Contaminants of Emerging Concern in the Great Lakes

Science to Inform Management Practices for Protecting the Health and Integrity of Wildlife Populations from Adverse Effects

GLRI Action Plan I, Focus Area 1, Goal 5

by

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Dedication


This report is dedicated to Tom Custer, in recognition of his 44 years of federal service, his impact and productivity as a scientist, and the inspiring energy and enthusiasm that he brought to all his work to protect Great Lakes ecosystems.

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Executive Summary

Under Action Plan I (2010-2014) of the Great Lakes Restoration Initiative (GLRI), Federal and Academic partners began an investigation of the presence and distribution of contaminants of emerging concern (CECs) in the Great Lakes and potential impacts on fish and wildlife. The term CECs is applied to a broad range of chemicals that are currently in use but for which we currently lack good understanding of whether fish, wildlife, or humans are being exposed and/or whether negative health or environmental effects are expected if exposure occurs. Pharmaceuticals, personal care products, flame retardants, many current use pesticides, and poly- and perfluorinated chemicals are some well-known groups of CECs, but there is no definitive or comprehensive list that can be used to support the management of CECs to reduce impacts on the Great Lakes ecosystem.

Four overarching goals were identified for this collaborative investigation:

1. Evaluate the sources, occurrence, and distribution of CECs across the Great Lakes Basin.
2. Examine associations between the distribution of CECs and land-use patterns.
3. Review both scientific literature and field-generated data to determine the potential for CECs to cause adverse effects on Great Lakes fish and wildlife populations.
4. Develop efficient strategies to survey and/or monitor for threats that CECs may pose in order to take effective management actions before those threats evolve into large scale impacts on Great Lakes ecosystems or the services they provide.

Achievement of these goals ensures progress towards Focus Area 1: Toxic Substances and Areas of Concern from GLRI Action Plan I, Goal 5: "*The health and integrity of wildlife populations and habitat are protected from adverse chemical and biological effects associated with the presence of toxic substances in the Great Lakes Basin*".

This large-scale research effort was comprised of individual and collaborative projects from multiple federal agencies and academic institutions, involving over 85 investigators, and overseen by the U.S. Environmental Protection Agency (EPA) Region 5, Great Lakes National Program Office. Partners include the United States Geological Survey, the National Oceanic and Atmospheric Administration, U.S. Fish and Wildlife Service, Saint Cloud State University, the U.S. EPA Office of Research and Development, and the U.S. Army Corps of Engineers.

Key findings:

1. ***Contaminants of emerging concern were found throughout the monitored Great Lakes tributaries, but types and concentrations vary in association with regional land use.*** CECs were detected in nearly all samples collected. The type and concentration of the specific contaminants detected varied considerably among field sites and in association with land use type, such as urban, agricultural, wetland,

or forest. Contaminants were detected in the water column, sediment, and tissues of all species surveyed in the current work (mussels, aquatic insects, fish, and insect-eating birds).

2. ***There were over 20 contaminants for which CEC concentrations approached or exceeded those reported to cause toxicity in laboratory experiments.*** This was based on detection in water, sediments and or biota at one or more field sites. These contaminants represent compounds that warrant further investigation and monitoring with respect to potential impacts in certain areas of the Great Lakes basin. Based on the present investigation, compounds of greatest concern include: polycyclic aromatic hydrocarbons, associated with oil-based products and combustion of organic matter; atrazine, an herbicide; dichlorvos, an insecticide; and ibuprofen and venlafaxine, both pharmaceuticals.

3. ***Results suggest that mixtures of CECs presently found in most Great Lakes tributary locations surveyed may elicit subtle biological effects, but likely are not, alone, causing obvious detriment to current communities of fish and wildlife.*** CECs detected in the Great Lakes were associated with subtle biological effects like changes in gene expression, altered circulating glucose, etc. in both wild-caught and laboratory-reared organisms. These effects were generally not indicative of reproductive failure or mortality. However, the effects may have more serious implications when combined with other sources of stress like habitat degradation, changing climate conditions, and competition with invasive species. Due to limited historical data, it is unknown whether severe CEC-related impacts have already affected aquatic communities in waterbodies that have received long-term inputs of these contaminants. Likewise, under Action Plan I, biological effects were not necessarily evaluated at the sites where CEC concentrations exceeding laboratory toxicity thresholds were detected. As a result, strategic, ongoing surveillance and monitoring of CECs is warranted.

