance. In addition, the group discussed the need for studies of non-chemical stressors, which present a difficult challenge. The group determined that a worthy long term goal would be the development of better parameters for non-chemical stressors (e.g., diet, age, stress, or lifestyle) and their incorporation into cumulative risk assessment paradigms. Currently, some non-chemical stressors are not amenable to dose-response evaluation, thus it is difficult to query interactions of non-chemical and chemical mixtures.

Another topic discussed was the evaluation of common downstream mechanisms of disease. The example of Parkinson’s disease was used. There are approximately 15 different animal models of Parkinson’s disease, which are all unique and yet produced similar or overlapping phenotypes of disease. If the common mechanisms could be determined, these key activities could be exploited in HTS to provide a signal for that disease.

Furthermore, the group mentioned that there is a great need for biomarkers of diseases with extended latencies. In many cases, there are no early markers of exposure for chronic diseases. A major challenge is how to incorporate these biomarkers into HTS in order to increase our ability to predict susceptibility to disease.

Group discussion results:

Low Impact/Short Timeframe

- More use of lower animals to bridge in vitro → in vivo
- QSAR
- Incorporating potency information
  - Secondary not necessarily low impact

The group discussed the need to incorporate intermediate assays between in vitro and in vivo formats. This would include the use of lower animal models or invertebrates and validate the use of these models for mixtures assessment. Another suggestion was to utilize quantitative structure activity relationship
(QSAR) databases to predict chemical profiles. Finally, the group discussed using HTS to generate potency information to be incorporated in higher order mixture assessments, which is a theme that has been presented throughout the workshop.

**Discussants:** Scott Auerbach (NIEHS DNTP), Mamta Behl (NIEHS DNTP, rapporteur), Christopher Borgert (Applied Pharmacology and Toxicology), Abee Boyles (NIEHS DNTP), David Christiani (Harvard School of Public Health), Deborah Cory-Slechta (University of Rochester, chairperson), Kevin Crofton (EPA NHEERL), Paul Feder (Battelle), Amy Herring University of North Carolina, Chapel Hill), Andrew Hotchkiss (EPA NCEA), Elizabeth Maull (NIEHS DNTP), David Lawrence (Wadsworth Center), Ronald Lorentzen (FDA), Keith Shockley (NIEHS DIR), Claudia Thompson (NIEHS DERT)
Group 5 Topic Description:

Mixtures research has often focused on concurrent exposures to multiple chemicals. However, constituent chemicals have different toxicokinetic and toxicodynamic properties, so that even concurrent exposures do not overlap entirely. An additional layer of complexity occurs with real-world exposures, as chemicals can be present at different life stages with their unique sensitivities (e.g., developmental versus adult exposures). In this breakout session, participants will address the types of studies required to elucidate principles of mixture exposures across time. General questions, example scenarios, and specific questions are provided below to help guide discussion.

General questions:

• What key questions need to be addressed to move forward on this topic?
• What scientific approaches could be used to address these questions?
• What are the scientific or technology barriers and anticipated limitations to implementation of these approaches?
• How does each key question/proposed approach rank according to time? Use the priority matrix provided (see instructions above).

Scenario 1 – Assumptions of averaging

A person is exposed to different magnitudes of various chemicals during different stages of their lives. However, regulatory agencies typically average exposures across a lifetime. What are the uncertainties associated with this practice? Are there specific chemical classes, life-stages, or health outcomes where averaging exposures is inappropriate? What are suggested practical alternative approaches for defining lifetime exposure?

Scenario 2 – Biomarkers for measuring aggregate exposures

Biomarkers are available to measure various exposures (e.g., micronucleus or comet assays for exposure to mutagenic chemicals). These biomarkers offer promise for assessing exposures across time. What should be considered in developing biomarkers for measuring exposures across time? Is it possible to tease apart the effects of chemical mixtures from non-chemical stressors (e.g., disease, psychosocial stress) using biomarkers? Will biomarkers be useful in elucidating principles related to exposures across time?

