Integrating Omic Technologies into Aquatic Ecological Risk Assessment and Environmental Monitoring: Hurdles, Achievements and Future Outlook.

Graham Van Aggelen[1], Gerald T. Ankley[2], William S. Baldwin[3], Daniel W. Bearden[4], William H. Benson[5], J. Kevin Chipman[6*], Tim W. Collette[7], John A. Craft[8], Nancy D. Denslow[9], Michael R. Embry[10], Francesco Falciani[6], Stephen G. George[11], Caren C. Helbing[12], Paul F. Hoekstra[13], Taisen Iguchi[14], Yoshi Kagami[15], Ioanna Katsiadaki[16], Peter Kille[17], Li Liu[9], Peter G. Lord[18], Terry McIntyre[1], Anne O'Neill[19], Heather Osachoff[1], Ed J. Perkins[20], Eduarda M. Santos[21], Rachel C. Skirrow[1], Jason R. Snape[22], Charles R. Tyler[21], Don Versteeg[23], Mark R. Viant[6], David C. Volz[24], Tim D. Williams[6] and Lorraine Yu[1]

[1] Environment Canada, Pacific Environmental Science Centre, Vancouver, BC, Canada

[2] US Environmental Protection Agency,Duluth, MN, USA

[3] Clemson University, Clemson, SC, USA

[4] NIST, Hollings Marine Laboratory, Charleston, SC, USA

[5] US Environmental Protection Agency, Gulf Ecology Division, Gulf Breeze, FL, USA

[6] The University of Birmingham, Birmingham, West Midlands, UK

[7]US Environmental Protection Agency, Athens, GA, USA

[8]Glasgow Caledonian University, Glasgow, Scotland, UK

[9]University of Florida, Gainesville, FL, USA

[10] Health and Environmental Sciences Institute (HESI), Washington, DC, USA

[11] Stirling University, Stirling, Scotland, UK

[12] University of Victoria, Greater Victoria, BC, Canada

[13] Syngenta Crop Protection Canada Inc., Guelph, ON, Canada

[14] Okazaki National Research Institutes, Okazaki, Aichi, Japan

[15] Ecogenomics Inc, Kurume, Fukuoka, Japan

[16] Centre for Environment, Fisheries and Aquaculture Science, Weymouth, Dorset, UK

[17] Cardiff University, Cardiff, Wales, UK

[18] Johnson & Johnson, New Brunswick, NJ, USA

[19] Environment Agency, Bristol, UK

[20] US Army Corps of Engineers, Vicksburg, MS, USA

[21] Exeter University, Exeter, Devon, UK

[22] Astrazeneca, Brixham Environmental Laboratory, Devon, UK

[23] Procter and Gamble, Cincinnati, OH, USA

[24] Syngenta Crop Protection Inc., Greensboro, NC, USA

* Corresponding Author

J.K. Chipman

School of Biosciences, The University of Birmingham, Birmingham, B15 2TT, UK

Tel: +0044 121 414 5422

Fax: +0044 121 414 5925

Email: j.k.chipman@bham.ac.uk

Running Title

Omics, Pollutants and the Environment

Key Words

environment, environmental monitoring, fish, metabolomics, microarray, regulatory toxicology, transcriptomics

Acknowledgements The authors thank staff at the Pacific Environmental Science Centre, Vancouver, Canada and Marilyn Cruz for hosting and co-organizing the workshop and the UK NERC for funding (Grant NE/D000793/1).

Competing Interests Declaration

A list of affiliations and potential financial interests has been submitted

List of Abbreviations

QA- quality assurance

NMR- nuclear magnetic resonance

PCR – polymerase chain reaction

SETAC - Society of Environmental Toxicology and Chemistry

US EPA – US Environmental Protection Agency

NERC - Natural Environment Research Council

GLP- good laboratory practice

Article Descriptor

Toxicogenomics**Section Headings**

Abstract

Background: In this commentary we present the findings from an international consortium on fish toxicogenomics sponsored by the UK Natural Environment Research Council (NERC) with

a remit of moving omic technologies into chemical risk assessment and environmental monitoring.

Objectives: The consortium from government agencies, academia and industry addressed three topics; progress in ecotoxicogenomics; regulatory perspectives on ‘roadblocks’ for practical implementation of toxicogenomics into risk assessment; and dealing with variability in datasets.

Discussion: Key points were: Examples of successful application of omic technologies have been identified. There remain critical studies to relate molecular changes to ecological adverse outcome. Recommendations were made for the management of technical and biological variation. The need for enhanced interdisciplinary training and communication. The need for considerable investment into the generation and curation of appropriate reference omic data.

Conclusions: The participants concluded that, while there are ‘hurdles’ to pass on the road to regulatory acceptance, omics technologies are already useful for elucidating modes of action of toxicants and can contribute to the risk assessment process as part of a ‘weight of evidence’ approach.

START OF MANUSCRIPT

The remit of this consortium was to assess current developments towards the incorporation of omic technologies into environmental risk assessment and environmental monitoring, particularly in relation to aquatic ecotoxicogenomics. Participants recognized that omic tools and associated endpoints are already significantly improving our understanding of how individual chemicals and mixtures affect organisms and could ultimately influence risk assessment and environmental management. While a significant amount of basic research and validation needs to be done before omic endpoints are incorporated as complementary data for routine assessments of environmental risk, it was generally agreed that there are no "*roadblocks*" for omics technology *per se*, but "*hurdles"* along the road of discovery, acceptance, and implementation of omic endpoints. Given the context of the workshop, the following quote is noteworthy: "the successful incorporation of toxicogenomics into regulatory frameworks may someday be regarded as the most important intellectual and practical contribution from this generation of ecotoxicologists" (Ankley et al. 2006).

Benefits and Successful Applications of Omics in Ecotoxicology and Ecological Risk Assessments

Historical Challenges and Recent Developments for Regulatory Implementation. Previous publications and workshops (e.g. Ankley et al. 2006; Boverhof and Zacharewski 2006) have discussed the potential application of omic technologies to risk assessment. The use of omic technology in toxicology (toxicogenomics) was initiated following the development of the first

high density techniques (microarrays). However, excitement surrounding this new technology generated "hype" which yielded unrealistic expectations of the timeline for incorporation into risk assessment. There is now a more realistic understanding of the potential contribution of omics to toxicology (NRC 2007). It is realized that a multi-level, systems biology approach to safety assessment, combining molecular- (including mRNA, protein and metabolites), cellular-, tissue-, individual- and population-level data represents a powerful new multidisciplinary approach that identifies biomarkers with much improved predictive capacity.

A number of initial concerns and difficulties have been overcome. The high cost of microarrays imposed severe restrictions on the number of doses, replicates and time points assessed following chemical administration to biological systems. As a consequence, reported omic responses frequently reflected pathological change with no evident predictive value. Methodology has now been improved, costs reduced, and microarrays are commercially available for a range of species. In the context of fish transcriptomics, a special issue of the Journal of Fish Biology (Miller and Maclean 2008) illustrates the progress that has been made with non-model organisms.

Successful Prospective and Diagnostic Case Studies. Transcriptomic experiments in aquatic toxicology have been diverse, encompassing different microarray platforms, test species and exposure routes, emphasising their use as "case studies" rather than standardized tools. This wide-ranging approach contributes to the elucidation of mechanisms of toxicity, including; dose-response relationships, differential species-sensitivity, and classification of chemical–specific biological responses. This approach also provides leads for identification of novel biomarkers of exposure and adverse effect.

Omic and bioinformatic tools offer substantial promise for discovery of gene, protein and/or metabolite alterations indicative of the mode of action (MOA) of chemicals and improved understanding of mechanisms in prospective studies (Ankley et al. 2006). Knowing MOA can reduce uncertainties in chemical risk assessments providing, for example, a basis for extrapolating effects across species (Benson and Di Giulio 2007). There is ongoing debate as to the appropriate role of biomarker data in ecological risk assessments (Forbes et al. 2006). Historically, most biomarker data employed in ecotoxicology was indicative of exposure, but had limited prediction of deleterious effects meaningful to risk assessment, namely; survival, growth and development, and reproduction (Forbes et al. 2006). This is largely due to a lack of mechanistic knowledge concerning linkages between molecular alterations and outcomes in the whole organism. Ideally, omics data would reflect both MOA and deleterious outcome(s). To achieve this, it is necessary to define the cascade of pathways associated with toxicity; from a molecular initiating event (e.g. receptor-binding) through subsequent biological alterations (reflected by omic and cellular changes) that culminate in a deleterious outcome (NRC 2007). However, as indicated below, there is also potential for a contribution to understanding ecological impacts. Furthermore, omic approaches can contribute to the reduction of animal usage and of severity of treatments, as more subtle changes can be identified and a more complete assessment of the health of individual animals or cell cultures can be achieved.

A recent example of how a toxicity pathway approach can be used to establish quantitative linkages across biological levels of organization was provided by Miller et al. (2007) and Ankley et al. (2008a), who investigated the consequences of molecular changes in the fish hypothalamic-

pituitary-gonadal (HPG) axis in terms of reproductive and population effects. Production of vitellogenin (VTG; an oocyte lipoprotein produced in the liver of oviparous female vertebrates) can be affected by a range of signaling events that alter steroid hormone production and activity. Analysis of an integrated dataset derived from fathead minnow (*Pimephales promelas*) reproduction studies, with five chemicals that decrease VTG and fecundity, but affect the HPG axis through different discrete mechanisms, demonstrated robust associations between steroid and VTG concentrations in female fish. This was predictive of egg production and, via modeling, could be used to tentatively forecast fathead minnow population status. Thus, through understanding the biological pathways leading to vitellogenesis, mechanistic molecular responses were successfully related to potential adverse outcomes meaningful to risk assessors.