This collaborative investigation resulted in new tools, approaches, and data that can be used to inform and support the management of CECs to reduce their impacts on Great Lakes natural resources. The following products of this research effort are available through https://communities.geoplatform.gov/glri/ or by contacting the investigators (see technical chapters found in Appendices A-F):

1. **Database of CEC occurrence and concentrations in US tributary streams**. The database includes CEC detections in water, sediment, and fish and wildlife tissues, and represents the most comprehensive survey of CECs in the Great Lakes Region.
2. **Synopses of results and key findings.** Integrated summaries of results, conclusions, and management implications of the CEC research are available through reports, topical fact sheets, and presentations.
3. **Technical publications**: This collaborative research effort has resulted in over 50 peer-reviewed publications, agency reports, and data releases that can be of use to resource managers, the scientific community, and members of the public.
4. **Innovative tools.** Innovative monitoring devices, sampling equipment, conceptual frameworks, and software applications were developed over the course of this

research. These tools are transferable to stakeholders via internet accessibility or via specifications, instructions, and demonstration detailed in technical publications.

Hypotheses to guide CECs research under Action Plan II. Findings from 2010-2014 were used to guide further research in 2015-2018 for basin-wide surveillance of CECs and for sites warranting further study of potential biological impacts of CECs. Additional surveillance included both evaluation of additional classes of contaminants and expanded lists for chemical classes shown to be of greatest concern. Mixtures of some of the most frequently detected contaminants were also tested in laboratory studies to understand whether long term exposures to multiple contaminants may result in effects not evident from uncontrolled, short-term field experiments.

Introduction

The Great Lakes Restoration Initiative (GLRI) accelerates efforts to protect and restore the Great Lakes, the largest system of fresh surface water in the world. Built upon the foundation of the Great Lakes Regional Collaboration Strategy, GLRI answered a challenge from the governors of the Great Lakes states to commit to creating a new standard of care that will leave the Great Lakes better for the next generation. Since 2010, the multi-agency GLRI has provided funding to strategically target the greatest threats to the Great Lakes ecosystem and to accelerate progress toward achieving long term goals, including protecting the health and integrity of wildlife populations and habitat from adverse chemical and biological effects associated with the presence of toxic substances.

Under GLRI Action Plan I, two broad classes of toxic substances were identified:

1. Legacy contaminants –identified as persistent, bioaccumulative, and toxic pollutants largely left over from past practices, but that continue to recirculate through the ecosystem (GLRI 2010). Examples of legacy contaminants include polychlorinated biphenyls, mercury, polychlorinated dioxins and furans, and several highly persistent pesticides (e.g., DDT, aldrin, dieldrin, chlordane, etc.). These chemicals are known to be persistent, bioaccumulative, and toxic.

2. Contaminants of emerging concern (CECs) – identified as chemicals detected in the Great Lakes over the past several years that may pose threats to the ecosystem (GLRI 2010). As it is used here, the term CECs applies to a broad diversity of chemical structures used in a range of commercial products including flame retardants (e.g., polybrominated diphenyl ethers; PBDEs), household chemicals, pharmaceuticals and personal care products (PPCPs), current use pest control agents, as well as a variety of industrial chemicals. Broadly speaking, some unifying attributes of CECs include a lack of regulatory standards, recent discovery/detection of occurrence in the environment, and limited information regarding effects on aquatic life at environmentally relevant concentrations. A few CECs, such as bisphenol A, 4-nonylphenol, and some per- and polyfluorinated alkyl substances (PFAS), have received considerable public attention and a greater level of study. Additionally, compounds including PAHs and widely-used agricultural chemicals, including atrazine, have also been well-studied and have existing regulatory standards, but are included as CECs for the purposes of this report due to their on-going input into aquatic systems.