Scenario 3 – Sensitive life stages

Endocrine active compounds can have organizational and activational effects depending on the timing of exposure. What experimental approaches would be best to elucidate the combined effects of exposure to endocrine active chemicals during development, puberty, and adulthood? Besides endocrine disrupting chemicals, what other types of targets will provide useful scenarios to address this issue (e.g., initiators and promoters in cancer, developmental versus adult neurotoxicity) and which cases offer the most public health impact (i.e., available experimental model, relevance to human exposures/disease)?
**Approaches suggested by the group:**

This group thought it was very interesting that the topic of temporality was included in the workshop, because they did not feel that we understand the temporality issues associated with single chemicals. Therefore, this represented a very challenging topic.

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**High Impact/Short Timeframe**

- *In vivo* animal studies for determining latent effects of exposures including mixtures during developmental periods.
  - Ensure animal models are appropriate for the endpoint studied and for aging
  - Conduct non-contemporaneous relevant co-challenges that alter susceptibility or induce persistent or subclinical effects
  - Evaluate subtle genetic changes from existing low dose mixture studies (epigenetic and genetic)
- Continue to implement exposure averaging ensuring the averages are over times of clinical significance (include metabolites and persistent effects)
- Couple whole or partial mixture ‘exposures’ to functional assays and *in silico* models but anchor to *in vivo* assays
- Define exposure mixtures using long term individual and population longitudinal and spatial data
  - Explore unconventional partnerships (e.g., grocery sales information, industry product databases, bar code ingredient databases)
  - Mine data from existing longitudinal studies and integrate birth cohorts with child cohorts and healthy aging populations
  - Piggy back onto existing longitudinal studies to include exposure data or use archived samples (e.g., GEN-R) potentially forming consortia
- Longitudinal qualitative and quantitative analyses of common matrices or mixtures
- Laboratory capacity-building to enable high throughput analyses with appropriate QA/QC
- Use of pattern recognition techniques to identify changes in whole or partial mixture patterns using chromatograms
- Use exposure models along with monitoring data, fate and transport and HAP to understands co-exposures
- Statistical strategies to cluster population with similar exposures
  - Couple with metabolomics to determine any prominent effects
- Infrastructure-related issues
  - Encourage transparency in product ingredient lists
  - Funding for rapid, HTS, comprehensive analytic technologies for monitoring
  - Develop these technologies
  - Standardize national database variables (e.g., NHANES and U.S. census sociodemographic variables differ)
The group began its discussion by recognizing the need for cross-disciplinary collaboration in order to make progress on the challenge of evaluating mixtures over time. The group focused primarily on two particular issues, which were both considered to be high impact and have a short timeframe. Although, it might be difficult to conduct studies on “mixtures across time” in a short timeframe, the group attempted to leverage current systems to reduce the proposed study timeframe.

The first issue that was discussed was related to how toxicology can be used to study “mixtures across time.” They advocated the use of in vivo animal studies to determine latent effects of exposures during developmental periods. Those developmental periods would include not only the in utero period, but early childhood and adolescent stages, as well as the process of aging. The group discussed the need for animal models that are appropriate for the endpoints at each stage of the life cycle. The group also suggested that researchers should conduct non-contemporaneous, relevant co-challenges that alter susceptibility or induce persistent or subclinical effects. In other words, this would consist of a primary exposure to a single chemical or a mixture of chemicals at one timepoint that might alter the susceptibility or an effect; this would be followed by a secondary exposure or co-challenge (e.g., allergen) that would change the effect or the timing of the effect of the primary exposure. The group also thought that researchers should evaluate subtle genetic changes from existing low-dose mixture studies. The information gleaned from those studies could provide clues as to changes in susceptibility over time.

The group expressed the need to implement exposure averaging during periods of clinical significance. One of the charge questions for the group was whether or not exposures should be averaged over time. The group agreed that it was appropriate in most instances, as long as it was over a critical time period. The group thought it was important to include not only the chemicals of exposure, but also active metabolites or persistent effects that might occur.

There was mention of the need to couple whole or partial mixture exposures to functional assays and in silico models, while anchoring findings to the in vivo assays that are considered apical.

Moving on to population-based and human exposure studies, the group thought exposure mixtures should be defined using long-term individual, population, longitudinal, and spatial data. One example would be to explore unconventional partnerships, such as obtaining grocery sales information or gaining access to industry product or ingredient databases that may help the research community to understand some of the current widespread exposures.