Omics data can be used in diagnostic studies to determine the efficacy of pollution remediation as part of a weight-of-evidence approach (Roling et al. 2007). Furthermore, omic profiling can be used to identify chemical causation of effects induced by complex mixtures (Garcia-Reyero et al. 2008a). For example, in studies with the fathead minnow Filby et al. (2007) applied multiple quantitative PCR to identify diagnostic signatures from different chemicals that induce the same phenotypic effects. There were common features in the responses of fish exposed to estrogenic waste-water effluent and individual steroid estrogens. These data demonstrate that patterns of gene expression induced by estrogenic effluents, although complex, can be diagnostic for some of the estrogens they contain and could be used by regulators to determine the primary contaminant.

The Utility of Omics in Studies on Environmental Sites

The influence of the local environment on an organism's transcriptome or metabolome can be exploited in environmental monitoring to characterise the effects of anthropogenic stressors such as pollution. In European flounder (*Platichthys flesus)* (Falciani et al. 2008), transcriptomics and genetic algorithm bioinformatic approaches were used to predict the site of origin of fish from the environment based on stress-responsive genes. Thus, although gene expression is affected by many environmental factors, a subset of genes with altered expression can inform on stress responses. The potential utility here is to improve biomarker identification and to identify patterns of gene expression associated with different types of pollution. Bundy et al. (2007) sampled an earthworm species (*Lumbricus rubellus*) from seven sites with different levels of metal contamination. Using NMR metabolomics it was shown that metabolic profiles of the earthworms could resolve individual sites. Despite the confounding influences of site parameters, specific metabolites were correlated with zinc, the major contaminant, across all seven sites. Another NMR metabolomics study, involving flounder sampled from industrialised and reference sites in the UK showed that water composition had a significant effect on the fish liver metabolome (Parsons et al. 2007). Uses of "omic" data for prognostic and diagnostic studies are summarized in Supplemental Material, Table 1. These important observations lend support to the implementation of omics as diagnostic tools in ecotoxicology.

The Current Hurdles from a Regulatory Viewpoint

General Technical Hurdles in the Application of Toxicogenomics. A proposed time-frame for realizing the utility of the omics in tiered testing was shown by Ankley et al. (2006). However, there are a number of factors limiting widespread acceptance for regulatory applications. As well

as complex relationships between omic responses and ecological outcome, there is a lack of standardized, validated exposure, assay and analysis procedures (Ankley et al. 2006). Omics technologies can be viewed as complementary testing procedures that can improve understanding of biological systems and can lead to development of simpler individual assays with defined endpoints. This approach, while valuable, does not exploit the full capabilities of omics, particularly their open nature, allowing discovery of unexpected changes. Intermediate technologies such as PCR-arrays have been proposed for use in clinical diagnosis (Bustin and Mueller 2005), but are not open systems. The validation required for uptake of multi-biomarker techniques for routine testing is time-consuming and complex. In the clinical setting some targeted microarray applications have now been approved by the FDA and EU for diagnostic use. These include the Amplichip CYP450® (Roche, Indiana, USA) for genotyping of human cytochromes P450 and Mammaprint® (Agendia, Amsterdam, Netherlands), a gene-expression microarray for breast cancer prognosis (Glas et al. 2006). Mammaprint validation required the testing of over 1,000 patient samples in 12,000 assays, highlighting the effort and investment necessary for such accreditation. Validation of an assay is a complex procedure encompassing the determination of its reliability and relevance. Hartung et al (2004) discussed a modular approach to validation of alternative tests as part of an initiative by the European Centre for Validation of Alternative Methods. This approach (Supplemental Material, Figure 1) is applicable in general terms to omics-based assays. Key to this procedure is defining a relevant endpoint. Biomarkers can be of exposure or of effect, and the choice between these, and endpoints they aim to predict, must be informed by the requirements of the regulators.

Corvi et al. (2006) suggested requirements for validation of transcriptomics in regulatory toxicology and it is unlikely that this will be rapid. However, a realistic application of omics techniques may be their use in pre-screening chemicals and mixtures for prioritization in further tests (Ankley et al. 2006). The ToxCast programme (Dix et al. 2007), employed a diverse selection of tests, including toxicogenomics, and showed the potential application of this approach. As discussed later, the interpretation of omic data is highly reliant on advanced computational and statistical methods which are still being developed. While QA procedures are paramount, at the pre-screening tier more flexibility is permitted. The US EPA (2002) currently accepts toxicogenomics data as part of a weight-of-evidence approach for establishing mechanisms of toxicity for regulated substances. While we discuss experimental variation below, it should be noted that data capture and archiving is an essential mechanism for highlighting and avoiding the pitfalls of inappropriate experimental design, such as the introduction of systematic variation during omics experiments. While transcriptomics databases are well-established, toxicology-specific omics databases are now emerging (Waters et al. 2008).

Environmental metabolomics recently benefited from the first inter-laboratory intercomparison exercise to evaluate the accuracy, precision and efficacy of ^{1}H NMR metabolomics (Viant et al. 2009). Flounder liver extracts from contaminated and reference sites were analysed, and multivariate statistical analyses confirmed high reproducibility across all seven laboratories. Furthermore, the same metabolic biomarkers that discriminated fish from the two sites were discovered by all the laboratories (see Supplemental Material). For transcriptomics in fish, diversity of microarray platforms has precluded inter-laboratory comparisons, but inter-laboratory microarray comparison has been successful for mammalian species (e.g. Mattes 2008;

Shi et al. 2006). It is likely that in future, improvements in the technologies for assaying gene expression, such as high-throughput pyrosequencing and digital transcriptomics (Nielsen et al. 2006), will replace microarrays. Already pyrosequencing allows for fast construction of high quality oligonucleotide microarrays for non-model species (Garcia-Reyero et al. 2008b). The technology is constantly evolving, but the key question that must be addressed, whatever technology is in use, is: How do gene and protein expression and metabolite concentrations relate to ecological outcome? Progress has been made on this question and initial studies on population bases are now being published (Supplemental Material, Table 1). It should be recognized that, although biomarkers are valuable in regulatory and monitoring contexts, the meanings of such changes must be clarified, to allow efficient use in regulatory decision-making (Adelman 2005; Boverhof and Zacharewski 2006).

Identification of Sources of Variation and Minimising their Effects. Variability in omics data is an ongoing concern particularly in relation to multiple individual manipulations between biological sampling and data interpretation. The workshop identified sources of technical and biological variation and made recommendations on how these should be managed in terms of experimental design (Supplemental Material). Study design, inadequate sample numbers, methods of sample acquisition, preparation, storage, processing and analysis are key areas of possible technical artefacts. Methods of normalisation and statistical interpretation are major sources of variability. Careful study design is essential to minimise biological variability "intra-class" (e.g. stage of reproduction in a control group) thus maximising "inter-class" differences (e.g. control versus exposed groups). It is noted that inter-individual variability within a

population is essential for ecological health and therefore an impact on such variability from a stressor can be very important.

Training and Communication. To advance the application of omics technology into regulatory ecotoxicology and water quality policy, there is a need for effective scientific communication amongst academia, industry and regulators (Blunt et al. 2007). The benefits and limitations of these techniques need to be candidly discussed so that tools with the potentially greatest return-on-investment (both financial and knowledge-based) may be prioritized for utilization.. Multidisciplinary workshops allow continued dialogue to inform all stakeholders of developments. These cross-functional meetings also provide researchers with an understanding of the priorities of regulatory authorities in order to discover practical ways of solving issues.

Advances in omics have significant implications for risk assessment practice and regulatory decision-making. The use of genomics technologies generates a large volume of data and the field of bioinformatics is evolving rapidly to meet data analysis needs. A Genomics White Paper identified areas likely to be influenced by omics (US EPA 2004). The Genomics Task Force recommended that the Agency develops training materials and modules to prepare risk assessors and decision makers who will be faced with the challenge of interpreting and applying omics information. Participants of this workshop also believed that training was critical for furthering the application of genomics technologies into monitoring and regulation, particularly as a means of interpreting and applying genomics data for risk assessment (Dearfield et al. 2008). Risk assessors must be able to communicate to managers and stakeholders both the underlying science and the interpretive tools and models used to develop the risk assessment. Likewise, it will be

important to provide training to risk managers regarding the benefits and limitations of genomics in risk assessments Haymes et al. 2008).

It was also recognized that there is a need to build capacity within academia, the private sector and government agencies to implement omic tools and to evaluate omics data, particularly with respect to biological and ecological significance. These institutions will require resources, support, and targeted training to bring scientists and decision makers within their organizations to a point where these tools can be effectively used in regulatory decision making, especially risk assessment (US EPA 2004). National and sub-national programs and agencies should apply strategic hiring practices to recruit individuals who possess omics skills. It would be useful to develop and initiate training in the near future to prepare risk assessors and risk managers to enable them to evaluate and incorporate omic data into environmental decision-making. Initial training could address basic omics concepts, technologies and potential applications and include the basic steps necessary to interpret and apply genomics data to risk assessment.

Research Needs for Regulatory Implementation. Research needs were reviewed in two successive SETAC Pellston workshops held in 2004 and 2005 (Ankley et al 2006; Ankley et al, 2008b; Benson and Di Giulio 2007). These efforts identified both short- and long-term needs which, due to resource constraints, have not yet been fully addressed.