To better understand the potential threats CECs may pose to the health and integrity of Great Lakes wildlife populations, several specific research objectives were defined:

1. ***Evaluate the sources, occurrence, and distribution of CECs across the Great Lakes Basin.*** This effort involved using analytical chemistry methods to quantify concentrations of selected CECs in water, sediment, and wildlife tissues that were collected from sites across the Great Lakes basin. Emphasis was

placed on tributary streams rather than open lake or nearshore habitats, as tributaries provide pathways for CECs to enter the Great Lakes.

2. ***Examine associations between the distribution of CECs and land-use patterns.*** Land use in the Great Lakes Basin varies, and a range of environments (e.g., urban, agricultural, wetland) can be found. Evaluating CECs in relationship to land use helps to identify predictive approaches for identifying locations around the Great Lakes region that might be more, or less, likely to have certain types and concentrations of CECs. The ability to associate CECs with specific land use types may also inform development of CEC source reduction strategies.

3. ***Review both existing data and research and novel field-generated data to determine the potential for adverse effects from CECs on Great Lakes fish and wildlife populations.*** Existing toxicity information was used in conjunction with new sources of data or models in order to better understand potential differences in sensitivity among species.

4. ***Develop efficient strategies to survey and/or monitor environmental threats from CECs, thereby allowing management actions to be implemented before threats evolve into large-scale impacts on Great Lakes ecosystems or the services they provide****.* Effects-based monitoring approaches can be used in order to evaluate potential threats from CECs. Effects-based monitoring refers to use of biological responses observed in cell cultures in water samples, laboratory-reared organisms, and/or wild-caught organisms as an indication of exposure to contaminants and/or potential harm to wildlife resources and the ecosystem functions they provide.

To address these objectives, a multi-partner research effort was developed and implemented. The project team included several federal agency and academic partners and was facilitated by the U.S. Environmental Protection Agency (EPA) Great Lakes National Program Office (GLNPO). Collectively, these efforts employed a range of complementary approaches that addressed the objectives and the overall goal of understanding the potential threats CECs may pose to the health and integrity of Great Lakes wildlife populations.

- The U.S. Geological Survey (USGS) Great Lakes Area Water Science Centers conducted a basin-wide survey of organic contaminants, microplastics, and pathogens and evaluated their association with land-use attributes. Both water and sediment were sampled.

- The USGS Upper Midwest Environmental Sciences Center expanded their historical monitoring of contaminant exposure and biological effects in tree swallows nesting at Great Lakes Areas of Concern (AOCs) to include an expanded range of CECs and a broader diversity of biological measurements. Tree swallow monitoring is well-suited to provide insight into potential transfer of CECs from the aquatic food base to terrestrial nesting birds.

- The National Oceanic and Atmospheric Administration Mussel Watch Program expanded their historical monitoring of persistent toxic substances in the Great Lakes basin to include monitoring of CECs and added several biological measurements. The sedentary lifestyle of dreissenid mussels provides for precise place-based monitoring and an examination of the effects of CEC exposure on biota. Although dreissenid mussels are non-native invasive species, they were used because of the extensive monitoring data available, abundant availability throughout the Great Lakes' Basin, lack of concern for removing individuals from wild populations for research use, and the possibility that they could be used as indicators for determining site-specific exposure.

- The U.S. Fish and Wildlife Service CEC Team and St. Cloud State University provided more in-depth local characterization of the concentrations and distribution of CECs within selected Great Lakes tributary streams. They coupled chemical monitoring in water, sediment, and resident fish tissues with biological analyses of both wild-caught and laboratory-reared caged fish.

- The U.S. EPA's Mid-Continent Ecology Division used cell culture-based assays to screen water samples for biological activities potentially associated with adverse health effects in fish and other wildlife. Additionally, laboratory-reared fish were caged at selected field locations and a variety of biological analyses were conducted to evaluate early warning signs indicative of exposure to CECs.

- The U.S. EPA's Exposure Methods and Measurements Division used advanced analytical instrumentation including nuclear magnetic resonance spectroscopy and high-resolution mass spectrometry to measure changes in the internal biochemistry of organisms following exposure to water from Great Lakes tributaries. These measurements provide insights into how the organism may be responding to stress or using energy following exposure to CECs and can provide an early indicator of damage to internal organs and tissues that would not be evident upon visual examination.