The group suggested mining data from existing longitudinal studies with integrated birth, child, and healthy aging population cohorts. These cohorts represent populations that are critical to understanding effects across time. It is also very important to leverage existing longitudinal studies to include exposure data, or to enhance the exposure data previously collected. This includes the use of archived samples, especially from studies that did not originally contain an environmental component. One example is the Generation R study, a prospective cohort study from fetal life to young adulthood. The group recommended development of consortia (Jaddoe et al. 2008). Members of the consortia could analyze a set of archival samples for environmental exposure data (e.g., from the Generation R Study) and make those data available to multiple researchers looking at various health endpoints.
Longitudinal qualitative and quantitative analyses of mixtures in common matrices would help to determine relevant mixtures of exposure. This would include capacity building of laboratories to enable high throughput analyses and would require appropriate quality assurance/control systems.

Another idea presented was to use pattern recognition techniques (i.e., chemical fingerprints) to identify changes in whole or partial mixtures using either qualitative or quantitative analyses from chromatographic data.

Approaches that can be used to understand co-exposures include exposure models, monitoring data, fate and transport, and human activity patterns (HAP). The group recognized that exposure modeling is critical to characterizing exposures across time.

The group suggested that statistical strategies could be used to cluster populations with similar exposures and possibly collapse these populations into 10 to 20 different sub-types. The population sub-types would be characterized by their co-exposures. Samples from the sub-types could eventually be compared using “omics” technologies to determine prominent effects that differ from one population to another.

The group also discussed infrastructure-related issues. For example, researchers should encourage manufacturers to be transparent in listing product ingredients. In addition, it is challenging to obtain proprietary information in commercial products (e.g., fragrance).

The group recommended increased support for the development and use of rapid, high throughput, comprehensive analytic technologies for monitoring exposure over time. The group believed that this is a long-term goal.

As discussed previously by other groups, variables reported in national databases should be standardized, so that these databases can be merged. For example, NHANES data and U.S. Census sociodemographic data variables differ, which complicates merging of variables across these databases.

**Discussants:** Dana Boyd Barr (Emory University, chairperson), Chad Blystone (NIEHS DNTP), George Bollweg (EPA Region 5), Herb Buxton (U.S.G.S), Danielle Carlin (NIEHS CERT), Stephanie Cormier (Louisiana State University), June Dunnick (NIEHS DNTP), Russ Hauser (Harvard School of Public Health), Jerry Heindel (NIEHS DERT), Shyamal Peddada (NIEHS DIR), Joanna Matheson (CPSC), Andrew Salmon (California EPA), Jane Ellen Simmons (EPA NHEERL), Linda Teuschler (EPA NCEA), Sheetal Thakur (NIEHS DNTP, rapporteur), Tracey Woodruff (University of California, San Francisco)
OPEN DISCUSSION

Claudia Thompson: Thank you to each of the groups. Those were very succinct recommendations from complicated group discussions. There are some common themes including understanding exposure, thinking about databases with various levels of information that can be merged to get a better handle on mixtures behavior from exposure to biological response and disease, and intermediate markers that might be useful in being able to look both temporally and spatially at effects of mixtures.

Raymond Yang: I would like to specifically talk about the suggestion for establishing an NIEHS mixtures studies section. I have been doing mixtures work since 1983 and throughout the years, I have become more and more aware of the fact that to evaluate other people’s mixtures studies, you really need to have a very open mind. Therefore, my suggestion is that you should form the panel with very open-minded people, and not those totally immersed in one approach.

Linda Birnbaum: We are familiar with the problems associated with study sections, and I will not spend a lot of time commenting on them. However, there is going to be a new head of CSR, so there may be some changes. They are interested in how study sections can be improved. NIEHS is not the only institute that has had challenges with the way study sections have been formulated, but I would challenge the academics to remember that we could clearly have a special emphasis panel to deal with mixtures. That is something that we can do, but it probably would not be a standard Research Project Grant (R01) mechanism, which is the most popular mechanism for investigators.