The short-term needs identified were (1) formal standardization and validation of data collection, analysis, and presentation for standard test species, and (2) generation of libraries of gene expression, proteomic, or metabolite profiling data based on a set of reference chemicals with

well-defined, relevant MOAs. As explained above, there have been important advances in recent years in the context of both of these needs. The long-term needs identified were (1) generation of genome sequence data for ecologically relevant species, and (2) linkage of molecular and biochemical responses to adverse alterations in survival, growth and development, and reproduction.

Significant advances have also been made towards obtaining data for the development of reference gene expression profiling databases from species commonly used for regulatory assessments (Ankley et al. 2008b) although, much work remains. As toxicogenomics data will be most valuable for predictive toxicology and elucidating toxicologically-relevant MOAs for additional chemicals, a future database should be based on toxicity testing and monitoring protocols commonly used for regulatory purposes (eg. global pesticide registrations), as well as chemicals with well-known MOAs such as 17ß-estradiol and dioxin.

Funding. Chemical production is highest in the Organization for Economic Cooperation and Development (OECD) countries, particularly in specialty chemicals and the life science sectors. Moreover, innovation in new chemical development and manufacturing practices is extremely high due to advances in combinatorial chemistry, nanotechnology, and biotechnology. These changes question the sustainability of current approaches to prioritization, monitoring, and risk assessment. It may be difficult to allocate additional resources required to efficiently incorporate and understand omics data. It is important for programs and agencies to focus on these needs and to ensure that adequate funds and people are brought to bear on this need.

The OECD Environment, Health and Safety Programme has started cooperative work for the use of genomic information for risk assessment of chemicals. The scope of this activity is to explore and evaluate regulatory application of genomic methods in chemical hazard/risk assessment. To reduce redundancy and minimize the funding to develop these omic technologies, international cooperation and a common data-base are essential. Target and cross-species omic information and technologies should be developed to monitor animal species relevant to disparate countries. There are many international examples of initiatives to enhance ecotoxicogenomics e.g. Ministry of Environment of Japan, Canadian Federal Government Interdepartmental Genomics initiative, the US EPA initiatives and the UK NERC Postgenomics and Proteomics research program.

To address the toxicogenomics needs, a considerable investment into the generation and curation of appropriate reference exposure data is required. For example, for most chemical registrations, chronic aquatic toxicity data are required for one freshwater fish species (early life-stage toxicity), one freshwater invertebrate species (full life-cycle toxicity), and, in most cases, one saltwater fish species (early life-stage toxicity) and one saltwater invertebrate species (full life-cycle toxicity). This leads to a large number of GLP-compliant studies and such a reference database would likely require ~$10 million over 3 to 5 years. While this is a substantial cost, a reference database is arguably the only means for successful and appropriate implementation of toxicogenomics data into the current ecological risk assessment paradigm. Without a database to compare chemicals with unknown MOAs, the risk assessor will not be able to interpret the significance of the gene expression responses within the context of characterizing ecological risk because many gene expression changes are not anchored to adverse effect and the risk-assessment requires knowledge of the MOA and the dose-response relationship. A good example

of the utility of a comprehensive database is (ToxRefDB; Martin et al. 2009), containing mammalian toxicity data.

The most feasible way forward would be funding through a multi-stakeholder consortium, such as has been achieved in the ACToR database (Judson et al. 2008). The final reference database would be open source and accessible through a website. The reference database could also be integrated into a tool similar to the U.S. Food and Drug Agency's ArrayTrack to allow regulatory agencies to easily manage, analyze, and interpret omics data submitted by registrants or other government or academic laboratories using similar ecological species, testing protocols, and microarray, proteomic, or metabolomic platforms.

Conclusions

The omic technologies have advanced over recent years and continue to become more efficient, data-rich and economical in use. Proof of principle has been achieved in terms of potential application to environmental toxicology, and specifically the assessment of environmental pollution impacts in non-model organisms. The key needs to progress the utilization are in relation to the expansion of a reference database of ecotoxicogenomics and a better understanding of the relationships between specific responses and biomarkers to ecological adverse events. Through improved communication between the sectors, the aim of assisting in regulatory decisions can be expedited through the utilization of omic techniques.

Supplemental Material

Supplemental Material discusses variability in the omics. Supplemental Material, Table 1 summarizes key examples of the successful use of toxicogenomics data in ecotoxicology. Supplemental Material, Figure 1 illustrates the modular approach for applying the ECVAM principles on test validity.

References

Adelman DE. 2005. The false promise of the genomics revolution for environmental law. Harvard Environmental Law Review 29(1):117-177.

Ankley GT, Daston GP, Degitz SJ, Denslow ND, Hoke RA, Kennedy SW, et al. 2006. Toxicogenomics in regulatory ecotoxicology. Environ Sci Technol 40(13):4055-4065.

Ankley GT, Miller DH, Jensen KM, Villeneuve DL, Martinovic D. 2008a. Relationship of plasma sex steroid concentrations in female fathead minnows to reproductive success and population status. Aquat Toxicol 88(1):69-74.

Ankley G, Miracle A, Perkins E, Daston G. 2008b. Genomics in regulatory ecotoxicology. Pensacola, FL.: SETAC Press.

Benson W, Di Guilio R. 2007. Genomic approaches for cross-species extrapolation in toxicology. Pensacola, FL: SETAC Press.

Blunt RE, Walsh KA, Ashton DK, Viant MR, Chipman JK. 2007. Knowledge transfer initiative between molecular biologists and environmental researchers and regulators. Environ Sci Pollut Res Int 14(5):293-296.

Boverhof DR, Zacharewski TR. 2006. Toxicogenomics in risk assessment: applications and needs. Toxicol Sci 89(2):352-360.

Bundy JG, Keun HC, Sidhu JK, Spurgeon DJ, Svendsen C, Kille P, et al. 2007. Metabolic profile biomarkers of metal contamination in a sentinel terrestrial species are applicable across multiple sites. Environ Sci Technol 41(12):4458-4464.

Bustin SA, Mueller R. 2005. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin Sci (Lond) 109(4):365-379.

Corvi R, Ahr HJ, Albertini S, Blakey DH, Clerici L, Coecke S, et al. 2006. Meeting report: Validation of toxicogenomics-based test systems: ECVAM-ICCVAM/NICEATM considerations for regulatory use. Environ Health Perspect 114(3):420-429.

Dearfield KL, Benson WH, Gallagher K, Johnson J. In Press. Activities to address genomics needs at the U.S. Environmental Protection Agency. In: Genetics and Environmental Regulation: Ethical, Legal and Policy Issues (Marchant G, Sharp R, Grodsky J, eds): Johns Hopkins University Press.

Dix DJ, Houck KA, Martin MT, Richard AM, Setzer RW, Kavlock RJ. 2007. The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol Sci 95(1):5-12.

Falciani F, Diab AM, Sabine V, Williams TD, Ortega F, George SG, et al. 2008. Hepatic transcriptomic profiles of European flounder (*Platichthys flesus*) from field sites and computational approaches to predict site from stress gene responses following exposure to model toxicants. Aquat Toxicol 90(2):92-101.

Filby AL, Santos EM, Thorpe KL, Maack G, Tyler CR. 2007b. Gene expression profiling for understanding chemical causation of biological effects for complex mixtures: a case study on estrogens. Environ Sci Technol 41(23):8187-8194.

Forbes VE, Palmqvist A, Bach L. 2006. The use and misuse of biomarkers in ecotoxicology. Environ Toxicol Chem 25(1):272-280.

Garcia-Reyero N, Adelman I, Liu L, Denslow N. 2008a. Gene expression profiles of fathead minnows exposed to surface waters above and below a sewage treatment plant in Minnesota. Mar Environ Res 66(1):134-136.

Garcia-Reyero N, Griffitt RJ, Liu L, Kroll KJ, Farmerie WG, Barber DS, et al. 2008b. Construction of a robust microarray from a non-model species largemouth bass, *Micropterus salmoides*, using pyrosequencing technology. Journal of Fish Biology 72(9):2354-2376.

Glas AM, Floore A, Delahaye LJ, Witteveen AT, Pover RC, Bakx N, et al. 2006. Converting a breast cancer microarray signature into a high-throughput diagnostic test. BMC Genomics 7:278.

Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortaner S, et al. 2004. A modular approach to the ECVAM principles on test validity. Altern Lab Anim 32(5):467-472.

Haymes KM, Hester SD, Benson WH, Thomas JT. In Press. Potential use of microarray technology at the U.S. Environmental Protection Agency. In: Genomics: Fundamentals and Applications (Choudhuri S, Carlson DB, eds): Informa Healthcare USA, Inc.

Judson R, Richard A, Dix D, Houck K, Elloumi F, Martin M, et al. 2008. ACToR--Aggregated Computational Toxicology Resource. Toxicol Appl Pharmacol 233(1):7-13.

Martin MT, Judson RS, Reif DM, Kavlock RJ, Dix DJ. 2009. Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef Database. Environ Health Perspect 117(3):392-399.

Mattes WB. 2008. Public consortium efforts in toxicogenomics. Methods Mol Biol 460:221-238.

Miller DH, Jensen KM, Villeneuve DL, Kahl MD, Makynen EA, Durhan EJ, et al. 2007. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 26(3):521-527.

