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# Molecular Assays for Environmental Endpoints

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Without precise estimates of toxicity, any method will lack the accuracy necessary to identify the cost/benefit ratio of proposed remedial actions intended to identify true chemicals of concern and reduce environmental hazards. This lack of precision results in agreement between environmentalists, government agencies, and industry that we are currently wasting large sums of taxpayer money on present methods of environmental hazard analyses. I believe we can do the job more accurately and less expensively.

A great deal of energy is being devoted to identification and remediation of sites containing potentially hazardous materials. Environmental engineers are developing remarkable technologies for finding hidden waste sites, including the use of LANSAT satellites and ground penetrating radar probes. Similarly, there are numerous technologies being developed for disposal, incineration, or encapsulation of that material once it has been found. Unfortunately, there has been little progress in developing rapid testing procedures to determine if the material is toxic, and therefore, in need of remedial attention in the first place. Even a rudimentary economic analysis shows that the cost of remediating every site known to contain environmentally hazardous material is astronomical, and in fact, prohibitive. There is no economic or environmental justification for remedial actions at sites that pose no real biological threat. On the other hand, we must find ways of prioritizing which sites shall receive attention.

One of the most important factors to be considered in such prioritization is whether or not a site actually contains bioactive/toxic materials which pose risks to human health and the environment. Few methods are currently available that can monitor the degree of toxicity, or determine the mechanisms whereby mixtures of chemicals may be toxic beyond that of the naturally occurring bioactive/toxic materials. Predictions of toxicity based on a subset of identified chemicals occurring in a sample fall short of the goal of protecting the environment and saving money. Determination of the true toxicity can best be achieved by monitoring molecular responses to environmental mixtures or pure compounds in living organisms.

The method currently employed to assess human health and environmental risks associated with contaminants usually relies on physical and chemical analysis of soil, water, and air samples. Samples are analyzed for the presence of approximately 400 chemicals that have been declared "toxic" based upon toxicity tests in whole animals using high concentrations of the pure form of a compound. If the sample analysis indicates the presence of compounds above a certain threshold limit, the site is then considered to pose a human health or environmental hazard. The cost of remediation usually depends upon the concentration of the contaminants found at the site, the contaminated area, and relative toxicity of those contaminants as determined by testing pure chemicals in whole animals. Therefore, several critical sources of error can lead to high uncertainty in predictions based on chemical analyses which include: (1) the presence of chemicals that were not identified, (2) the presence of chemicals that lack toxicity data, (3) the effects of synergy or antagonism in mixtures of contaminants, (4) bioavailability, and (5) the effectiveness

of treatment methods which may generate toxic substances as a result of remediation.

While this analytical chemistry approach to environmental monitoring was the best available technology twenty years ago, there are five shortcomings. These shortcomings are briefly described below:

1) There are approximately 120,000 chemicals manufactured world-wide. The toxic potential of which is largely unknown. If a given environmental sample does not contain any of the 400-plus toxins on the Priority List of Hazardous Substances published by the U.S. Environmental Protection Agency (EPA) above the allowable level, the sample receives a clean bill of health. It is impossible to monitor all 120,000 compounds by current physical chemical analysis and thus the search is conducted for only a small percentage of known toxin compounds. Such analysis may vastly underestimate the toxic potential of a site because it only looks for only 0.003 of the potential "man-made" toxins. If cost were not an issue, physical-chemical analysis could still only detect and identify approximately 5% of the known man-made compounds.
2) The physical-chemical approach to hazard analysis ignores most naturally occurring chemical hazards such as heavy metals and organic toxins. Therefore, it may underestimate toxic potential of any particular site. In addition, the turn-around time between collecting samples and receiving analytical data may be several months.
3) The physical-chemical approach to hazard analysis cannot detect novel compounds formed by the interaction of manmade compounds with each other or with naturally occurring compounds. This is likely to result in novel compounds that are impossible, not just costly, in determining their toxic potential.
4) The means by which environmental "toxins" have been designated as such is questionable. Pure compounds suspected of being toxic are tested in a limited number of whole animals at high concentrations. For economic reasons, classical toxicologists have had to assume that high concentrations in a small cohort of test animals give the same results as low concentrations in a large cohort! Furthermore, they generally extrapolate to expected responses at low doses using a linear dose response curve, when in fact, most compounds show a threshold level below which there is no detectable effect. Thus, if the compound is found to be toxic in test animals, then with the appropriate safety factor, it is assumed to be toxic in humans. This represents a vast assumption.
5) Finally, previous animal studies as well as the few animal studies used today in environmental analysis focus almost exclusively on cancer potential while ignoring most of the other noncarcinogenic toxic endpoints.