Regarding the presentations today, there were some of take-home messages that I wanted to reflect on. We really do need to develop a database where we can gather all the information together, so that all the information that relates to mixtures could be made searchable. Second, we need to be sure to always have a phenotype that we can link in vitro responses to. Another theme that I thought was important and also came up at the International Society for Environmental Epidemiology (ISEE) meeting is that toxicologists and epidemiologists need to communicate more and work together. Furthermore, the idea that we should at least develop some competency in other areas could be an advantage for all of us.

I really like the idea of binning or sufficient similarity or pattern recognition. Those things all go together and provide approaches for prioritizing because we can’t study everything. Regarding novel partners, I think it is a great idea to brainstorm different people to work with. We often tend to go to the same sources again and again.

Lastly, there are a couple of things I want to challenge you to think about. I wonder whether there are opportunities, for example, to incorporate more environmental measurements into NHANES. That’s a program that provides statistically-based sampling. We heard that it would be really nice if all of the chemicals could be measured in a single sample, so that for all 8,000 people included in NHANES who are measured every two years, you would have all of the information. There is a lot of information that perhaps we could start gathering, such as taking hand wipe samples and analyzing those for chemicals. We need to begin to think outside of the box.
There has been a lot of focus on exposure and I want to ask a question: do we really need to know exposure first or do we want to have a non-biased way of looking at things? We need to think about the GWAS-EWAS kind of approach.

Andreas Kortenkamp: I wanted to ask the last group whether you discussed the problem of sequential exposures? I haven’t heard anything about it. We are all very obsessed with simultaneous exposures, and quite rightly so, but the conceptual framework for dealing with sequential exposures is not really worked out, and I think there is a need for that.

Audience Member: One of the things that comes up in having cross-disciplinary communication is that different disciplines have their own good reasons for the way they do things, and sometimes, there is going to be a need for doing things a little differently than your discipline has trained you to in order to take advantage of the wisdom that is available from other disciplines.

Among the things that I think that breaks down in communication between basic experimental biologists and toxicologists and the statistics/epidemiological communities is the tendency on the part of the epidemiologists to have models that are as simple as possible, whereas incorporating the wisdom, or at least the prior understanding from the biologists, involves making models that can be more complicated because they attach prior weights to different kinds of things. They might have different potency information or they might have different mathematical forms than a simple linear form.

This collaboration involves changes to the way things are traditionally done within different disciplines, and that is why we have to encourage people to be open to that and for the people who review papers and review grants, to also be open to those kinds of interdisciplinary ideas.

Linda Teuschler: I wanted to make a couple of comments about the methodology of sufficient similarities. It is really great that this group has seen the utility of the idea of sufficient similarity and recognized that it could be useful in looking at whole mixtures. It could also be useful in looking at defined mixtures as well, but it is important to note that sufficient similarity is not totally worked out. There is still a lot of work that needs to be done in that area.

We worked on it for disinfection byproducts and have published multiple papers on methods. Paul Feder, Chris Gennings, and others have worked on some statistical techniques for assessing sufficient similarity. We found that the methodology is somewhat mixture-specific. In our work, we developed certain statistical methods for assessing the data. However, there are also some biological and toxicological criteria involved. The idea is that there are certain chemical characterizations that would lead you to believe that a mixture would have certain toxicology properties. If you come to the next related mixture from another source, you would look at those same parameters to see if they still apply to that mixture.

Additional research in this area is needed. If we take a chemical class like PAHs, we would have to spend some time looking at the statistical properties, the chemical analysis of the PAH mixtures, and the toxicological properties that are associated with that the mixtures. I think it is doable, but it takes some time and some effort and publications on these things in order to work that out. There is always
toxicological research and epidemiological research, but there is also an area for statistical research, and you should build that into your ideas when you're going forth with your program.

Richard Hertzberg: I was extremely pleased to hear the discussion by one of the other groups of including homeland security and defense as partners and then the mention of occupational data as another resource. It has been easy, in the last 20 years or so, to keep thinking about mixtures issues in terms of the Hazard Index and what Superfund does, as well as how low do we need to go to be clean and to assure everybody that they are safe.