Miller KM, Maclean N. 2008. Teleost microarrays: development in a broad phylogenetic range reflecting diverse applications. Journal of Fish Biology 72(9):2039-2050.

Nielsen KL, Hogh AL, Emmersen J. 2006. DeepSAGE--digital transcriptomics with high sensitivity, simple experimental protocol and multiplexing of samples. Nucleic Acids Res 34(19):e133.

NRC. 2007. Toxicity testing in the twenty-first century: A vision and a strategy: The National Academies Press Washington, DC.

Parsons HM, Ludwig C, Gunther UL, Viant MR. 2007. Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. BMC Bioinformatics 8:234.

Shi L, Reid LH, Jones WD, Shippy R, Warrington JA, Baker SC, et al. 2006. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 24(9):1151-1161.

Roling JA, Bain LJ, Gardea-Torresdey J, Key PB, Baldwin WS. 2007. Using mummichog (*Fundulus heteroclitus*) arrays to monitor the effectiveness of remediation at a superfund site in Charleston, South Carolina, U.S.A. Environ Toxicol Chem 26(6):1205-1213.

USEPA. 2002. Interim policy on genomics: Science Policy Council.

USEPA. 2004. Potential implications of genomics for regulatory and risk assessment applications at EPA EPA Publication No. EPA 100/B-04/002.

Viant MR, Bearden DW, Bundy JG, Burton IW, Collette TW, Ekman DR et al. 2009. International NMR-based environmental metabolomics intercomparison exercise. Environ Sci Technol 43(1):219-225

Waters M, Stasiewicz S, Merrick BA, Tomer K, Bushel P, Paules R, et al. 2008. CEBS--Chemical Effects in Biological Systems: a public data repository integrating study design and toxicity data with microarray and proteomics data. Nucleic Acids Res 36(Database issue):D892-900.

Supplementary Text 1 – Variability in the omics

Sources of Technical Variability

Transcriptomics: The complex nature of microarray experiments, with many individual manipulations between biological sampling and data interpretation, make technical variability a serious concern. The process of interpretation is especially vulnerable to technical variability because of the simultaneous measurement of hundreds or thousands of endpoints and these are compounded by the high cost of the technique which has often led to the use of suboptimal sample sizes.

Technical variation in transcriptomics measurements can arise from many sources, including sampling processes, non-ideal RNA isolation and storage, variations in RNA labeling (including lability of probes), imperfect hybridization and subsequent data analysis methodologies. Transcriptomics has been the subject of quite extensive evaluations of technical variation (reviewed in detail in (de Koning et al. 2007; Fuscoe, et al. 2007; Mattes 2008; Thompson and Hackett 2008; Walker and Hughes 2008). Several consortia have recently examined technical variability within laboratories, between laboratories and between different expression monitoring platforms (Bammler et al. 2005; Kuo et al. 2006; Shi et al. 2006; Beyer et al. 2007; Chen et al. 2007; Arikawa et al. 2008; Fielden et al. 2008; Kohlmann et al. 2008). These large scale studies have demonstrated that microarray analysis can be performed with good intra- and inter-laboratory reproducibility for genes that have copy numbers of at least 5 to 10-fold above

their detection limits and this has been confirmed by alternative methods such as quantitative real time PCR (Canales et al. 2006).

Several key areas have been identified that can cause technical artefacts. These include study design, inadequate numbers of samples, and methods for sample acquisition, preparation, storage, processing and analysis. Once capture of the biological target has been achieved it is important that equivalent portions of tissue are dissected because of the likelihood of cellular and functional heterogeneity of the tissue and zonation of gene expression (Gebhardt 1992; Oinonen and Lindros 1998; Boedigheimer et al. 2008). It is important to recognize that batch effect as a source of error is likely and that biological samples are processed at random in each batch rather than each batch analyzing biological material from a single sampling event. Sample integrity is critical to successful analysis. Tissue thawing can lead to a rapid loss of RNA integrity thereby dramatically affecting array quality especially if probes are located more than 1000 bp from the transcriptional end of the mRNA (Thompson et al. 2007). Standardized protocols and external controls for quality control must be established to assure reliability. The quality of microarray analysis can be greatly improved by inclusion of adequate documentation that capture important aspects of experimental variables such as treatments, sample quality and methods, therefore helping to assess microarray quality issues due to protocol variation.

Design of microarrays can introduce technical variation especially if multiple platforms are used for analysis. Disagreement in results from different platforms can result from

probes hybridizing with different efficiencies to non-overlapping target sequences and in the case of cDNA probes, this may be compounded by cross hybridization with multigene family members. The format of the microarrays can affect variability with commercial products having better quality control and reproducibility with different sample labeling protocols contributing negligible variations in results (Kuo et al., 2006, Patterson et al., 2006). While different formats give similar results in terms of expression patterns, variation in signal intensity and resulting expression values can be obtained between different laboratories and microarray formats (Chen et al. 2007).

Perhaps the most important technical variable is in the normalization and statistical interpretation of array data. Preliminary steps should be employed to eliminate outliers (excessive chip-to-chip variation) that may be as high as 5-10% (Boedigheimer et al. 2008; Hershey et al. 2008). Normalization is essential to eliminate the effects of variable cDNA labeling but different normalization protocols and statistical analysis algorithms generate different lists of differentially expressed genes. For chip-based arrays, normalization by robust multichip average (RMA) is the usual approach and may have advantages over traditional normalization with housekeeping genes (Irizarry et al. 2003; Hershey et al. 2008), especially since in the present context toxicants are being studied and by definition these will adversely affect cellular architecture and basal metabolic processes. While some groups advocate simple cut off rules based on fold-change versus controls and significance tests (Shi et al. 2006), this can lead to high false positive errors if a no-treatment effect is not considered (Chen et al. 2007). Reproducibility can be maximized if analyses are based on biological functions such as defined by gene

ontology terms and toxicological function (Bammler et al. 2005; Fielden et al. 2008). Analysis of species of ecological interest is complicated by lack of genome sequence and poor annotation.

Proteomics: Global analyses of the proteome are now becoming possible with new technological innovations, however, sample preparation and manipulations can be exceedingly complex and thus variability is of considerable concern. Recently there have been a few attempts to ascertain intra-individual variability but these have not, so far, been extended to inter-laboratory comparisons, possibly due to the lack of established standards for representing proteomic data.

Inter-experimental reproducibility is not good using 2-dimensional polyacrylamide gel electrophoresis approaches, however, an excellent differential display technique (DIGE) allows multiple samples to be compared on the same gel and is a powerful tool in biomarker discovery for laboratory exposure studies which is quantifiable. Potentially useful biomarker candidates must be carefully selected from proteins that are not subject to sexual, nutritional and naturally high variations in expression, moreover, they must be sufficiently abundant and resolvable from other proteins for reliable quantitation. Recent studies indicate that a sample size of some 7 replicates creates reliable data. There is obviously a pressing need for establishment of standardized selection procedures. Whilst proteins of genomically characterized animals can be identified relatively easily by mass spectrometry of excised proteins using MALDI-TOF instruments, protein identification in non-characterized organisms requires *de novo* sequencing methods, precluding routine use until sufficiently comprehensive databases have been assembled.

Increasingly, proteomic techniques use liquid-chromatography (LC) separations coupled with electrospray ionization (ESI) MS and tandem mass spectrometry (MS/MS) for the characterization of the separated peptides or proteins. Sample preparation procedures are potentially much less variable and theoretically they should be able to analyze a larger proportion of the proteome, however, extensive sequence databases of the study organism are required and methods for accurate inter-individual quantitation are lacking and still under development.

Metabolomics. The ultimate expression of an organisms' phenotype is the profile of metabolites in its cells and bodily fluids. Often their existence is transient as many are rapidly biotransformed therefore sampling, sample preservation and extraction techniques are critical for high reproducibility. Standardisation of methods is therefore essential and progress is being made on this front.

Keun et al. (2002) assessed the analytical reproducibility of an NMR metabolomics experiment by analysing two identical sets of rat urine samples from an acute toxicity study. The analyses were performed at two sites and principal components analyses (PCA) revealed extremely similar descriptions of the metabolic responses to hydrazine. In one study (Bertram et al. 2007), identical sets of human urine before and after dietary intervention were measured using 250, 400, 500 and 800 MHz NMR spectrometers. When analysed by partial least squares discriminant analysis (PLS-DA), the loadings were found to comprise of the same spectral regions implying that the same metabolites

were discriminating pre- and post-dietary intervention, independent of magnetic field strength. Most recently, an intercomparison exercise involving seven laboratories evaluated the accuracy, precision and efficacy of ^{1}H NMR metabolomics for environmental research (Viant et al. 2009).

The study comprised the analysis of both synthetic metabolite mixtures as well as European flounder (*Platichthys flesus*) liver extracts from clean and contaminated sites. For both sample types, PCA revealed highly similar scores plots across all laboratories. Furthermore, the same metabolic biomarkers that discriminated fish from clean and contaminated sites were discovered by all the laboratories. Taken together, these studies clearly demonstrate that NMR-based metabolomics can generate data that are sufficiently reproducible between laboratories to support its use in regulatory studies. No such intercomparison exercises have yet to be reported for mass spectrometry based metabolomics studies.

Sources of biological variability

Biological variation can be conveniently sub-categorised into variation within the control group or population (i.e. intra-class variation that is often unrelated to the toxic stressor being studied) and variation between the control and exposed groups (i.e. inter-class variation). In general, experiments should be designed to minimise both technical and intra-class variation, thereby maximising inter-class differences that can be explored

using data mining techniques. Meaningful results depend on technical variability being less than biological variability.

