Literature Search on Effects-Based Biomonitoring Tools for Ecological Risk Assessment

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DRAFT March 11, 2011

Introduction

Historically, a majority of the ecological risk assessment efforts for pollutants in the Great Lakes have focused on monitoring programs that rely on chemical measurements in different matrices (e.g., water, sediments, tissues), primarily providing spatio-temporal information. However, relatively little work has been conducted systematically to determine the biological effects that may be occurring as a result of exposure to potentially toxic substances in the Great Lakes. There are also some important drawbacks to only using analytical approaches to monitor for contaminants of possible concern, including (1) lack of detection of "unknown" chemicals possibly responsible for adverse biological effects, (2) uncertainty that measurements of known chemicals have adequate detection limits compared to concentrations that elicit possible biological effects, and (3) inability to reconstruct possible biological effects of chemical mixtures. Past regulatory and monitoring efforts have recognized these drawbacks and addressed them through the use of biological effects-based testing to complement chemical analyses. For example, whole effluent toxicity testing is routinely used in permitting surface water discharges to address the uncertainties associated with chemical monitoring alone.

To address the lack of ecological effects data for compounds of emerging concern, the US Environmental Protection Agency (EPA) prepared an initial draft Strategy for Assessing Exposure to and Effects of Toxic Substances in the Great Lakes (Strategy). The Strategy describes actions that need to be taken to improve the ecological risk assessment of potentially toxic substances in the aquatic environment of the Great Lakes. To complete the draft Strategy, a more thorough understanding was needed of available effects-based monitoring tools for ecological risk assessment. A review of the available literature was conducted to collect more comprehensive information that will be used to inform Strategy rationale and approaches for the use of systematic, effects-based methods for monitoring the ecological risk of compounds of emerging concern in the Great Lakes aquatic environment.

Method

Battelle conducted a review of the published scientific literature to identify biological effects-based tests and endpoints that have been or can be used for environmental monitoring of aquatic vertebrates and invertebrates. Targeted searches of the published and 'grey' literature were conducted in June 2010 and repeated in October 2010. For the published literature, scientific citation indexing and search services were used (Web of Knowledge and Science Direct databases) to identify articles dating from 1995 to the present. Table 1 lists the key search terms, reference databases searched, and number of citations that met the search criteria for each database or web resource searched. For the grey literature, websites of agencies and organizations involved in ecological monitoring were searched. A summary of online ecotoxicology resources searched is presented as Attachment 1.

Table 1. Key Search Terms, Reference Databases Searched, and Number of Citations That Met the Search Criteria

Source	Concepts/Search Statements	Hits
ISI Web of Knowledge/Science http://www.isiwebofknowledge.com/ Limits for all searches: 1995 to October 2, 2010, English only, Databases: Science Citation Index Expanded (SCI-EXPANDED), Conference Proceedings Citation Index-Science (CPCI-S)	TS=(great lakes) AND TS=(tox* OR ecotox*) AND TS=(aquatic animal* OR aquatic vertebrate* OR aquatic invertebrate* OR fish OR aquatic microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*) AND TS=(assay* OR bioassay* OR endpoint* OR biomarker* OR biotest* OR "in vivo" OR "in vitro" OR molecular endpoint* OR biochemical endpoint* OR histological endpoint* OR apical endpoint* OR acute toxicity OR chronic toxicity OR ecological risk assess* OR environmental risk assess* OR hazard assess*)	136
	TI=(ecotox*) AND TS=(aquatic animal* OR aquatic vertebrate* OR aquatic invertebrate* OR fish OR aquatic microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*) AND TS=(tox* or ecotox*)	521
	TI=(assay* OR bioassay* OR endpoint* OR biomarker* OR biotest* OR "in vivo" OR "in vitro" OR molecular endpoint* OR biochemical endpoint* OR histological endpoint* OR apical endpoint* OR acute toxicity OR chronic toxicity OR ecological risk assess* OR environmental risk assess* OR hazard assess*) AND TS=(aquatic invertebrate* OR aquatic microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton) AND TS=(tox* or ecotox*)	1529
	TI=(assay* OR bioassay* OR endpoint* OR biomarker* OR biotest* OR "in vivo" OR "in vitro" OR molecular endpoint* OR biochemical endpoint* OR histological endpoint* OR apical endpoint* OR acute toxicity OR chronic toxicity OR ecological risk assess* OR environmental risk assess* OR hazard assess*) AND TS=(fish) AND TS=(tox* or ecotox*)	1250
	TI=(assay* OR bioassay* OR endpoint* OR biomarker* OR biotest* OR "in vivo" OR "in vitro" OR molecular endpoint* OR biochemical endpoint* OR histological endpoint* OR apical endpoint* OR acute toxicity OR chronic toxicity OR ecological risk assess* OR environmental risk assess* OR hazard assess*) AND TS=(aquatic animal OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*) AND TS=(tox* or ecotox*)	227
	TS=(steroid* OR endocrin* OR estrogen* OR androgen* OR ethinylestradiol OR estradiol OR testosterone) AND TS=(tox* OR ecotox* OR intox*) AND (TS=(aquatic OR marine OR ocean OR freshwater OR saltwater OR seawater OR water* OR lake* OR surface water* OR sediment* OR benthic OR effluent* OR discharge* OR wastewater*) AND TS=(aquatic animal* OR aquatic vertebrate* OR aquatic invertebrate* OR fish OR aquatic microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*))	814
	TS=("mode of action" OR "mechanism of action" OR MOA OR "Adverse Outcome Pathway" OR AOP) AND TS=(aquatic animal* OR aquatic vertebrate* OR aquatic invertebrate* OR fish OR aquatic microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*) AND TS=(tox* OR ecotox*)	486
	TS/TI=("aryl hydrocarbon receptor" OR ArH OR EROD OR ethoxyresorufin-O-deethylase OR H42E OR H4IIE OR vitellogenin OR VTG) AND TS=(aquatic animal* OR aquatic vertebrate* OR aquatic invertebrate* OR fish OR aquatic microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*) AND TS=(tox* OR ecotox*)	869
	TI=(Genom* OR toxicogenom* OR transcriptomics OR proteomics OR metabolomics OR gene* expression OR protein* expression) AND TS=(aquatic animal* OR aquatic vertebrate* OR aquatic invertebrate* OR fish OR aquatic	379