- The U.S. Army Corps of Engineers Research and Development Center employed cutting-edge biotechnologies to examine the effects of CECs occurrence and concentrations in Great Lakes water on molecular responses in exposed fish.

At the onset of Action Plan I, the agencies planned and executed CEC studies independently. Consequently, the technical appendices (Appendices A-F) were organized by partner organization. As work progressed, US EPA GLNPO Focus Area leads/sub-leads coordinated biannual meetings that provided a platform for partners to share their approaches and results and identify opportunities for collaboration and coordination. By the end of Action Plan I, the partners were organized into an interagency CECs workgroup. Overarching results of the entire research effort and resulting management implications are presented in an integrated fashion in Chapters 2 and 3, respectively. These integrated results served as the foundation for a more coordinated and targeted research effort carried out under Action Plan II. Additional details of the research are presented in over 50 peer-reviewed journal articles and technical reports that resulted from the interagency CEC workgroups efforts under Action Plan I.

Integrated Results and Discussion

Partners employed a diverse range of approaches to evaluate presence and variability of CECs across a representative range of Great Lakes tributary streams, and the potential biological effects of the contaminant mixtures present at selected sites. Water and sediment samples from Great Lakes tributaries were collected and analyzed for a broad suite of CECs to provide information on occurrence and distribution (Appendices A, D). Wildlife tissues were examined for presence of CECs, including those from dreissenid mussels (Appendix B), egg, plasma, and other tissues from tree swallows (Appendix C), and fish tissues (Appendix D). Biological effects along the continuum from molecular and biochemical to physiological to individual level outcomes (e.g., impacts on survival, growth, and/or reproduction) were also evaluated on dreissenid mussels, fish, and birds (Appendices B-E). Collectively, these studies have produced complementary information and a multi-faceted data matrix on CECs that provides evidence for initial screening and prioritization of CECs that are of biological concern in Great Lakes tributaries.

CEC Occurrence

Contaminants of emerging concern are ubiquitous in monitored Great Lakes tributaries and vary according to association with land use. Analysis of water and sediment samples indicated that CECs were present in nearly all tributaries sampled, but the actual contaminants detected varied substantially by site (Baldwin et al. 2016; Elliot et al. 2017; Dila et al. 2018). The contaminants detected most commonly in water samples (Table 1) included PAHs, herbicides and insecticides, contaminants commonly associated with wastewater (e.g., alkylphenols, dyes and pigments, fecal indicators, flavors and fragrances, PPCPs) and flame retardants (PBDEs). Contaminants were generally most prevalent in urban areas, followed by agricultural areas. Pesticides were evenly associated with agricultural and urban land use, although with herbicides more dominant in agricultural area and insecticides more prevalent in urban watersheds. Watersheds dominated by forest and wetlands had the lowest concentrations of CECs.

The most prevalent contaminants (> 30% occurrence) detected in sediment samples included PAHs, industrial chemicals (e.g., 9,10-anthraquinone), wastewater contaminants (e.g., alkylphenols, fecal indicators, hormones, PPCPs, solvents) components of plastic, and phenolic chemicals (which have natural and anthropogenic sources; Elliot et al. 2017, Table 1). PAHs were the most common class of contaminants and were observed at the greatest concentrations among all the contaminants detected in sediment.

Fish liver tissues were sampled for CECs at a subset of locations studied (Appendix D). Plasticizers and flame retardants were detected in all fish liver samples, while all other chemical classes had at least one sample with non-detects observed. Atrazine, 17-alpha-ethynylestradiol,17-alpha-estradiol, androstenedione, carbamazepine, diazepam, diclofenac, estrone, meprobamate, naproxen, sulfamethoxazole, and estriol were not detected in any benthic or pelagic fish samples (Choy et al. 2017), even though they were commonly detected in water and sediment samples (Table 1). The most commonly detected chemical class in fish liver tissue was PFAS while PBDEs were detected in just a few samples.