As a prelude to this discussion, two distinct scenarios must be considered. In chemical testing (e.g. OECD) a limited number of model organisms are used worldwide for controlled laboratory studies. In environmental monitoring, locally relevant sentinel species, with little supporting genomics information, are typically used and sampled directly from the environment. These two scenarios will be associated with significantly different degrees of biological variation.

Variability between individuals within a given population is an essential component of population health and sustainability as it encodes for phenotypic flexibility and ability to acclimate to changing conditions and is the vehicle of evolution. Environmental stressors can impact this and affect the phenotypic variation between individuals. Reduced variability is potentially adverse to the sustainability of the population. Little is understood about the stressor-induced changes on these parameters in an environmentally relevant context. Some examples in the literature indicate that individuals within a population may have different degrees of susceptibility to estrogenic exposure, resulting in a large spread in the degree of response at low concentrations of estrogens and a more consistent response between individuals at high concentrations (Thorpe et al. 2001; Thorpe et al. 2003). The implications of the decreased variability caused by chemical exposure for the sustainability of wild fish populations are unknown. Potentially, if such an effect was also occurring in the wild, it could impact on the ability of the population to

cope with changing environmental conditions and compromise its sustainability. It is clear that individual variation might be considered as an endpoint in toxicological studies when population level effects are being addressed as chemicals may impact on this parameter and compromise the ability of populations to survive in environments constantly under pressure by changes in natural factors and anthropogenic stressors.

Inter-individual biological variation can be associated with two levels of organisation, generally defined as genotypic and phenotypic variation. Genetic variation is inherent in individuals within populations and accounts for the phenotypic plasticity allowing individuals to acclimate and populations to adapt to changing conditions. Genetic variation within a group of organisms is apparent at several levels, including polymorphisms, copy-number variants, alternative splicing, post-transcriptional and post-translational regulation and epigenetic modifications. Furthermore, organisms may vary in strain or clonal line. In general, genetic variability will be considerably higher (and less well characterised) for environmentally sampled organisms compared to model organisms unless out-bred colonies have been maintained. Furthermore the identification of closely related species (eg *Mytilus spp.*) is sometimes difficult for environmentally sampled organisms, which can be a major source of error and a confounding issue in the interpretation of ecotoxicogenomics data. Phenotypic variation is strongly influenced by the environment and its interaction with the unique physiological conditions associated with individual organisms. This form of variation can arise from factors such as age, stage of life cycle and reproductive cycle, sex, nutritional status and general health. Phenotypic variation is also likely to be lower in studies of model organisms under

laboratory conditions where it is practical to use standardized experimental conditions. Many of these factors cannot be controlled for environmentally-sampled animals, but recommendations applied to current biological-effects monitoring regarding sampling and documentation (by EPA, ICES etc) should be adhered to. The NERC Environmental Bioinformatics Centre (NEBC) has recommended those parameters that should be recorded and reported for transcriptomic submissions (envgen.nox.ac.uk/posters/MIAME_Env.ppt) as has the Environmental Context working subgroup of the Metabolomics Standards Initiative for a metabolomics study (Morrison et al., 2007). While it might be anticipated that model organisms raised in a controlled laboratory exhibit less environment- and capture-induced variation than similar animals living in the wild, the situation is far less clear for wild animals housed within the laboratory for short periods. Hines et al. (2007) showed that direct sampling of *Mytilus galloprovincialis*, a marine mussel, from the environment resulted in less metabolic variation than for animals from the same cohort that were returned to the laboratory in an attempt to allow their metabolome to equilibrate in controlled conditions.

Mammalian toxicogenomics studies have provided valuable information on baseline fluctuations in gene expression due to study condition and/or endogenous factors. A consortial effort was recently undertaken by the HESI Genomics Technical Committee to examine microarray data from control animals from toxicogenomics studies of rat liver and kidney (Boedigheimer et al. 2008). Gender, organ section, fasting state, and strain emerged as study factors that contributed highly to variability in gene expression, whereas other factors, such as age, vehicle administration route, sacrifice method, and

dose frequency were not major contributors to baseline variance. Genes with high variability, identified in the study, include many of interest in toxicology, such as those involved in xenobiotic metabolism, androgen and estrogen metabolism, steroid biosynthesis, and antigen processing and presentation. Low variance genes were also identified, and included those involved in protein metabolism and immune response. Such low variance genes may prove valuable as study controls. This collaborative effort determined the impacts of key study factors on measured gene expression in a toxicogenomics study and illustrated the importance of defining the baseline gene expression against which stressor-induced gene expression changes are to be evaluated.

Others have considered expression level variation as a genetic Quantitative Trait that can be used to identify loci that regulate gene expression and thus explain the mechanisms behind the variation (Williams et al. 2007). While it is possible to establish the importance of genetic factors in dictating mRNA expression level, variation between independent studies frequently generates conflicting data which have sometimes been attributed to a technical failure of array technology from differential hybridization of array probes by polymorphic transcripts (Alberts et al. 2007). In the area of proteomics, Hu et al. (2005) investigated variability in expression of proteins of human cerebrospinal fluid and Zhang et al. (2006) reported variability in protein expression between 12 human liver samples. These, and other studies on fish (George et al. pers. comm.), identified a very large dynamic range of variation in protein expression.

Experimental design and biological variation

Here we highlight recent findings from studies of variability within both the chemical risk assessment and environmental monitoring scenarios. Hines et al. (pers. comm.) recently conducted an extensive investigation into sources of metabolic variation in marine mussels sampled from the environment. An initial analysis of the entire metabolic dataset showed large variability but the majority of this could be rationalised in terms of season (i.e. month when animal was sampled), sex, species and site effects. This data suggests that information about these parameters is necessary for interpretation of inter-individual biological variation and to potentially reveal pollutant effects.

Similarly, many studies in both fish and rodents have highlighted the strong influence that parameters such as sex and stage of the reproductive cycle have on an individual's transcriptome and proteome, not only in the reproductive tissues but also in other commonly studied tissues such as the brain and liver. In many cases this can be attributed directly to interaction with sex hormone signalling pathways; however, more subtle effects can also be due to cross-talk between nuclear transcription factors. Therefore, it is essential to consider and document both the sex and the stage of the reproductive cycle in toxicological studies even when assessing chemicals which are not suspected of causing endocrine disruption. While differences in the gonad are to be expected, extensive sexually dimorphic gene expression is also found in somatic tissues in rodents (Boyle and Craft 2000; Waxman and O'Connor 2006; Yang et al. 2006), zebrafish (Robison et al. 2008; Santos et al. 2008; Sreenivasan et al. 2008) and *M. edulis* (Brown et al. 2006) and

is likely to occur across species and in particular in organisms developing as gonochoristic males or females. Environmental factors such as temperature and hypoxia have also been shown to strongly influence the transcriptomic and proteomic profiles in poikilotherms. Indeed the expression of several enzymes and proteins which activate and detoxify important chemical toxins, as well as those involved in RNA processing, translation initiation, mitochondrial metabolism, proteasomal function, and essential fatty acid synthesis in fish show a clear temperature dependence (Gracey et al. 2004). Thus the response to chemical exposure may be significantly modulated by the ambient temperature and this must be taken in to account in field sampling. The general recommendation of European legislative studies of chemical impacts is to standardise the sampling season to minimise effects of temperature and the nutritional status and to utilise animals (usually males) when they are either sexually immature or gonadally quiescent.

Key components that require attention to minimise variability, as discussed above, are sampling processes, sample isolation and storage, preparation of samples for analyses, numbers of replicates, design of platforms and methods of normalisation. Particularly important are study design and statistical analyses. Only with biological replicates is it possible to apply statistical tests. Statistical justification and formula presented by Kendziorski et al. (2003) and Peng et al. (2003) can be used to calculate the number of biological samples and pools needed for an appropriately powered analysis. For omics based studies the challenge in power analysis is how to determine the appropriate variance since tens of thousands of elements (i.e. genes, proteins or metabolites) are

measured simultaneously and each one possesses its own variance. One option is to use a pooled variance, such as the value estimated from PCA or other error pooling algorithms. The second option is to calculate the variance for each element and take the n^{th} percentile as the value to use. Usually, detection of small changes for elements with large variances is unlikely. By adjusting the variance value and the effect size (i.e. fold change), power analysis can be tailored to choose the sample size that fits the purpose and significance of an experiment. Power analysis requires a pre-existing data set that resembles the proposed project as much as possible in order to derive an accurately estimated variance. The perfect data set comes from a pilot study for the proposed project. When that is impossible, we recommend matching the biological aspects (e.g. species and tissues) before matching the technical aspects (e.g. technologies and platforms), assuming the technical variance is smaller than the biological variance.

References

de Koning DJ, Jaffrezic F, Lund MS, Watson M, Channing C, Hulsegge I, et al. 2007. The EADGENE Microarray Data Analysis Workshop. Genet Sel Evol 39(6): 621-631.

Fuscoe JC, Tong W, Shi L. 2007. QA/QC issues to aid regulatory acceptance of microarray gene expression data. Environ Mol Mutagen 48(5): 349-353.

Mattes WB. 2008. Public consortium efforts in toxicogenomics. Methods Mol Biol 460: 221-238.

Thompson KL, Hackett J. 2008. Quality control of microarray assays for toxicogenomic and in vitro diagnostic applications. Methods Mol Biol 460: 45-68

Walker MS, Hughes TA. 2008. Messenger RNA expression profiling using DNA microarray technology: diagnostic tool, scientific analysis or un-interpretable data? Int J Mol Med 21(1): 13-17.