Source	Concepts/Search Statements	Hits
	microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*) AND TS=(tox* OR ecotox*)	
	TS=(("Toxicity Identification Evaluation" TIE OR effect*-directed analysis OR bioassay-directed analysis) AND (aquatic OR marine OR ocean OR freshwater OR saltwater OR seawater OR water* OR lake* OR surface water OR sediment* OR benthic or effluent* OR discharge*))	225
	TS=(pharmaceutical* OR personal care product* OR flame retardant* OR endocrine disrupt*) AND TS=(aquatic animal* OR aquatic vertebrate* OR aquatic invertebrate* OR fish OR aquatic microorganism* OR aquatic arthropod* OR aquatic insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR aquatic bird* OR avian OR amphibian* OR aquatic mammal* OR mink* OR beaver* OR otter*) AND TS=(tox* or ecotox*) AND TI=(assay* OR bioassay* OR endpoint* OR biomarker* OR biotest* OR "in vivo" OR "in vitro" OR molecular endpoint* OR biochemical endpoint* OR histological endpoint* OR apical endpoint* OR acute toxicity OR chronic toxicity OR ecological risk assess* OR environmental risk assess* OR hazard assess*)	159
	TOTAL ISI WOS MERGED	6708
	Duplicates	1508
	TOTAL ISI WOS before SCREENING	5200
Science Direct http://www.sciencedirect.com/ Limits for all searches: 1995 to October 6, 2010, English only [* = wildcard]	TITLE(aquatic OR marine OR ocean OR *water* OR lake* OR sediment* OR benthic OR effluent* OR discharge* OR wastewater*) and TITLE(*tox*) and TITLE(assay* OR bioassay* OR *endpoint* OR biomarker* OR biotest* OR {in vivo} OR {in vitro} OR {acute toxicity} OR {chronic toxicity} OR {ecological risk assess*} OR {environmental risk assess*})	340
	TITLE(aquatic OR marine OR ocean OR *water* OR lake* OR sediment* OR benthic OR effluent* OR discharge* OR wastewater*) and TITLE(*tox*) and TITLE-ABSTR-KEY(assay* OR bioassay* OR *endpoint* OR biomarker* OR biotest* OR {in vivo} OR {in vitro} OR {acute toxicity} OR {chronic toxicity} OR {ecological risk assess*} OR {environmental risk assess*}) and TITLE-ABSTR-KEY(animal* OR *vertebrate* OR fish OR *organism* OR arthropod* OR insect* OR amphipod* OR daphni* OR mysid* OR mussel* OR urchin* OR polychaete* OR larva* OR zooplankton OR bird* OR avian OR amphibian* OR mammal* OR mink* OR beaver* OR otter*)	594
	TITLE(aquatic OR marine OR ocean OR *water* OR lake* OR sediment* OR benthic OR effluent* OR discharge* OR wastewater*) and TITLE(*tox*) and TITLE-ABSTR-KEY(assay* OR bioassay* OR *endpoint* OR biomarker* OR biotest* OR {in vivo} OR {in vitro} OR {acute toxicity} OR {chronic toxicity} OR {ecological risk assess*} OR {environmental risk assess*}) and TITLE-ABSTR-KEY(hormone* OR steroid* OR endocrin* OR estrogen* OR androgen* OR *estradiol OR testosterone)	47
	TITLE(aquatic OR marine OR ocean OR *water* OR lake* OR sediment* OR benthic OR effluent* OR discharge* OR wastewater*) and TITLE(*tox*) and TITLE-ABSTR-KEY(assay* OR bioassay* OR *endpoint* OR biomarker* OR biotest* OR {in vivo} OR {in vitro} OR {acute toxicity} OR {chronic toxicity} OR {ecological risk assess*} OR {environmental risk assess*}) and TITLE-ABSTR-KEY({mode of action} OR {mechanism of action} OR MOA OR {Adverse Outcome Pathway} OR AOP)	21
	TITLE(aquatic OR marine OR ocean OR *water* OR lake* OR sediment* OR benthic OR effluent* OR discharge* OR wastewater*) and TITLE(*tox*) and TITLE-ABSTR-KEY(assay* OR bioassay* OR *endpoint* OR biomarker* OR biotest* OR {in vivo} OR {in vitro} OR {acute toxicity} OR {chronic toxicity} OR {ecological risk assess*} OR {environmental risk assess*}) and TITLE-ABSTR-KEY({aryl hydrocarbon receptor} OR ArH OR EROD OR {ethoxyresorufin-O-deethylase} OR H42E OR H4IIE OR vitellogenin OR VTG)	33
	TITLE(aquatic OR marine OR ocean OR *water* OR lake* OR sediment* OR benthic OR effluent* OR discharge* OR	14