We also have Katrina, floods, explosions and tsunamis, and we are reminded that we are not always exposed to low levels of everything constantly over a long period. Many of the interactions that are well documented are those that are high, short-term exposure spikes. The average time is in the matter of minutes; it is not in the matter of years. It was extremely gratifying to me to hear that there is still concern and recognition that the mixtures issues are not just the safe levels of long-term exposures.

I’m reminded of the surprising presentation by a woman from NIOSH in Cincinnati after the BP Oil spill that the primary acute toxic endpoint was from overdose of Gatorade. The point is that lifestyle factors and personal choice can be extremely important, at least in the short term.

Tom Webster: There is one other thing I was really encouraged by based on the interdisciplinary discussion in our group. I think the thing I would really encourage NIEHS to think about is forming collaborative teams through your granting procedures where you integrate in-house with extramural resources and look across groups.

The example that I would use is the four-lab project that EPA undertook. We had many different disciplines, engineers, chemists, toxicologists, and risk assessors who came together and worked together for a long period of time. It was about an 8–10 year project. This is not a short-term thing, but I think the advantage of doing something like that is that you really have a very good understanding and a solid experimental design.

J.B. Brown (Kyoto University): I am a chemo-informatician. I was invited at the last minute to attend this meeting and I want to say that from the perspective of chemoinformatics and drug design and systems biology, this was really eye-opening to me because the approaches discussed in this workshop provide a totally different perspective than that used in drug design.

One of the things that I heard a lot about during this meeting is the integration of systems biology. In drug design, we are also starting to move in that direction. My laboratory in particular has started to embrace that and I wanted to make this group at this meeting aware of an interesting concept that I think would be very useful.

First of all, I appreciate the breakout session format that was used in this workshop, which is not commonly used in Japan. One approach that was used by Hiroaki Kitano, who coined the term “systems biology,” was to bring a multidisciplinary group of 150 or 200 people to India for a 4-day workshop focused on using systems biology tools to create the theoretical metabolic pathways in tuberculosis. I
think that the problem-solving method used by Hiroaki Kitano offers promise for use with the issues discussed at this workshop.

Claudia Thompson: Thank you for those thoughts. It’s always good to have other perspectives that are looking at the issues from a very different angle from which we can borrow.

Paul Price: One other group that you might not ordinarily think of is the chemical industry, which is now moving towards the idea of designing our products so that they fit the environment and meet people’s needs, so they’re greener and more sustainable. These are buzzwords, but what they translate into is thinking about the lifecycle of the product and whether it achieves what you want it to achieve, while avoiding all of the things that you are concerned about.

There is a growing group inside of industry who are very interested in mixtures and mixture-related issues as a way of making our products better upfront, rather than just dealing with these issues in terms of cleanup. There is a growing resource here that could be tapped into for mixtures issues. How do we optimize formulations that achieve goals? There is also a blurring between the chemical industry and the drug industry and the food industry, where we are now interested in trying to design agricultural products that improve human health as well.

Raymond Yang: I would like to congratulate NIEHS from Linda Birnbaum on down, and all the colleagues who put this workshop together. When I began working in the mixture area in 1983, one thing I felt very strongly is loneliness – there were not many people willing to tackle such a difficult issue at the time. Therefore, I really appreciate Linda and colleagues at the NIEHS putting so much effort into this workshop.

I also want to tell NIEHS not to be discouraged that only 29 people responded to the RFI. You didn’t reach the right people. My advice to you is to write e-mails to the people who are publishing in the area in order to get responses from experts.

Cynthia Rider: Thank you, Ray. When we began putting this meeting together, we thought we would have a small group of 30 or 40 people. It was very encouraging to see such an overwhelming response of interest in this area. Mixtures is an issue that has been discussed for a long time, and we now have a critical mass of people and tools to make excellent progress in this area. Thank you all for your interest.
CLOSING REMARKS  RICHARD WOYCHIK, Ph.D., DEPUTY DIRECTOR OF NIEHS

Richard Woychik, Ph.D., provided a summary of the major themes resulting from workshop presentations and discussions. The workshop coincides with the development of the 2012-2017 NIEHS Strategic Plan (http://www.niehs.nih.gov/about/od/strategicplan/index.cfm), which is aimed at evaluating the scientific direction of the Institute. Through the Strategic Plan development process, we will be evaluating new and exciting research directions and continuing the important research currently being conducted at NIEHS. The NIEHS Mixtures Workshop provides one of many opportunities to receive input from the extramural community and the public on the NIEHS Strategic Plan.