Bammler T, Beyer RP, Bhattacharya S, Boorman GA, Boyles A, Bradford BU, et al. 2005. Standardizing global gene expression analysis between laboratories and across platforms. Nat Methods 2(5): 351-356

Kuo WP, Liu F, Trimarchi J, Punzo C, Lombardi M, Sarang J, et al. 2006. A sequence-oriented comparison of gene expression measurements across different hybridization-based technologies. Nat Biotechnol 24(7): 832-840

Shi L, Reid LH, Jones WD, Shippy R, Warrington JA, Baker SC, et al. 2006. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 24(9): 1151-1161.

Beyer RP, Fry RC, Lasarev MR, McConnachie LA, Meira LB, Palmer VS, et al. 2007. Multicenter study of acetaminophen hepatotoxicity reveals the importance of biological endpoints in genomic analyses. Toxicol Sci 99(1): 326-337.

Chen JJ, Hsueh HM, Delongchamp RR, Lin CJ, Tsai CA. 2007. Reproducibility of microarray data: a further analysis of microarray quality control (MAQC) data. BMC Bioinformatics 8: 412.

Arikawa E, Sun Y, Wang J, Zhou Q, Ning B, Dial SL, et al. 2008. Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. BMC Genomics 9: 328.

Fielden MR, Nie A, McMillian M, Elangbam CS, Trela BA, Yang Y, et al. 2008. Interlaboratory evaluation of genomic signatures for predicting carcinogenicity in the rat. Toxicol Sci 103(1): 28-34.

Kohlmann A, Kipps TJ, Rassenti LZ, Downing JR, Shurtleff SA, Mills KI, et al. 2008. An international standardization programme towards the application of gene expression profiling in routine leukaemia diagnostics: the Microarray Innovations in LEukemia study prephase. Br J Haematol 142(5): 802-807.

Canales RD, Luo Y, Willey JC, Austermiller B, Barbacioru CC, Boysen C, et al. 2006. Evaluation of DNA microarray results with quantitative gene expression platforms. Nat Biotechnol 24(9): 1115-1122.

Gebhardt R. 1992. Metabolic zonation of the liver: regulation and implications for liver function. Pharmacol Ther 53(3): 275-354.

Oinonen T, Lindros KO. 1998. Zonation of hepatic cytochrome P-450 expression and regulation. Biochem J 329 (Pt 1): 17-35.

Boedigheimer MJ, Wolfinger RD, Bass MB, Bushel PR, Chou JW, Cooper M, et al. 2008. Sources of variation in baseline gene expression levels from toxicogenomics study control animals across multiple laboratories. BMC Genomics 9: 285.

Thompson KL, Pine PS, Rosenzweig BA, Turpaz Y, Retief J. 2007. Characterization of the effect of sample quality on high density oligonucleotide microarray data using progressively degraded rat liver RNA. BMC Biotechnol 7: 57.

Patterson TA, Lobenhofer EK, Fulmer-Smentek SB, Collins PJ, Chu TM, Bao W, et al. 2006. Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project. Nat Biotechnol 24(9): 1140-1150.

Hershey AD, Burdine D, Liu C, Nick TG, Gilbert DL, Glauser TA. 2008. Assessing quality and normalization of microarrays: case studies using neurological genomic data. Acta Neurol Scand 118(1): 29-41.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4(2): 249-264.

Keun HC, Ebbels TM, Antti H, Bollard ME, Beckonert O, Schlotterbeck G, et al. 2002. Analytical reproducibility in (1)H NMR-based metabonomic urinalysis. Chem Res Toxicol 15(11): 1380-1386.

Bertram HC, Malmendal A, Petersen BO, Madsen JC, Pedersen H, Nielsen NC, et al. 2007. Effect of magnetic field strength on NMR-based metabonomic human urine data. Comparative study of 250, 400, 500, and 800 MHz. Anal Chem 79(18): 7110-7115.

Viant MR, Bearden DW, Bundy JG, Burton IW, Collette TW, Ekman DR, et al. 2009. International NMR-based environmental metabolomics intercomparison exercise. Environ Sci Technol 43(1): 219-225.

Thorpe KL, Hutchinson TH, Hetheridge MJ, Scholze M, Sumpter JP, Tyler CR. 2001. Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (Oncorhynchus mykiss). Environ Sci Technol 35(12): 2476-2481.

Thorpe KL, Cummings RI, Hutchinson TH, Scholze M, Brighty G, Sumpter JP, et al. 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ Sci Technol 37(6): 1142-1149.

Morrison N, Bearden D, Bundy JG, Collette T, Currie F, Davey P et al. 2007. Standard reporting requirements for biological samples in metabolomics experiments: environmental context. Metabolomics 3(3): 203-210.

Hines A, Oladiran GS, Bignell JP, Stentiford GD, Viant MR. 2007. Direct sampling of organisms from the field and knowledge of their phenotype: key recommendations for environmental metabolomics. Environ Sci Technol 41(9): 3375-3381.

Williams RB, Chan EK, Cowley MJ, Little PF. 2007. The influence of genetic variation on gene expression. Genome Res 17(12): 1707-1716.

Alberts R, Terpstra P, Li Y, Breitling R, Nap JP, Jansen RC. 2007. Sequence polymorphisms cause many false cis eQTLs. PLoS ONE 2(7): e622.

Hu Y, Malone JP, Fagan AM, Townsend RR, Holtzman DM. 2005. Comparative proteomic analysis of intra- and interindividual variation in human cerebrospinal fluid. Mol Cell Proteomics 4(12): 2000-2009.

Zhang X, Guo Y, Song Y, Sun W, Yu C, Zhao X, et al. 2006. Proteomic analysis of individual variation in normal livers of human beings using difference gel electrophoresis. Proteomics 6(19): 5260-5268.

Boyle SP, Craft JA. 2000. Gender-specific metabolism of benz[a]anthracene in hepatic microsomes from Long-Evans and Hooded Lister rats. Chem Biol Interact 125(3): 209-220

Waxman DJ, O'Connor C. 2006. Growth hormone regulation of sex-dependent liver gene expression. Mol Endocrinol 20(11): 2613-2629.

Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, et al. 2006. Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res 16(8): 995-1004.

Robison BD, Drew RE, Murdoch GK, Powell M, Rodnick KJ, Settles M, et al. 2008. Sexual dimorphism in hepatic gene expression and the response to dietary carbohydrate manipulation in the zebrafish (Danio rerio). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 3(2): 141-154.

Santos EM, Kille P, Workman VL, Paull GC, Tyler CR. 2008. Sexually dimorphic gene expression in the brains of mature zebrafish. Comp Biochem Physiol A Mol Integr Physiol 149(3): 314-324.

.

Sreenivasan R, Cai M, Bartfai R, Wang X, Christoffels A, Orban L. 2008. Transcriptomic analyses reveal novel genes with sexually dimorphic expression in the zebrafish gonad and brain. PLoS ONE 3(3): e1791.

Brown M, Davies IM, Moffat CF, Craft JA. 2006. Application of SSH and a macroarray to investigate altered gene expression in Mytilus edulis in response to exposure to benzo[a]pyrene. Mar Environ Res 62 Suppl: S128-135.

Gracey AY, Fraser EJ, Li W, Fang Y, Taylor RR, Rogers J, et al. 2004. Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. Proc Natl Acad Sci U S A 101(48): 16970-16975.

Kendziorski CM, Zhang Y, Lan H, Attie AD. 2003. The efficiency of pooling mRNA in microarray experiments. Biostatistics 4(3): 465-477.

Peng X, Wood CL, Blalock EM, Chen KC, Landfield PW, Stromberg AJ. 2003. Statistical implications of pooling RNA samples for microarray experiments. BMC Bioinformatics 4: 26.

Supplementary Table 1: Key examples of the successful use of toxicogenomics data in ecotoxicology.

A. Prospective Studies

APPLICATION	NATURE OF EXPOSURE	APPROACHES	SIGNIFICANCE OF RESULTS	REFERENCES
Chemical Signatures	Diet, static renewal, semi-static renewal or *ip* injection	Microarray	Demonstrates chemical class specific gene expression, and/or chemical specific signatures	Benninghoff and Williams 2008; Larkin et al. 2003; Hamadeh et al. 2002; McMillian et al 2005; Moens et al. 2006; Hook et al. 2006; Ellinger-Ziegelbauer et al. 2008
Mixtures	Hepatocytes in vitro	Microarray	Demonstrates that mixtures show attenuated biomarker responses compared to those of the chemical signatures of classical environmental toxicants	Finne et al. 2007
Endocrine Disruption Pathways	Flow-through	Microarray	Links reduced fecundity/population decline to perturbations in endocrine pathways, vitellogenin, steroid concentrations, gonad weight, and specific perturbations in gene expression	Miller et al. 2007; Ankley et al. 2008; Villeneuve et al. 2007; Hoffmann et al. 2008
Pathways of Toxicity	Injection	Microarray	Demonstrates that TCDD perturbs fin regeneration by impacting the expression of genes involved in extracellular matrix composition and cellular differentiation	Andreasen et al. 2006
Pathways of Toxicity	Static exposure	Microarray	Demonstrates heart-specific mechanisms of AhR/TCDD-mediated toxicity	Handley-Goldstone et al.