Source	Concepts/Search Statements	Hits
	wastewater*) and TITLE(*tox*) and TITLE-ABSTR-KEY(assay* OR bioassay* OR *endpoint* OR biomarker* OR biotest* OR {in vivo} OR {in vitro} OR {acute toxicity} OR {chronic toxicity} OR {ecological risk assess*} OR {environmental risk assess*}) and TITLE-ABSTR-KEY(Genom* OR toxicogenom* OR transcriptomic* OR proteomic* OR metabolomic* OR {gene* expression} OR {protein* expression})	
	TITLE(aquatic OR marine OR ocean OR *water* OR lake* OR sediment* OR benthic OR effluent* OR discharge* OR wastewater*) and TITLE(*tox*) and TITLE-ABSTR-KEY({Toxicity Identification Evaluation} TIE OR {effect*-directed analysis} OR {bioassay-directed analysis})	40
	TOTAL SciDirect	1092
	TOTAL SciDirect AFTER REMOVAL OF DUPLICATES	783

The relevance of the studies and reports results (citations and abstracts) was determined based on the following criteria:

1. Whether the study/report addresses the toxicity of substances found in the environmental media of interest (fresh/saltwater, sediments/porewater, effluent, tissues/tissue extracts), AND
2. Whether the study/report addresses biological tests/endpoints that measure toxic effects in aquatic invertebrates and vertebrates (including fish, amphibians, freshwater aquatic mammals, and fish-eating birds), AND
3. Whether the study/report includes a focus on some aspect of the test/method/endpoint – such as method comparisons, investigation of the ecological relevance of a method, new method development, or validation or use of an existing method for a new application or chemical of emerging concern – rather than simply a toxicological characterization of a particular chemical or mixture. Literature was not considered relevant if it primarily reported an investigation of the toxicity of a specific chemical or mixture for reporting/data collection purposes.

If all three relevance criteria above were met, the study/report was considered a key reference and was reviewed for the literature assessment. If one or two of the relevance criteria were not met, the study/report was considered lower priority for inclusion in the literature review, but the reference citation itself was retained in the database/library of literature search results. If none of the relevance criteria were met, the study/report was not considered relevant and it was not included in the database/library of literature search results. Mathematical/statistical model-based tools for estimating toxicity based on predicted mode of action (MOA) (e.g., quantitative structure-activity relationships) were not considered within the scope of the project.

Relevant references were then reviewed with the following primary objectives:

- Identify and summarize in vivo and in vitro assays/endpoints that have been used for effects-based environmental monitoring.
- Assess the degree to which the endpoint(s) reflects generic versus specific effects (including, for specific endpoints, the pathway(s) affected, e.g., chemical activation of the estrogen receptor for vitellogenin in male fish).
- Assess the degree to which the assay/endpoints can be related to an adverse response at the individual or population level.
- Assess whether the endpoint/assay would be considered an "off the shelf" measurement (e.g., validated system with strong scientific underpinnings, readily available through commercial, government, and/or other sources, etc.) versus a research tool that may not be ready for broad deployment or use (this point also should consider the degree of validation and standardization that has been conducted).
- Assess the types of matrices to which the assay/endpoint has been successfully applied (e.g., fresh- versus salt-water; whole waters, effluents, sediments, tissues—or extracts thereof, etc.).
- Consider the relative sensitivity of the assay/endpoint. Different endpoints will differ in sensitivity (e.g., lethality versus activation of a biochemical receptor), and consideration of varying sensitivities may help in the selection of monitoring tools for the Strategy.

Results

Search Results

Searches of the scientific and grey literature returned over 6,000 articles and reports that were published since 1995. Approximately 3,000 articles were published between 2005 and 2010. Due to resource constraints, only review articles published prior to 2005 were reviewed. A full listing of the 6,193 bibliographic citations obtained during the literature search is provided in Appendix A (an EndNote® library of the citations is available upon request).

Of the 3,000 articles published since 2005, methods and endpoints for ecological risk assessment were sorted into the following major categories of methods:

1. Traditional survival, growth, development, reproduction endpoints
2. Methods focused on organ-based system responses
3. Biochemical markers/enzyme activity/protein-based measurements
4. "Omic" technologies and global assessment of mRNA, protein and metabolite abundance
5. Genotoxicity and mutagenicity
6. Behavioral endpoints

Some methods were relevant to more than one category. The methods identified are catalogued in an Excel spreadsheet presented in Appendix B. For each test method, the Excel spreadsheet identifies the test type (e.g., in vitro or in vivo, lab or field) target organism(s), exposure media, test duration, biological level(s) of effect, endpoint specificity, endpoint category, key literature references, and other information pertaining to the test method. Appendix C contains the abstracts of articles represented in the Excel spreadsheet.

Among the methods and endpoints extracted from the literature, some general findings are as follows:

- The status of method validation varied from extensively validated to unvalidated, proposed new method.
- Methods ranged from those used for regulatory purposes (e.g., NPDES, TMDLs, EDSP, SIDS, TIEs) to proposed new research tools.
- Many studies incorporated a variety of different methods for purposes of trying to validate or compare new methods to well-established standard methods.
- There was a trend toward approaches that integrate a variety of endpoints (e.g., tying gene expression to physiological endpoints).
- Incorporating or linking behavioral endpoints with other endpoints may be increasing. There are many studies in the recent literature describing integrated behavioral endpoints.
- A majority of methods involved laboratory exposures; few assessed in situ field exposures.
- Biological level of effect varied, ranging from sub-cellular to tissue/organ level to individual and population level.
- Studies addressing population-level effects appear to be relatively limited.

Results for each major category or organizing theme are presented below.

Traditional Survival, Growth, Development, and Reproduction Endpoints

Traditional methods that assess survival, growth, development, and reproduction endpoints were grouped into one category. These methods include standard acute and chronic toxicity tests that are applicable to fresh and saltwater fish and invertebrates (e.g., crustaceans, insects), amphibians, and birds associated with aquatic systems (e.g., mallard duck, quail). Table 2 presents a few examples of traditional methods for assessing ecotoxicity in freshwater. The examples presented focus on freshwater for their relevance to the Great Lakes. The methods in this category largely include assessments of controlled laboratory exposures but also organisms exposed in situ. Many are widely used, validated tests used for regulatory purposes.