NIEHS Strategic Plan

There are three different phases of the NIEHS strategic planning process. The first phase included the publication of a Request for Information (RFI) in the Federal Register (http://edocket.access.gpo.gov/2011/pdf/2011-5503.pdf) in which responses were accepted from the public through April 30, 2011. This phase was followed by a stakeholder community workshop in July 2011 in Research Triangle Park, North Carolina. This workshop attempted to bring in a cross-section of approximately 170 different individuals from the global environment health sciences community to discuss issues they considered important in environmental health research. As a result of that workshop, there were 97 different white papers and reports, which contained themes that have been combined and prioritized. These reports are available on the NIEHS strategic planning website (http://www.niehs.nih.gov/about/od/strategicplan/index.cfm).

Several important topics emerged from Web-based input and the stakeholder meeting. These topics included:

- Basic research on human health and disease
- Exposure science and the exposome
- Translational science
- Collaboration and integrative approaches for conducting environmental health science research
- Knowledge management and data analysis
- Environmental health disparities, environmental justice, climate change, and global environmental health
- Training the environmental science workforce
- Effective communication and outreach of environmental health science to other scientists and non-scientists

The next phase of the strategic planning process is a two-day workshop that will be held on October 13–14, 2011, in Research Triangle Park, North Carolina. There will be approximately 50 experts in attendance, selected from the intramural and extramural community. This meeting will consist of development of a mission, vision, and draft of the Strategic Plan goals and will also provide the directional framework of the Institute over the next five years. Subsequent to the workshop in October,
Linda Birnbaum and the NIEHS leadership team will be examining the mission, vision, and the strategic goals, and then developing implementation strategies. The NIEHS will announce the final Strategic Plan in May 2012. Woychik emphasized that he is accepting comments from the public throughout the NIEHS strategic planning process and welcomes input from the participants of the NIEHS Mixtures Workshop.

**Cross-cutting Themes from the NIEHS Mixtures Workshop**

There were nine major themes of the NIEHS Mixtures Workshop.

**Theme 1: The concept of mixtures is not new to NIEHS.**

NIEHS has been supporting mixtures projects for many years, such as the Gulf Oil Study and research on disinfection by-products. Mixtures research has a long history. However, there is a current need to evaluate what has been done and think about future mixtures research needs. The field of mixtures needs to move beyond the current concept of mixtures. For example, researchers should begin to think about the exposome, which is a term that has been brought forth by Stephen Rapport, Ph.D., of University of California, Berkeley. The exposome allows us to evaluate how all exposures come together rather than considering specific mixtures, such as pesticides, tobacco smoke, or air pollution. If we want to understand the role of the environment on health, we have to be able to put all of this information together.

**Theme 2: The study of mixtures is an enormous challenge.**

It is encouraging to know that researchers do not have to evaluate all possible permutations of mixtures that occur. Rather, it is imperative that researchers study those mixtures that are relevant to real-world conditions. Some of the questions that need to be addressed include: what do we know; what do we not know; and what would we like to know about mixtures. To help answer these questions, new tools are being developed to measure exposure. One example is subdermal microchips to measure glucose, lead, and other parameters. Scientists are developing new analytical tools, utilizing sophisticated computational analysis, and adapting everyday technologies such as iPhones® and other portable electronic devices to measure our exposure to mixtures. These tools are necessary to provide us with a better sense of real-world mixture exposures and the development of these new technologies will be a very high priority.

**Theme 3: Address internal versus external exposure.**

Another challenge for researchers is addressing internal versus external exposure. For example, are the mixtures that reside in the environment the same mixtures that affect the cells in the body? Addressing this issue is critical if researchers want to interpret their results in model organisms to explain what is occurring in humans.
Theme 4: More innovative approaches should be developed for analyzing and modeling the biological effects of mixtures.