				2005; Carney et al. 2006
Pathways of Toxicity/ Biomarkers of Effect and Temporal Changes	Flow-through	Microarray	Used cDNA arrays and Q-PCR to identify potential indicators of thyroid axis and metamorphosis disruption in frogs	Helbing et al. 2007a; Helbing et al. 2007b
Dose-Response, Adaptive/Toxic Response	Static renewal	Microarray	Links gene expression changes to reduced growth.	Roling et al. 2006
Temporal and Adaptive Changes	Injection	Microarray	Demonstrates time-dependent adaptive changes prior to toxicity following TCDD treatment	Volz et al. 2006
Screening of Emerging Chemicals; Predicting Adverse Outcomes	Flow-through	Microarray	Uses microarrays to determine the potential mechanisms of PFOA toxicity	Wei et al. 2008
Emerging Chemicals	Static	Microarray	Uses microarrays to demonstrate differential effects of nanoparticles and their constituents	Griffitt et al. 2007
Inter-laboratory Comparisons	Flow-through, other	Microarray	Meta-analysis of data from environmental estrogen exposure that produced new, sensitive biomarkers of exposure	Gunnarsson et al. 2007
Adaptive/Toxic Response	Static renewal	Microarray	Demonstrates metal specific gene expression in response to copper, cadmium, and zinc. Proposes novel modes of toxicant action.	Poynton et al. 2007
Computational	Flow-through	Microarray	Assesses sources of variation in fish microarray experiments. Chemical class prediction using bioinformatic classification software such as Support vector machines	Wang et al. 2008a, Wang et al. 2008b
Chemical Signatures/ Adaptive Response	Flow-through	Metabolomics	Demonstrates compensatory mechanisms and adaptive recovery from an estrogen. Also demonstrates the potential of metabolomics in	Ekman et al. 2007

			ecotoxicology.	
Temporal Changes	Flow-through	Proteomics	Provides early response indicators to thyroid hormones in frogs	Domanski and Helbing 2007
Chemical signatures	laboratory	Metabolomics	A mechanism of action from combination of NMR metabolite profiling and neural network classification	Ott et al 2003

B. Diagnostic Studies

APPLICATION	NATURE OF EXPOSURE	APPROACHES	SIGNIFICANCE OF RESULTS	REFERENCES
Population Genetics	None/unknown field	Microarray	Demonstrate variation in gene expression and potential difficulties of using particular genes as biomarkers	Oleksiak et al. 2002; Oleksiak et al. 2005
Sample Monitoring	Wastewater treatment	Microarray	Effect of Mixtures; endocrine disruptors and no-endocrine disruptors	Filby et al. 2007b; Garcia-Reyero et al. 2008; Filby et al. 2007c
Sample Monitoring	Field	qRT-PCR	Demonstrates that seal thyroid hormones are sensitive to disruption by pollutant stress	Tabuchi et al. 2006
Population Genetics	Field	Microarray	Demonstrates differential adaptation to distinctly different field sites	Larsen et al. 2007
Chemical Remediation	Field	Microarray	Demonstrates the utility of microarrays as an additional weight-of-evidence approach to monitor remediation at a polluted site	Roling et al. 2007
Site Monitoring and computational	Field combined with laboratory	Microarray, Differential display	Demonstrates differential expression at polluted and reference sites, and demonstrates distinct differences between males and females in the pollutant responses. Prediction of environmental source of fish by stress gene responses	Williams et al. 2003; Falciani et al 2008, Meyer et al. 2005
Site Monitoring	Field	Subtractive hybridization, Differential display	Demonstrates differential expression at polluted and references site. Links to lab work to field sampling	Maples and Bain 2004; Roling et al. 2004
Computational	Field	Metabolomics	Improved discrimination between sample	Parsons et al. 2007

			classes compared to unscaled, autoscaled or Pareto scaled data. Leads to better site classification	
Site monitoring	Field	Metabolomics	Demonstrates differential metabolic fingerprints in earthworms (*Lumbricus rubellus*) from sites with differing metal contamination.	Bundy et al. 2007
Site monitoring	Field	Metabolomics	Demonstrates differential metabolic fingerprints in marine mussels (*Mytilus edulis*) at polluted and reference sites.	Viant, unpublished

REFERENCES

Benninghoff AD, Williams DE. 2008. Identification of a transcriptional fingerprint of estrogen exposure in rainbow trout liver. Toxicol Sci 101(1): 65-80.

Larkin P, Folmar LC, Hemmer MJ, Poston AJ, Denslow ND. 2003. Expression profiling of estrogenic compounds using a sheepshead minnow cDNA macroarray. EHP Toxicogenomics 111(1T): 29-36.

Hamadeh HK, Bushel PR, Jayadev S, Martin K, DiSorbo O, Sieber S, et al. 2002. Gene expression analysis reveals chemical-specific profiles. Toxicol Sci 67(2): 219-231.

McMillian M, Nie A, Parker JB, Leone A, Kemmerer M, Bryant S, et al. 2005. Drug-induced oxidative stress in rat liver from a toxicogenomics perspective. Toxicol Appl Pharmacol 207(2 Suppl): 171-178.

Moens LN, van der Ven K, Van Remortel P, Del-Favero J, De Coen WM. 2006. Expression profiling of endocrine-disrupting compounds using a customized Cyprinus carpio cDNA microarray. Toxicol Sci 93(2): 298-310.

Hook SE, Skillman AD, Small JA, Schultz IR. 2006. Gene expression patterns in rainbow trout, Oncorhynchus mykiss, exposed to a suite of model toxicants. Aquat Toxicol 77(4): 372-385.

Ellinger-Ziegelbauer H, Gmuender H, Bandenburg A, Ahr HJ. 2008. Prediction of a carcinogenic potential of rat hepatocarcinogens using toxicogenomics analysis of short-term in vivo studies. Mutat Res 637(1-2): 23-39.

Finne EF, Cooper GA, Koop BF, Hylland K, Tollefsen KE. 2007. Toxicogenomic responses in rainbow trout (Oncorhynchus mykiss) hepatocytes exposed to model chemicals and a synthetic mixture. Aquat Toxicol 81(3): 293-303.

Miller DH, Jensen KM, Villeneuve DL, Kahl MD, Makynen EA, Durhan EJ, et al. 2007. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (Pimephales promelas). Environ Toxicol Chem 26(3): 521-527.

Ankley GT, Miller DH, Jensen KM, Villeneuve DL, Martinovic D. 2008. Relationship of plasma sex steroid concentrations in female fathead minnows to reproductive success and population status. Aquat Toxicol 88(1): 69-74

Villeneuve DL, Larkin P, Knoebl I, Miracle AL, Kahl MD, Jensen KM, et al. 2007. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. Environ Sci Technol 41(1): 321-330.

Hoffmann JL, Thomason RG, Lee DM, Brill JL, Price BB, Carr GJ, et al. 2008. Hepatic gene expression profiling using genechips in zebrafish exposed to 17alpha-methyldihydrotestosterone. Aquat Toxicol 87(2): 69-80.

Andreasen EA, Mathew LK, Tanguay RL. 2006. Regenerative growth is impacted by TCDD: gene expression analysis reveals extracellular matrix modulation. Toxicol Sci 92(1): 254-269.

Handley-Goldstone HM, Grow MW, Stegeman JJ. 2005. Cardiovascular gene expression profiles of dioxin exposure in zebrafish embryos. Toxicol Sci 85(1): 683-693.

Carney SA, Chen J, Burns CG, Xiong KM, Peterson RE, Heideman W. 2006. Aryl hydrocarbon receptor activation produces heart-specific transcriptional and toxic responses in developing zebrafish. Mol Pharmacol 70(2): 549-561.

Helbing CC, Bailey CM, Ji L, Gunderson MP, Zhang F, Veldhoen N, et al. 2007. Identification of gene expression indicators for thyroid axis disruption in a Xenopus laevis metamorphosis screening assay. Part 1. Effects on the brain. Aquat Toxicol 82(4): 227-241.

Helbing CC, Ji L, Bailey CM, Veldhoen N, Zhang F, Holcombe GW, et al. 2007. Identification of gene expression indicators for thyroid axis disruption in a Xenopus laevis metamorphosis screening assay. Part 2. Effects on the tail and hindlimb. Aquat Toxicol 82(4): 215-226.

Roling JA, Bain LJ, Gardea-Torresdey J, Bader J, Baldwin WS. 2006. Hexavalent chromium reduces larval growth and alters gene expression in mummichog (Fundulus heteroclitus). Environ Toxicol Chem 25(10): 2725-2733.

Volz DC, Hinton DE, Law JM, Kullman SW. 2006. Dynamic gene expression changes precede dioxin-induced liver pathogenesis in medaka fish. Toxicol Sci 89(2): 524-534.

Wei Y, Liu Y, Wang J, Tao Y, Dai J. 2008. Toxicogenomic analysis of the hepatic effects of perfluorooctanoic acid on rare minnows (Gobiocypris rarus). Toxicol Appl Pharmacol 226(3): 285-297.

Griffitt RJ, Weil R, Hyndman KA, Denslow ND, Powers K, Taylor D, et al. 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (Danio rerio). Environ Sci Technol 41(23): 8178-8186.

Gunnarsson L, Kristiansson E, Forlin L, Nerman O, Larsson DG. 2007. Sensitive and robust gene expression changes in fish exposed to estrogen--a microarray approach. BMC Genomics 8: 149.

Poynton HC, Varshavsky JR, Chang B, Cavigiolio G, Chan S, Holman PS, et al. 2007. Daphnia magna ecotoxicogenomics provides mechanistic insights into metal toxicity. Environ Sci Technol 41(3): 1044-1050.