Short term reproduction assays are typically not used for regulatory purposes but can be a sensitive "effects window" for certain toxicants. The traditional acute and chronic toxicity tests in fish are sensitive to a wide variety of chemicals but are particularly sensitive to certain classes such as organochlorine and pyrethroid pesticides, and certain metals. Protocols for freshwater amphipod sediment toxicity with *Hyalella azteca* are among the most sensitive available for sediment quality assessments.

Table 2. Examples of Traditional Survival, Growth, Development, and Reproduction Endpoints

Test Method	Target Organism	Test Type (Duration)	Biological Level of Effect	Test Status
Cladoceran Acute and Chronic Neonate Toxicity Test	Water flea, daphnid, cladoceran	In vivo lab (24 h to 21 d)	Whole organism	Validated, widely used
Migratory Bird Acute Toxicity	Mallard ducks	In vivo lab (30 d)	Whole organism	Validated
Freshwater Insect Sediment/Water Toxicity Test	Midge, mayfly	In vivo lab (10-65 d)	Whole organism	Validated, widely used
Freshwater Amphipod Sediment Toxicity Test	Amphipod	In vivo lab (10-42 d)	Whole organism	Validated, widely used
Fish Embryo/Larvae Acute and Chronic Toxicity Test	Many species of freshwater & saltwater fish	In vivo lab (varies)	Whole organism	Validated, widely used

Methods Focused on Organ-Based System Responses

This category includes methods that assess endpoints focused on biological system-based responses such as Hypothalamic-Pituitary-Adrenal (HPA) axis, Hypothalamic-Pituitary-Gonadal (HPG) axis, Hypothalamic-Pituitary-Thyroid (HPT) axis, and other endocrine system responses. Methods in this category also assess neurotoxicity, which has a role in determining some behavioral effects. Immune function and disease susceptibility are also important and are considered an "organ-based response." Endpoints at this biological level provide a bridge between cellular/sub-cellular endpoints and whole-organism endpoints. A given system or axis may involve several modes of action. For endocrine responses, several amphibian and fish methodologies are well-developed or validated, and are available for regulatory purposes. For

example, the Amphibian Metamorphosis Assay is an in vivo screening assay that identifies substances that interfere with the HPT axis in the African clawed frog (*Xenopus laevis*). The assay incorporates measurements of thyroid hormones in addition to external morphological and histological evaluations.

Emerging responses include olfactory toxicity (which might also be considered a form of neurotoxicity) and its association with food and predator recognition. For endocrine-associated toxicity, circulating levels of specific hormones can be useful if they are measured in conjunction with other phenotypic markers such as reproductive status, condition index, and histopathology (when available). Endocrine associated toxicity may be especially important in organisms transitioning between life history stages (e.g., larval to juvenile, and juvenile to reproductively active adult).

Table 3 presents a few examples of methods focused on organ-based system responses. Some methods can assess effects at multiple levels of biological organization. For example, fish full-lifecycle/multi-generational studies of reproductive success represent the most comprehensive approach to detect possible effects on the whole organism but are more directly applicable to population-level effects. These studies are also very resource intensive; assays can take 6 to 9 months to complete, compared to other methods that can be completed within a few weeks.

Table 3. Examples of Methods Focused on Organ-Based System Responses

Test Method	Target Organism	Test Type (Duration)	Biological Level of Effect	Test Status
EDSP Amphibian Metamorphosis Assays	Various species of amphibians	In vivo lab (21 d)	Organ system, Whole organism	Validated
Fish Integrated Reproductive Toxicity Test	Fathead minnow, medaka, zebrafish	In vivo lab (21 d)	Whole organism	Validated
Fish Full-lifecycle / Multi-generational Studies of Reproductive Success	Fathead minnow, medaka, zebrafish, rainbow trout	In vivo lab (weeks to months)	Whole organism, population level	Not yet validated

Biochemical Markers/Enzyme Activity/Protein-Based Measurements

Biochemical markers such as enzyme activity or protein-based measurements are widely used and developed for environmental monitoring. Measurements in this category range from markers related to redox status (e.g., GSH/GSSG, superoxide dismutase activity), reproduction-associated proteins (e.g., vitellogenin or VTG), and stress response pathways (e.g., heat shock protein) to cell proliferation and differentiation (e.g., AhR receptor).

The endpoints assessed typically provide information on specific aspects of cellular/sub-cellular function that are associated with a toxic MOA or are more reflective of cell death/necrosis (e.g., acute toxicity). Examples of endpoints associated with a toxic MOA would include Ah receptor-mediated induction of P450 enzymes (similar to the MOA for dioxin), metallothionein induction (as in cadmium and certain metal toxicities), circulating levels of VTG in males, and many others. Examples of endpoints that reflect acute toxicity would include measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), which reflect acute liver toxicity and cell death.

Table 4 presents a few examples of biochemical markers, enzyme activity, and protein-based measurements. VTG is among the best established biomarkers, useful for detecting contaminants acting as an estrogen agonist (e.g., when VTG is induced in males) or antagonist (when decreased synthesis is observed in reproductively active females). VTG can also be measured in vitro, using cultured hepatocytes. For example, the estrogenic activity of phthalate esters was evaluated using a VTG assay with amphibian hepatocytes (Nomura et al. 2006), and the anti-estrogenic activity of two pharmaceuticals, letrozole and tamoxifen, were evaluated in zebrafish using changes in VTG synthesis (Sun et al. 2010). VTG is often measured in conjunction with observations such as gross reproductive organ morphology and secondary sex characteristics. Some fish such as the Japanese medaka may show less sensitivity towards VTG responses when exposed to estrogens compared to other fish, such as zebrafish (Ma et al. 2007; Orn et al. 2006).