There are a number of ways in which the tools of modem molecular biology can aid in the assessment of risk posed by chemicals in pure form *and* in mixtures. Physical-chemical analysis of site samples is useful for detecting the presence of only a limited number of known toxic agents. Because such an analysis overlooks so many potential toxins, it may underestimate the true toxicity of a site. Conversely, because these analyses base toxicity analysis on whole animal exposures to pure compounds at extremely high doses, it may also dramatically overestimate the health hazards of a site. As you can see, the room for error using current techniques and models is so great that its value is highly questionable. We must identify new methods to correctly identify those sites that pose a legitimate toxic threat to humans versus those that contain biologically insignificant levels of compounds found to be toxic only in test animals at high doses. At the risk of being redundant, there is simply no justification for remedial actions at sites that pose no biological threat.

How do we improve our ability to accurately estimate the health hazard potential of an environmental site? First, we do not attempt to ascertain toxicity by physical-chemical means alone. Rather, we measure more direct end points, namely, the toxic effects on living organisms. If our end goal is to determine the health effect that a certain environment poses on living organisms,

the most direct and accurate method is to expose living organisms to that environment (or a sample thereof), and ask if there are observable toxic manifestations. Unfortunately, while whole animal assays would certainly improve our ability to predict human health impacts over physical-chemical analyses, whole animal (mammal) tests are extremely expensive and time-consuming, not to mention politically unpopular, and ethically suspect. Furthermore, they do not generally provide mechanistic information about the biochemical event that causes harm to the cell.

Leading molecular toxicologists have developed a battery of *in vitro* and transgenic assays for the rapid, inexpensive, and technically-simple collection of toxicological information. This technology utilizes a panel of bacterial, yeast, insect, and mammalian (including human) cell assays. Unlike existing *in vitro* toxicity assays, this panel of assays provides results which are integrated, and thus allow a thorough and mutually confirming analysis of relative toxicity. In addition, these assays are *directly relevant to humans* because the tests are performed on human cells.

One example of how we are employing the power of modem molecular biology toward assessing environmental toxicity is as follows. In order to rapidly assess the bioactivity/toxicity to humans of a complex mixture, realistic of most environmental samples, we have taken advantage of the fact that individual human cells respond to toxic stimuli *in vitro* in most cases identically to the way they respond *in vivo*. Part of that response is an induction in the transcriptional activity of specific genes with well defined functions. The genes that can be directly monitored encode proteins that can detoxify the toxic chemical, repair the damage that the toxic chemical causes to cell components (a toxin is toxic because it damages one or more cell components), or reduce the bioavailability by binding or excretion. The stress/damage genes that are induced are highly specific for the type of stress/damage caused by a given class of environmental toxins, and any given class of toxins induces a "signature" subset of stress genes.

Several published papers indicate that this assay system can provide the most accurate assessment of both the degree and mechanism of toxicity available in an *in vitro* assay. The advantages of such an assay are as follows: 1 ) the cost of this assay is in the range of hundreds of dollars versus tens of thousands of dollars using traditional whole animals, 2) the time required to run this assay is hours versus months for traditional assays, 3) this assay provides useful data about the level and mechanisms of toxicity; information that is rarely provided by whole animal tests, and 4) this assay dramatically decreases the reliance on whole animal tests. Thus, these assays represent the broadest range of molecular biological approaches to human toxicology available.