Wang RL, Bencic D, Biales A, Lattier D, Kostich M, Villeneuve D, et al. 2008. DNA microarray-based ecotoxicological biomarker discovery in a small fish model species. Environ Toxicol Chem 27(3): 664-675.

Wang RL, Biales A, Bencic D, Lattier D, Kostich M, Villeneuve D, Ankley GT, Lazorchak J, Toth G. 2008. DNA microarray application in ecotoxicology: experimental design, microarray scanning, and factors affecting transcriptional profiles in a small fish species. Environ Toxicol Chem; 27: 652-663.

Ekman DR, Teng Q, Jensen KM, Martinovic D, Villeneuve DL, Ankley GT, et al. 2007. NMR analysis of male fathead minnow urinary metabolites: a potential approach for studying impacts of chemical exposures. Aquat Toxicol 85(2): 104-112.

Domanski D, Helbing CC. 2007. Analysis of the Rana catesbeiana tadpole tail fin proteome and phosphoproteome during T3-induced apoptosis: identification of a novel type I keratin. BMC Dev Biol 7: 94.

Ott KH, Aranibar N, Singh B, Stockton GW. Metabonomics classifies pathways affected by bioactive compounds. Artificial neural network classification of NMR spectra of plant extracts. Phytochemistry 2003; 62: 971-985.

Oleksiak MF, Churchill GA, Crawford DL. 2002. Variation in gene expression within and among natural populations. Nat Genet 32(2): 261-266.
Oleksiak MF, Roach JL, Crawford DL. 2005. Natural variation in cardiac metabolism and gene expression in Fundulus heteroclitus. Nat Genet 37(1): 67-72.

Filby AL, Santos EM, Thorpe KL, Maack G, Tyler CR. 2007b. Gene expression profiling for understanding chemical causation of biological effects for complex mixtures: a case study on estrogens. Environ Sci Technol 41(23): 8187-8194.

Filby AL, Thorpe KL, Maack G, Tyler CR. 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. Aquat Toxicol 81(2): 219-231.

Garcia-Reyero N, Adelman I, Liu L, Denslow N. 2008. Gene expression profiles of fathead minnows exposed to surface waters above and below a sewage treatment plant in Minnesota. Mar Environ Res 66(1): 134-136.

Tabuchi M, Veldhoen N, Dangerfield N, Jeffries S, Helbing CC, Ross PS. 2006. PCB-related alteration of thyroid hormones and thyroid hormone receptor gene expression in free-ranging harbor seals (Phoca vitulina). Environ Health Perspect 114(7): 1024-1031.

Larsen PF, Nielsen EE, Williams TD, Hemmer-Hansen J, Chipman JK, Kruhoffer M, et al. 2007. Adaptive differences in gene expression in European flounder (Platichthys flesus). Mol Ecol 16(22): 4674-4683.

Roling JA, Bain LJ, Gardea-Torresdey J, Key PB, Baldwin WS. 2007. Using mummichog (Fundulus heteroclitus) arrays to monitor the effectiveness of remediation at a superfund site in Charleston, South Carolina, U.S.A. Environ Toxicol Chem 26(6): 1205-1213.

Williams TD, Gensberg K, Minchin SD, Chipman JK. 2003. A DNA expression array to detect toxic stress response in European flounder (Platichthys flesus). Aquat Toxicol 65(2): 141-157.

Falciani F, Diab AM, Sabine V, Williams TD, Ortega F, George SG, et al. 2008. Hepatic transcriptomic profiles of European flounder (Platichthys flesus) from field sites and computational approaches to predict site from stress gene responses following exposure to model toxicants. Aquat Toxicol 90(2): 92-101

Meyer JN, Volz DC, Freedman JH, Giulio RT. 2005. Differential display of hepatic mRNA from killifish (Fundulus heteroclitus) inhabiting a Superfund estuary. Aquat Toxicol 73(4): 327-341.

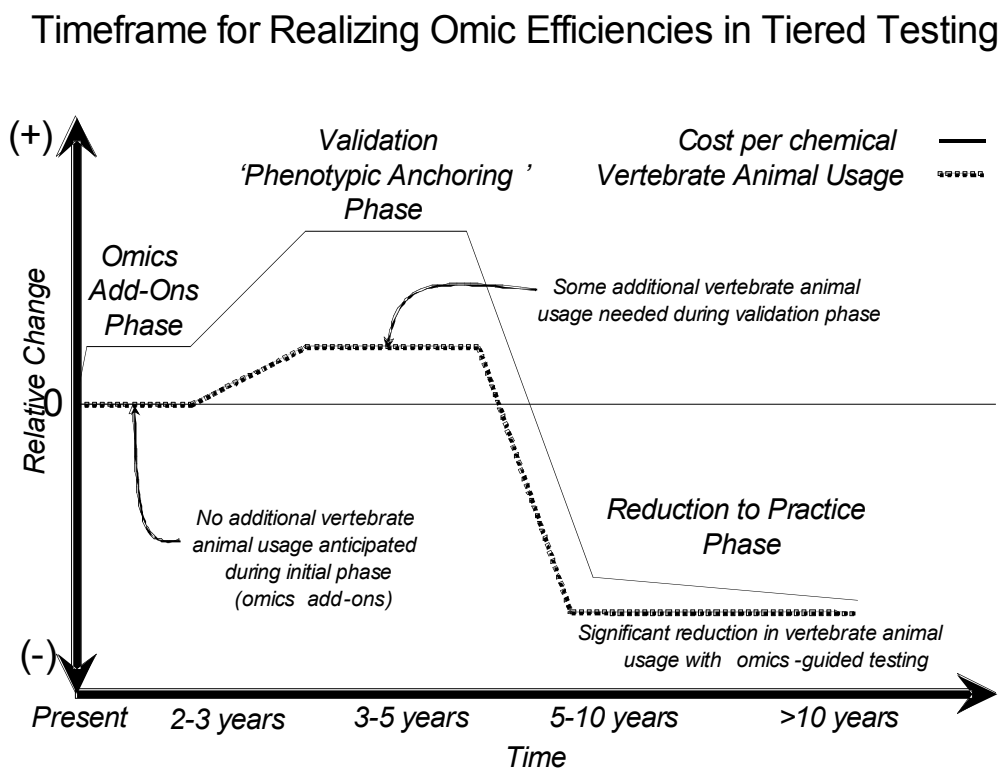
Maples NL, Bain LJ. 2004. Trivalent chromium alters gene expression in the mummichog (Fundulus heteroclitus). Environ Toxicol Chem 23(3): 626-631.

Roling JA, Bain LJ, Baldwin WS. 2004. Differential gene expression in mummichogs (Fundulus heteroclitus) following treatment with pyrene: comparison to a creosote contaminated site. Mar Environ Res 57(5): 377-395.

Parsons HM, Ludwig C, Gunther UL, Viant MR. 2007. Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. BMC Bioinformatics 8: 234

Bundy JG, Keun HC, Sidhu JK, Spurgeon DJ, Svendsen C, Kille P, et al. 2007. Metabolic profile biomarkers of metal contamination in a sentinel terrestrial species are applicable across multiple sites. Environ Sci Technol 41(12): 4458-4464.

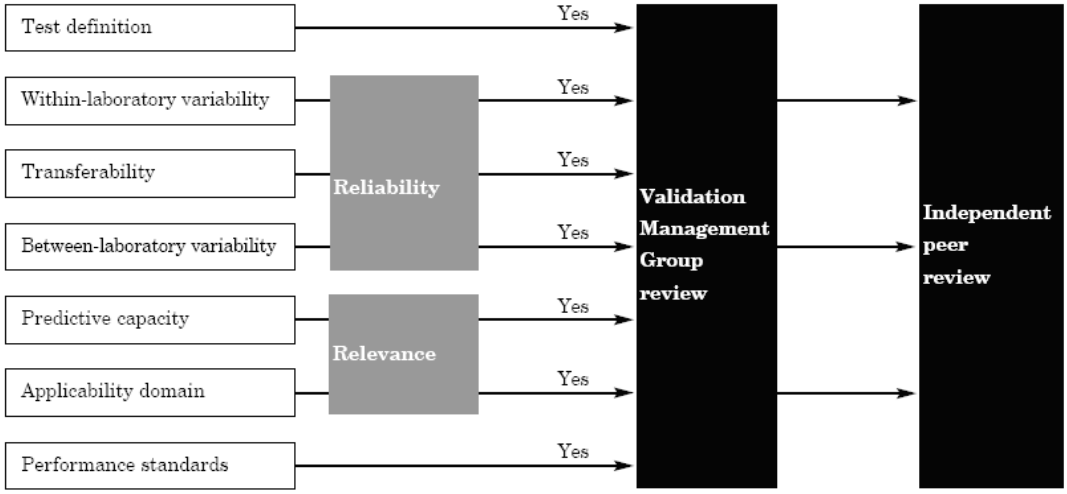
Supplementary Figure 1.



Reproduced from Ankley et al (2006), with permission.

Supplementary Figure 2.

The modular approach for applying the ECVAM principles on test validity



A "yes" indicates that the appropriate information for the module is adequate for entrance into the peer-review process. All seven modules have to be satisfactorily completed, as judged by the Validation Management Group, before a method can enter the peer-review process.

ECVAM = European Centre for the Validation of Alternative Methods.

Reproduced from Hartung et al (2004), with permission.