Induction of Ethoxyresorufin O-deethylase (EROD) can be an extremely sensitive indicator of environmental alterations and is usually one of the first detectable, quantifiable responses to exposure of substances that have dioxin-like activity. EROD activity describes the rate of the CYP1A-mediated deethylation of the substrate 7-ER to form resorufin, which indicates the potential for AhR ligands to induce biochemical change (enzyme induction by planar hydrocarbons). EROD measurement techniques have been employed in many diverse species of fish from a broad range of habitat types. EROD is now a widely measured endpoint, as it can represent the cumulative impact of all inducing chemicals with a similar MOA, whether or not they are detected analytically (Whyte et al. 2000). EROD measurements are relatively simple to perform and thus can provide a relatively rapid indication of the bioaccumulation of important classes of toxicants such as co-planar PCBs. For this reason, EROD is often termed an "early warning system" (Whyte et al. 2000).

Note that there are in vitro systems, like the H4IIE cell bioassay, which can be used in combination with other biomarkers such as in vivo measurements of CYP1A1 induction to help pinpoint the sources and identities of chemical contaminants. The H4IIE bioassay is used in the Biomonitoring of Environmental Status and Trends (BEST) program as a Tier I method for screening environmental samples for dioxin-like activity (BEST 1996).

An additional set of biochemical markers that are important for assessing oxidative stress in organisms include glutathione status (GSH/GSSG; ratio of reduced vs. oxidized glutathione) and the activities of several enzymes directly or indirectly involved in protecting against cellular oxidative damage (superoxide dismutase, catalase, glutathione s-transferase (GST), glutathione peroxidise and glutathione reductase (GR)). Other commonly used markers of oxidative stress reflect oxidative changes to lipids. One of the more widely applied assays is TBARS (2-thibarbituric acid reactive substance). For example, Oakes et al. (2005) demonstrated elevated hepatic TBARS in rainbow trout exposed to kraft pulp-mill effluents.

Table 4. Examples of Biochemical Markers/Enzyme Activity/Protein-Based Measurements

Test Method	Target Organism	Test Type (Duration)	Biological Level of Effect	Test Status
Vitellogenin (VTG) in Fish	Many species of freshwater and marine fish	Lab or field (varies)	Whole organism, Cellular/sub-cellular	Validated, widely used
Ethoxyresorufin O-deethylase (EROD) Assay	Fish, birds (Great Blue Heron), invertebrates (mussels, crayfish)	Lab	Cellular/sub-cellular	Validated, widely used
Oxidative stress biomarkers (e.g., GST)	Freshwater fish (guppy) & invertebrates (mussel, crayfish, Daphnia)	Lab/field	Cellular/sub-cellular, Organ system	Widely used

"Omic" Technologies and Global Assessment of mRNA, Protein and Metabolite Abundance

During the past five years, emerging "omic" technologies that measure global changes in gene expression (transcriptomics), protein (proteomics) and metabolite abundance (metabolomics) have seen a rapid increase in ecotoxicological applications. For example, of approximately 350 articles containing "omic" in the title or abstract (e.g., genomics, ecotoxicogenomics, proteomics), approximately 100 were published prior to 2005 and 250 were published in the past 5 years (2005 to 2010). The large majority of these studies were laboratory exposures using a few, well-established model organisms. However, in the past 2-3 years, a growing number of studies used an "omic" method on aquatic organisms collected or exposed in the field. For example, Yudkovski et al. (2010) assessed the differences in the hepatic transcriptome of striped seabream (*Lithognathus mormyrus*) to distinguish between six different contaminated field sites. Another example is by Garcia-Reyero et al. (2009), who measured transcriptomic changes in the liver and gonad of fathead minnows that were exposed in-situ (by caging) to five different waste water treatment effluents.

Among the various "omic" methodologies, microarray-based transcriptomic studies were the most typical type of method used. Several studies were able to demonstrate the diagnostic and predictive capability of transcriptomics for identifying specific class of toxicant or mode of action. For example, Popesku et al. (2010) used a microarray approach to identify certain pulp mill effluents that altered dopaminergic sensitive pathways in the hypothalamus of fathead minnows. Another good example is a recent study by Taylor et al. (2010), which demonstrated the diagnostic capability of metabolomic analysis from studies with *Daphnia* exposed to four different types of model toxicants. It was also noted that an increasing number of studies combined "omic" measurements with endpoints measured at higher levels of biological organization, which strengthened the correlation between gene expression or protein abundance and toxicity or survival. One of the most ambitious efforts in this respect is the study by Cooke et al. (2008), who combined measurements of gene expression with whole animal measurements and migratory behavior of salmon to provide analysis that included population-level effects. Another example is provided in the study by Bugiak and Weber (2010), which assessed changes in select mRNA levels in zebrafish larvae along with cardiac defects observed after various chemical exposures.

Table 5 presents a few examples of "omic" technologies and gene and protein expression bioassays.

Table 5. Examples of "Omic" Technologies and Gene and Protein Expression Bioassays

Test Method	Target Organism	Test Type (Duration)	Biological Level of Effect	Test Status
DNA Microarrays	Fish (e.g., fathead minnow, zebrafish, rainbow trout)	In vivo lab (acute / chronic) & field	Cellular/sub-cellular	Research/ Dev phase
Proteomics	Fish, invertebrates	In vivo lab & field	Cellular/sub-cellular, Organ system	Research/ Dev phase
Metabolomics	Fish, mollusks	In vivo lab & field	Cellular/sub-cellular, Organ system	Research/ Dev phase

Measurements of Genotoxicity and Mutagenicity

Genotoxicity and mutagenicity endpoints are another type of sub-cellular response that is often associated with chronic disease such as cancer or reproductive impairment (including gamete and embryo viability). Many types of in vitro and in vivo genotoxicity assays are included in this category. Many are well-established methods for detecting specific types of DNA damage such as base-pair modifications or single and double DNA strand breaks. For example, the 'comet' assay, also called the single cell gel electrophoresis assay, is now a widely used method to assess the extent of DNA damage within an individual cell. The comet assay and other genotoxicity assays are useful in a wide range of applications, including monitoring contamination of the environment by genotoxic agents.