Another example of a molecular toxicity assay is the utilization of transgenic nematodes for the detection of mutagenic potential contained in soil samples. Analysis of mutagenic potential is facilitated by the insertion of a mutation-reporter gene inserted into the genome of every cell in the nematode in the same location, as well as a facile means of monitoring mutations in that gene. These are but a few examples of several molecular toxicology assay systems available today that can dramatically reduce the cost and time of analysis while simultaneously increasing the quality and value of information available for risk assessment.

Pertaining to environmental toxicological endpoints, a review of current screening technologies relevant to the needs of the TSCA existing chemical program on the use of a battery of molecular toxicology methods as a prescreening technique to complement and guide chemical and whole animal tests.

## BEST TESTS TO IDENTIFY CHEMICALS OF CONCERN

Along with the limitations of chemical and whole animal testing discussed in the previous section, no test will provide accurate results in all cases, thereby supporting the validity of using a battery of assays for scientifically sound weight-of-evidence predictions.

- *Chemical tests* - most widely used, Confirms the presence of potential toxicants but may identify a subset of all substances in a sample and misrepresent the bioavailable components. Important to identify sources of toxicity predicted with animal and molecular toxicology tests so that remedial treatment technologies can be tested
- *Animal tests* - widely used. Useful to determine lethal effects and some gross sublethal effects such as weight loss, fertility, and behavioral changes.
- *Molecular toxicology tests* - Major technological advancement of recent development. Useful to quickly determine lethal effects and sublethal mechanisms that may explain why substance is toxic. Have also been used to determine if remedial treatment technologies are cost effective and fine tune environmental studies so that scientists can predict where to look for effects from contaminants.

## WHAT FASTER AND CHEAPER SCREENS ARE AVAILABLE

Two types of molecular assays are currently available. Immunoassay detect the presence of specific chemicals or specific effects from chemicals; and *in vitro* tests using genetically engineered organisms.

The tests with the highest cost/benefit ratio are stress gene assays that can quickly monitor many of the known primary and secondary mechanisms produced by toxins in a single test. Weight-of-evidence data from multiple species reduce the probability of false negatives and false positives. There, assays also provide information about many non-genotoxic endpoints.

A list of commercially available immunoassay include:

- ENSYS, INC.: produces immunoassay for the rapid detection of certain classes of chemicals such as PAHs.

Commercially available *in vitro* assays using genetically engineered cells and organisms include:

- *Pro-Tox Bacterial Stress Gene Assay:* prediets 16 mechanisms of toxicity and lethality.
- *CAT-Tox Mammalian Stress Gene Assay:* predicts 14 mechanisms of toxicity and lethality in human HepG2 liver cell lines.
- *Ames II Genotoxicity Assay:* predicts 8 specific genotoxic point mutation and frame shift types of DNA damage, as well as lethality in bacterial cell lines with improvements over the widely used Ames Assay.
- *Yeast DEL Genotoxicity Assay:* predicts DNA damages in a eukaryotic cell line that responds by a global recombination repair pathway more similar to that found in mammals than may be predicted by the Ames Assay.
- *E. coli TRP Assay:* predicts several types of genotoxic damage in a bacteria that has evolved closely with humans.
- *Mutametrix Nematode Assay:* predicts mutagenicity in a transgenic nematode for determination of mutagenicity.

## TRADEOFFS: CONFIDENCE, VALIDATION, AND REPRODUCIBILITY

Currently screening of samples can be achieved with a battery of molecular toxicology assays (using a minimum of two species) with capabilities to monitor both cytotoxic and genotoxic effects at the subcellular level. The assays evaluated were selected based on requirements that they can provide high precision at low cost, provide rapid turn-around or can be adaptable to field use, and predictive of potential mechanisms of toxicity. The rationale is that if the presence of bioactive/toxic materials cannot be demonstrated on the total sample then the hazard is minimal, whereas indications with multiple species that a sample can produce DNA damage or subcellular damage is a “red flag” warning that addition testing may be required. This approach allows fine-tuned site evaluations instead of the current “shotgun” technique that is costly, time consuming, and inaccurate.