Researchers can use either a “bottom-up” or a “top-down” approach, depending upon the situation. We need new mathematical, statistical, computational, and analytical tools for evaluating complex mixtures. We need to be able to estimate the concentration, potency, and synergism associated with different components of mixtures. Researchers need to disentangle the effects so that we may understand which components are responsible for which effects. The Environment-Wide Association Study (EWAS) approach presented by Chirag Patel, Ph.D., represents a very creative and novel approach for blending mathematical genomics, genetics, and exposure data. This approach utilizes complex data sets to better understand the role of the environment in human health.

Theme 5: Develop databases.

Thus far, individual laboratories or collaborating groups have typically developed their own databases, but these stand-alone databases should be “talking” to each other. In other words, researchers need to effectively collaborate and consider how best to combine their databases to avoid overlap and address larger questions that require a substantial amounts of data sharing. If databases exist separately, we need to develop guidelines such that there can be seamless integration of the data so that we, as a community, can use this information to better understand how the environment interfaces with human biology. Furthermore, we need to have user-friendly bioinformatics tools and interfaces so that biologists can effectively use data without being experts in computer science.

Theme 6: Use of reference populations by mixtures scientists.

Epidemiological studies with these populations will address the connection between the environment and mixtures exposures, and will be used to develop hypotheses about the possible dangers associated with mixtures and human health. These experiments factor in genetics and epigenetics. Individuals with different genetic makeups respond differentially to the environment, and the same is true for epigenetic susceptibilities. Therefore, epidemiological experiments should factor these into the equation by using broad-based sampling of humans or animals when conducting experiments. Other considerations for these studies include diet, physical activity, infectious diseases, as well as psychosocial stresses on the host, which can also factor into the response to environmental mixtures. Another notion that should be included in this theme is the use of better model organisms. We need to go beyond using inbred strains of mice or utilizing only the first generation of rodent models for studies of mixtures. Researchers should also be thinking about developing not only rodent models but also non-rodent models that can better capture the genetic diversity that exists within the human population.

Theme 7: “Windows” and routes of exposure are critical in the study of mixtures.

NIEHS is interested in understanding how prenatal and early-life exposures can give rise to effects much later in life as well as how different routes of exposure result in different health outcomes. There was much discussion about the in vitro versus the in vivo approaches. Researchers conducting in vitro and in vivo studies should work together, rather than continuing with an “us versus them” mindset. In reality, mixtures researchers need both in vitro and in vivo research in order to answer complex questions. We
need to recognize that there is no way that we will be able to handle all of the chemicals that need to be evaluated using the existing \textit{in vivo} tools. We should also recognize that the \textit{in vivo} tool is not always predictive of health effects in humans. On the other hand, we also have to be knowledgeable about the \textit{in vitro} tools we develop and understand the strengths and limitations of \textit{in vitro} approaches as well.

\textbf{Theme 8: Mixtures researchers should consider a systems-based approach for studying mixtures and the role of the environment.}

This work will inevitably require cross-disciplinary efforts. For example, rather than working in separate laboratories, toxicologists, geneticists, and exposure scientists should work together on collaborative teams to allow us to understand the role of the environment in human health.

\textbf{Theme 9: Mixtures researchers need to evaluate total exposure.}

Total exposure includes all routes of exposure, all sources of exposure, and all chemicals, whether they are endogenous or exogenous. We need to factor in the genetics, the epigenetics, the genomics, the nutritional status of the host, psychosocial factors, as well as infectious diseases. Once we begin to understand all of these different factors in the context of an exposome model, then we will truly begin to understand how the environment influences human health.

\textbf{Next Steps}

The organizers of this workshop will generate a workshop report that will capture the details within each of the themes discussed in this presentation. The knowledge gained from this workshop will be used in the future NIEHS mixtures research agenda. NIEHS asks those participants attending both this workshop and the NIEHS strategic planning workshop (next month) to represent the points of view that emerged from this Mixtures Workshop. Additionally, NIEHS will be gathering the output from other 2011 key mixtures workshops and meetings and incorporate their information into its Strategic Plan. Ultimately, all of this input will lead to a better understanding of the scientific direction of the Institute for the next 5–10 years.
## APPENDIX 1: INVITED PARTICIPANT AND REGISTERED OBSERVER DIRECTORY

### INVITED PARTICIPANT DIRECTORY

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