Table 6 presents a few examples of measurements of genotoxicity and mutagenicity. In many assessments, researchers use basic exposure protocols for sediment/water and then perform tests of DNA damage and repair.

Table 6. Examples of Measurements of Genotoxicity and Mutagenicity

Test Method	Target Organism	Test Type (Duration)	Biological Level of Effect	Test Status
Freshwater Mollusc genotoxicity assays (micronucleus assay; comet assay, DNA damage)	Zebra mussel, mussel	Lab (2 months)	Cellular/sub-cellular, Organ/ System	Research/Dev phase
Freshwater Amphibian genotoxicity (micronucleus assay; comet assay)	Green frog, American toad	Lab (7-14 d)	Cellular/sub-cellular, Whole organism	Widely used
Fish Genotoxicity Assays (comet and micronucleus assays)	Tilapia RTL-W1 fish cell line	Lab	Cellular/sub-cellular, Whole organism	Research/Dev phase
Genetic Ecotoxicology	Fish / invertebrates	In situ / field	Population	Research/Dev phase

Behavioral Endpoints

Measurements in this category include assessments of behavioral responses to exposure such as feeding, mating, nesting, parental care, and other behaviors. Studies to date primarily appear to focus on behaviors related to reproductive success (e.g., mating behaviors, aggression, nest building, choice of nest location, egg laying). Measurements include both short- and long-term in vivo lab assessments of individual behavior. A potential advantage of behavioral endpoints is in the detection of emerging chemicals or chemicals with neurological effects, which may elicit more pronounced behavioral changes before effects are detectable or recognized at other biological levels of organization. Several studies have been able to correlate behavioral changes with measurable physiological effects and pathway- or mechanism-specific modes of toxic action.

Table 7 presents examples of assessments of behavioral endpoints. Behavioral assessments in the field are difficult to conduct and, as a result, are not widely used. However, some methods that assess behavioral endpoints have found programmatic applications. For example, the Freshwater Oligochaete Sediment-Water Toxicity Test is included in the international Organization for Economic Development (OECD) test guidelines and USEPA Toxicity Identification Evaluation (TIE) procedures.

Behavioral endpoints may not always be the primary focus of a test, but behaviors may be observed as part of another category of test method. For example, the Freshwater Oligochaete Sediment-Water Toxicity Test assesses the effects of prolonged exposure to sediment-associated chemicals on reproduction, biomass, and mortality, in addition to abnormal behavior (e.g., sediment avoidance, failure to burrow or ingest sediment). The oligochaete used in this test, *Lumbriculus variegates,* is widely used in sediment toxicity tests, and its behavior can be compared to controls for additional information that may be helpful in the interpretation of test results with respect to exposure pathways.

Table 7. Examples of Behavioral Endpoints

Test Method	Target Organism	Test Type (Duration)	Biological Level of Effect	Test Status
Freshwater Oligochaete Sediment-Water Toxicity Test	Oligochaetes	In vivo lab (28 days)	Whole organism	Protocol developed
Freshwater Amphipod Acute Water Toxicity	Freshwater amphipod species (*Gammarus* genus, *Hyalella azteca*)	In vivo lab (96 hours)	Whole organism	Protocol developed
Freshwater Amphipod Behavioral Bioassays	*Gammarus pulex, Gammarus fossarum*	In situ lab or field	Whole organism	Research/ Dev phase

Conclusions

The best characterized (longest history of use) and most widely applied effects-based methods have been those that primarily rely on apical endpoints, measured at the whole organism level of biological organization. With regard to toxic mode of action, some apical endpoints are generic, such as acute lethality, or they can be more specific to an organ system, such as impaired reproduction. Some advantages of these types of endpoints are that they provide an immediate

and empirically verifiable toxic outcome of an exposure and are often associated with fully validated tests using standardized exposure protocols. Many of these tests and endpoints have been demonstrated to be effective at detecting diverse types of toxicants in a wide range of aquatic species. Acute toxicity tests or longer exposures that incorporate non-lethal apical endpoints appear to be most commonly used in the laboratory with single chemical exposures or with environmental media collected from the field (e.g., contaminated sediments). Less often, in situ exposures are employed using caged animals deployed at field sites. A comparatively fewer number of studies or tests were reported that directly studied feral animals. Some of the endpoints that were used in field studies were nutritional status (type and quantity of lipid stores), immune status as reflected by disease/parasite incidence, and in a few cases behavioral responses. An important value of field monitoring with feral or naturally occurring animals is the ability to directly assess toxicity at the population or community level of biological organization. The study by Wagner et al. (2010) is a good example of a field study attempting to link individual and population level effects.

A notable trend observed in the literature survey results was the increasing use of endpoints reflecting cellular or sub-cellular responses. Some of the more common endpoints were VTG induction in male animals, EROD activity as a biomarker of Ah receptor agonism, and endpoints reflecting redox status or oxidative stress (GSSG-GSH, GST activity, lipid oxidation products). In many cases, these endpoints were applied to archived tissue collected from feral animals in addition to laboratory-based studies. Endpoints and assays measured at the cellular/sub-cellular level can be useful for more specifically identifying the toxic mode of action. The integration of methods from different levels of organization (e.g., traditional apical endpoints and sub-cellular biomarkers) was thought to provide a time/cost-effective means of assessing multiple levels of sensitivity and biological organization. It would also appear that the more useful in vitro based assays are those that measure endpoints that are hardy and persistent in field situations, as opposed to those endpoints that are subject to postmortem degradation from factors such as temperature and time.