Table 1. Contaminant of emerging concern use categories sampled (X) for different environmental sample types, 2010-2014. NS = Not sampled. The analytes evaluated in tissues were more limited.

	Sample Type				
	Water	Sediment	Mussel Tissue	Fish Tissue	Tree Swallow Tissues
Alkyphenols [w,s]	x	x	x	NS	NS
Dyes and pigments [w]	x	NS	NS	NS	NS
Fecal indicators [w,s]	x	x	NS	NS	NS
Flame retardants[1] [w]	x	NS	NS	NS	NS
Flavors and fragrances [w]	x	x	NS	NS	NS
Herbicides [w]	x	x	NS	NS	NS
Hormones [s]	x	x	NS	x	NS
Industrial chemicals [s]	x	x	NS	NS	NS
Insecticides [w]	x	x	NS	NS	NS
Microplastics	x	NS	NS	NS	NS
PAHs [w,s]	x	x	x	NS	x
PBDEs	NS	NS	x	x	x
PFAS	NS	NS	NS	NS	x
Phenolic chemicals [s]	x	x	NS	NS	NS
Plastics components [s]	x	x	NS	x	NS
PPCPs [w,s]	x	x	x	x	NS
Solvents [w,s]	x	x	NS	NS	NS

[1]Refers to flame retardants other than PBDEs, which are identified separately.

[w] Contaminants commonly detected in water samples.

[s] Contaminants commonly detected in sediment samples.

Common contaminants reported in mussel tissue (Appendix B) included PAHs, PBDEs, PPCPs, alkylphenols and alkylphenol ethoxylates (Table 1). Results generally indicated that compounds were more concentrated in dreissenid mussels from harbor and tributary samples than nearshore or offshore samples; however harbor and tributary sites were limited in scope to the Milwaukee and Niagara River areas. Of the CECs detected in mussel tissues, PAHs had the greatest concentrations.

The inclusion of tree swallows in the study provided insights into the potential transfer of CECs from the aquatic to terrestrial environment via food web interactions. Among the suite of contaminants monitored in tree swallow samples and nestling diets (prey items as well as gut contents), compounds detected included PAHs, PBDEs, and PFAS

(Table 1, Appendix C). The sites with the greatest PAH concentrations were near industrial urban areas (Appendix C; Custer et al. 2017a). PBDEs and PFAS were detected in all samples, but at relatively low concentrations at most sites. The greatest concentrations of PBDEs in tree swallows were detected at industrial sites (Appendix C), and the greatest concentrations of PFAS were near airfields where aqueous film forming foam was used in fire-suppression training exercises (Custer et al. 2019; Custer et al. 2017; Nakayama et al. 2010).

Overall, monitored CECs varied across water, sediment, and wildlife tissues, with a common conclusion that PAHs were a primary concern due to their prevalence in many watersheds across the Basin. Water sample results indicated that wastewater-related chemicals and pesticides were prevalent at many sites and are important to consider when evaluating potential biological effects. Flame retardants such as PBDEs and phosphate-based chemicals were commonly detected, but at relatively low concentrations relative to reported biological effect concentrations.

Priority CECs

Data describing the presence of hundreds of contaminants of emerging concern in tributaries of the Great Lakes are becoming more available. However, understanding which of these contaminants may elicit negative consequences and at which locations is challenging. Nonetheless, identifying the CECs likely to be causing negative impacts is important in order to focus management and monitoring resources in a way that most effectively mitigates the threats that CECs may pose to Great Lakes fish and wildlife. The CECs for which there was compelling evidence associating environmental concentrations with the potential for negative biological effects include:

- Select PAHs, products of combustion
- Atrazine, an herbicide
- Dichlorvos, an insecticide
- Ibuprofen and Venlafaxine, pharmaceuticals

Identification of priority CECs was based on multiple approaches. Each approach utilized different types of input data and yielded different levels of uncertainty regarding the potential to detect negative effects. Consequently, the overall weight of evidence, considering lines of evidence from multiple approaches and sources, were used wherever possible to inform prioritization