A major advantage of molecular assays is the ability to control variables. Therefore, reproducibility of results is improved. The interpretation of results comes from comparisons to data on test chemicals that have known mechanisms of toxicity, as well as a thorough understanding of the causal relationship between the damage and the endpoint measured. Comparisons of gene inductions from chemicals used to validate the assays allow prediction of mechanisms of toxicity with mixtures of chemicals – the most difficult class of samples to evaluate. Using good laboratory procedures in the conduct of molecular assays provides high agreement in data. Currently, interlaboratory validation studies are ongoing.

## RECEPTOR-BASED, MECHANISM-BASED, AND SAR APPROACHES

Taken together, the three types of tests – chemical, whole animal, and molecular toxicology give scientifically strong, mutually confirming, weight-of-evidence evaluations. However, the cost of such an extensive test sequence is not justified at most contamination sites nor to evaluate most chemicals unless the risk to human health and the environment could be substantial and exposure widespread. The best application of these approaches is in a tiered battery starting with simple biological endpoints. One factor that may be overlooked in the evaluation of potential hazards from chemical tests is the time delays in reaching a decision on remedial actions. Since chemical tests are the most indirect approach to determine toxicity then it is logical to conclude that data from ambiguous chemical analyses may be the least precise of the three types of tests. As the complexity of the contaminant mixture increases the accuracy of using chemical tests to predict actual toxicity decreases. The *use* of chemical analyses in a SAR, a common practice, may benefit most from the additional use of molecular toxicology data.

The use of animal testing is the only method to detect systemic and chronic effects in multiple species. Many of these effects can be accurately predicted with chemical or molecular assays. Additionally, the expense, ethical concerns, and time interval to conduct many animal tests limits the utility of these options, and requires that they be used only if chemical and molecular toxicity assays fail to produce clear, unambiguous results. Much of the current animal testing may be replaced with molecular assays in the future.

The use of molecular testing is gaining widespread support due to qualities such as high precision, low cost, rapid analyses that indicate why substances may be expected to cause adverse effects. Classical dose-response curves using multiple species can be generated simultaneously for a chemical or mixture of chemicals in several hours to one or two days. The use of up to dozens of different genetically engineered cells, each monitoring the activity of a different gene with characterized functions, in a single assay is a powerful tool not previously available for prescreening chemicals to predict the probability of adverse effects.

## INTEGRATION INTO AN OVERALL SCREENING AND TEST STRATEGY

Much is already known of the tests available using chemical and whole animal tests. Less well known in the environmental community are the commercially available tests from several suppliers of molecular assays currently used by government agencies and laboratories, chemical and pharmaceutical industries, and research institutions to rapidly and quickly screen substances for toxicity. Molecular tests can be readily integrated with current test strategies to provide first-tier evidence indicating the potential of toxicity. These assays should be used as a prescreen prior to expensive animal tests or chemical tests that may indicate only a subset of contaminants in a mixture. The common endpoints in whole animal and molecular assays based in genetically engineered organisms are the lethal concentrations. When lethal concentrations indicate similar sensitivity between the whole animal test and molecular toxicology organisms to the test substance then the probability of predicting applicable mechanisms of toxicity may be improved. The

precision of molecular assays and results from validation tests may allow calculation of confidence limits with data. Therefore, the uncertainty of evaluations using molecular, whole animal, and chemical test is likely to within limits allowing meaningful predictions of risk to human health and the environment.

## NEW DEVELOPMENTS

It would not seem to be an overstatement to predict that major advancements in molecular toxicology will overshadow state-of-the-art advances in animal testing and chemical testing aimed at predicting toxicity. Since organisms are more accurate predictors of toxicity than indirect chemical tests we expect use of transgenic animal models to be the greatest contribution to the field of toxicology over the next decade.