It was also apparent from the literature review that a diverse range of mode of action terms have been used, which often varies by the applicable level of biological organization. For example, some mode of action terms such as CNS seizure, respiratory inhibition and narcosis can reflect broad impacts on organ systems while others such as acetylcholinesterase inhibition reflect a specific molecular target. Thus, it would be helpful for future diagnostic assessments to have a list of currently accepted mode of action terms and where possible, include chemical classes or specific chemical agents that are known to be representative of a particular mode of action.

Monitoring Ecotoxicological Effects in the Short Term

Based on the number and types of studies employing various endpoints and their potential for medium to high throughput testing, promising assays/endpoints in the short term for monitoring toxic chemical effects would include:

- In vitro cell cultures (e.g., H4IIE) or reporter gene bioassays (e.g., EPA method 4425) for specific types of receptor mediated toxicity are useful in multiple media (e.g., sediment, water extracts).

- In vitro hormone receptor binding assays can identify agonist/antagonist actions of a chemical or mixtures.
- The EROD assay has been widely used to detect Ah receptor interaction in fish, birds, and invertebrates.
- VTG induction in male fish or inhibition/decreased expression in female fish can detect estrogenic and anti-estrogenic substances. Measurements of the VTG protein in blood plasma, liver, and whole fish homogenates have been successfully used, depending on species.
- Histopathological analysis, especially assessment of gonad histology (e.g., inter-sex).
- External morphological changes such as those associated with sexual dimorphism or tumor formation.

Monitoring Ecotoxicological Effects in the Long Term

Some methods and endpoints offer high promise for high throughput testing and/or specificity towards toxic mode of action but appear to be still in the developmental phase and require further validation before routine use in effects-based monitoring. For example, several of the "omic" methods listed below have been developed to detect gene/protein/metabolite changes in response to exposure to pharmaceuticals and endocrine disruptors. These endpoints appear to be particularly valuable for early warning/detection of initial toxic events. However, additional research is needed to determine mechanisms of action for various chemical classes to enable detection of chemicals in complex mixtures.

- Transcriptomics methods, either DNA microarray or emerging RNA sequencing based. This endpoint would be considered the best established of the "omic" methods and has been used with the widest range of ecologically important species. These tools appear viable for non-model species. There is a need to better understand or differentiate generic stress responses from toxicant specific or mode of action specific changes.
- Proteomics methods, now most commonly employing mass spectroscopy, but can also rely on gel electrophoresis and immunological methods.
- Metabolomics, NMR or MS based, have seen recent increases in ecotoxicological applications.

For genomics and proteomics tools, a baseline understanding of genomes and biological processes is needed. However, the continued growth of bioinformatic knowledge for eco-relevant species is expected to improve this understanding and enhance the value for effects-based monitoring. Also, the use of "omic" endpoints with feral animals or in situ exposures would be enhanced by the incorporation of standardized toxico- "omic" protocols using specific species/life stage/gender to minimize confounding effects of inter-species differences and reproductive or developmental status. Phenotypic anchoring (integration of "omics" with higher level endpoints) appears to a valuable approach for establishing the diagnostic power of these endpoints. Based on the current survey of the literature, it has already been demonstrated that "omic" methods can be used in conjunction with lab-based in vitro or in vivo assays using complex mixtures from the field, such as discharge or receiving waters or sediments/sediment fractions. Samples also could be from organisms held in situ (e.g., caged animal studies) or animals collected from extant populations.

The results of the literature review suggest that there are available effects-based monitoring tools that can be used for systematic, effects-based monitoring of the ecological risk of compounds of emerging concern in the Great Lakes aquatic environment. Commonly used assays and methods can be employed in the short-term to support an effects-based monitoring program, and more cutting-edge methods and endpoints can be developed for incorporation into future monitoring efforts.

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Attachment 1. Summary of Online Ecotoxicology Resources Searched

Source	Details/Findings
General search terms only used: e.g., aquatic toxicology, (eco)toxicology, aquatic toxicity tests/bioassays/endpoints	
California Environmental Protection Agency (CalEPA)	- Office of Environmental Health Hazard Assessment http://www.oehha.ca.gov/index.html - (2004) Overview of Freshwater and Marine Toxicity Tests http://www.oehha.ca.gov/ecotox/pdf/marinetox_2004.pdf - Ecotoxicology Program http://www.oehha.ca.gov/ecotox.html - Cal/ECOTOX database http://www.oehha.ca.gov/cal_ecotox/
EC European Chemical Substances Information System (ESIS)	- Primarily access to programmatic data only on toxicity of specific chemicals http://ecb.jrc.ec.europa.eu/esis/
European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC)	http://www.ecetoc.org/index.php - Misc technical reports, workshop proceedings, etc related to ecotox and toxicogenomics (see publications catalog http://www.ecetoc.org/uploads/Publications/Catalogue%20of%20ECETOC%20Publications%20Dec-2010.pdf)
Environment Canada	http://www.ec.gc.ca/default.asp?lang=En&n=FD9B0E51-1 - EC Biological Test Method Series http://www.ec.gc.ca/faunescience-wildlifescience/default.asp?lang=En&n=0BB80E7B-1 (~30 publications re: traditional acute toxicity endpoints of survival, growth/development, reproduction)
National Environmental Methods Index (NEMI)	- Toxicity Methods Index: links back to EPA-OW methods https://www.nemi.gov/apex/f?p=237:1:414708723055592::NO::P1_CUR_TAB:T - Biological Methods index: links to misc (NOAA, USGS, etc) methods for community monitoring https://www.nemi.gov/apex/f?p=237:1:414708723055592::NO::P1_CUR_TAB:B
NIH National Toxicology Program (NTP)	Focus on human health http://ntp.niehs.nih.gov/ - NTP High Throughput Screening Initiative http://ntp.niehs.nih.gov/?objectid=05F80E15-F1F6-975E-77DDEDBDF3B941CD - NTP Roadmap http://ntp.niehs.nih.gov/files/NTPrdmp.pdf - NIEHS Strategic Plan http://www.niehs.nih.gov/about/od/strategicplan/docs/strategic-plan06.pdf - NTP Results and Status Search (National Toxicology Program) http://ntp.niehs.nih.gov:8080/index.html?col=010stat
Organization for Economic Co-operation and Development (OECD)	- Guidelines for the Testing of Chemicals http://www.oecd.org/document/40/0,3343,en_2649_34377_37051368_1_1_1_1,00.html: >25 methods relevant to toxicity assessment in aquatic biotic systems - HPV Chemical Screening Information Datasets (SIDS) supporting documentation -- various test method guidance documents http://www.oecd.org/document/21/0,3343,en_2649_34379_1939669_1_1_1_1,00.html - Annex I: OECD Test Guidelines for Studies Included in the SIDS (http://www.oecd.org/document/23/0,2340,en_2649_34379_1948503_1_1_1_1,00.html) - OECD Guidelines for the Testing of Chemicals (section included on biotic tox) http://www.oecd.org/document/40/0,3343,en_2649_34377_37051368_1_1_1_1,00.html) - OECD Fish Embryo Tox Testing document (http://www.oecd.org/dataoecd/39/62/36817242.pdf)
OPP Pesticide Ecotoxicity Database	- Chemical specific ecotoxicity data for pesticides http://www.ipmcenters.org/Ecotox/DataAccess.cfm;
Society of Environmental Toxicology and Chemistry (SETAC)	- Society of Environmental Toxicology and Chemistry (SETAC) http://www.setac.org/ - WET technical issue paper http://www.setac.org/sites/default/files/TIP-WET.pdf

Source	Details/Findings
Southern California Coastal Water Research Project (SCCWRP)	http://www.sccwrp.org/ - Contaminants research program http://www.sccwrp.org/ResearchAreas/Contaminants.aspx - Effects on Biota Group http://www.sccwrp.org/ResearchAreas/Contaminants/ContaminantsOfEmergingConcern/EffectsOnBiota.aspx - Gene microarray, effects of EDCs on fish http://www.sccwrp.org/ResearchAreas/Contaminants/ContaminantsOfEmergingConcern/EffectsOnBiota/CausesOfEndocrineDisruptionInFish.aspx - Molecular tools for TIES review http://www.sccwrp.org/ResearchAreas/Contaminants/ToxicityAssessmentAndIdentification/MolecularToolsForToxicityIdentificationEvaluation.aspx
TOXNET	- Covered via ISI Web of Science searches: NIH site http://toxnet.nlm.nih.gov/ for simultaneous searches of numerous chemical toxicity databases and clearinghouses, including HSDB, IRIS, GENE-TOX, CCRIS, TOXLINE, DART/ETIC, ITER PubMed; much included data are human health focused, but some ecotox information available.
USEPA ECOTOX Database	http://cfpub.epa.gov/ecotox/ - Toxicity data for aquatic life, terrestrial plants and wildlife; from papers published in the peer-reviewed literature, and data sets compiled by OECD member nations, EPA's Office of Pesticides, and Office of Research and Development, the US Geological Survey, and Russia. Updated quarterly.
USEPA Endocrine Disruptor Screening Program (EDSP)	http://www.epa.gov/endo/ - Includes documentation on status of assay development and validation (http://www.epa.gov/endo/pubs/assayvalidation/index.htm)
USEPA High Production Volume Chemical Information System (HPVIS)	http://www.epa.gov/hpvis/ - Aquatic Toxicity Endpoints used in this program overlap with OECD EDSP http://iaspub.epa.gov/oppthpv/metadata.homepage_metadata?p_discipline=EcoToxicity
USEPA/NCCT ACTOR: ACToR (Aggregated Computational Toxicology Resource)	- Collection of databases collated or developed by the US EPA National Center for Computational Toxicology (NCCT). More than 200 sources of publicly available data on environmental chemicals; searchable by in vitro assay data and in vivo toxicology data. http://actor.epa.gov/actor/faces/ACToRHome.jsp;jsessionid=315F08C3FB323C19680A82BE44587C74
US Geological Survey (USGS)	USGS Contaminant Biology Program http://biology.usgs.gov/contaminant/index.html - Toxicology Research Section http://biology.usgs.gov/contaminant/toxicology.html: >50 possibly relevant studies on development and validation o f Biomarkers and Other Methods for Toxicity Assessment, Aquatic Toxicology, Wildlife Toxicology, and Endocrine Disruption USGS Toxic Substances Hydrology Program - Emerging contaminants research http://toxics.usgs.gov/regional/emc/ - Endocrine Disruptors http://toxics.usgs.gov/regional/emc/endocrine_disruption.html USGS Columbia Environmental Research Center - Aquatic Toxicology Program http://www.ecrc.usgs.gov/ScienceTopics.aspx?ScienceTopicId=2: > 25 possibly relevant studies on development, application, and validation of methods to assess the effects of contaminants, degraded water quality, and other environmental stressors on aquatic organisms
Water Environment Research Foundation (WERF)	http://www.werf.org/ - Jan 2010 Workshop Report on Progress in Determining Aquatic Impacts of TOrCs http://www.werf.org/AM/Template.cfm?Section=Water_Reuse&CONTENTID=13398&TEMPLATE=/CM/HTMLDisplay.cfm