Screening Values (SV) and Benchmarks

In cases where toxicity information was available either from regulatory guideline studies or from the peer-reviewed scientific literature, concentrations of CECs detected in this study were compared to those previously demonstrated to adversely affect organisms (Appendices A, D). This was done by dividing concentration of the chemical required to produce a negative biological response by the concentration measured in the environment (i.e., calculating a toxicity quotient). Values greater than one indicate a strong likelihood of effect. Two complementary methods were used; either comparing water chemistry results to established water quality benchmarks (Appendix A; Baldwin et al. 2016) or comparing water chemistry results to custom developed water quality screening values (Appendix D; Gefell et al. 2019a). Established water quality benchmarks were compiled from agencies such as U.S. Environmental Protection Agency, Canadian Council of Ministers of the Environment, and Environment and Climate Change Canada (Baldwin et al. 2016) and were available for 27 CECs (Table 2). Screening values were developed for an additional 14 CECs that were detected frequently in water samples but for which benchmark standards did not already exist (Gefell et al. 2019a). Screening values were based on concentrations reported in the scientific literature to cause negative impacts on survival, growth, reproduction, or selected developmental processes or behaviors in fish that could be readily linked to population demographics (Gefell et al. 2019b). Screening values were developed for an additional seven effect categories related to fish health (endocrine, neurological, physiological/metabolic, etc.). Two types of screening values were derived; "SV_{LOW}" as a concentration below which no hazard to fish is expected and "SV_{HIGH}" as a concentration above which there is a strong expectation of adverse effects. Collectively, toxicity screening-based methods were applied for 41 unique CECs (Table 2).

One or more established water quality benchmarks were exceeded in samples from 20 of the 57 sites evaluated (Appendix A). In total, 11 unique chemicals including five PAHs, five pesticides, and one detergent metabolite (4-nonylphenol; Table 2; Baldwin et al. 2016) exceeded established water quality benchmarks at one or more sites. Chemicals with the most frequent exceedances were PAHs, 4-nonylphenol, and atrazine. Up to nine different compounds exceeded established water quality benchmarks at a single site.

Based on custom screening values (Appendix D; Gefell et al. 2019b), measured concentrations exceeded SV_{LOW} values for all but one of the 14 CECs evaluated (lidocaine). Exceedance of SV_{HIGH} values in population-relevant effect categories (e.g., survival, growth, reproduction) was detected for two of the 14 chemicals, both of which were pharmaceutical compounds (ibuprofen and venlafaxine). Each of the 24 project locations evaluated using SVs had at least one exceedance of a SV_{HIGH} and 17 of 24 locations had at least one SV_{HIGH} exceedance. Variability in the exceedance of SV_{HIGH} was observed between different tributaries and exceedance of screening values was not detected at all sites on the same tributary, suggesting spatial heterogeneity. As one might expect, within a given tributary system, exceedances were typically downstream of point sources.

Both methods indicated the potential for adverse biological effects at multiple sites, and both methods highlight the need to evaluate biological effects of co-occurring chemicals. Current methods using screening values or benchmarks only assess the risks of each individual chemical and do not address the cumulative risk associated with exposure to co-occurring mixtures of CECs. Consequently, complementary methods that can help to better understand the risk and effects of chemical mixtures on fish and wildlife are needed in addition to toxicity quotient-based approaches.

Use of established water quality benchmarks is of value for evaluating concentrations in a biological context, but these benchmarks are available for only fraction of the CECs detected in these studies. For example, of the organic waste compounds monitored by USGS water quality benchmarks were available for less than half (27/69). US Fish and Wildlife service derived SVs by drawing on a broader range of laboratory studies published in the peer-reviewed scientific literature, not just standardized regulatory toxicity tests. However, for the 25 most commonly detected chemicals, out of over 150 monitored, SVs could be developed for just 14 of the 25. An on-going and recognized challenge in assessing the biological significance of CECs is the lack of available toxicity information for many compounds. Because traditional laboratory-based toxicity testing data are lacking for many CECs, alternative approaches for associating CECs with biological effects that are less reliant on traditional toxicity data were also evaluated.

Table 2. Chemicals for which water chemistry data was evaluated using established water quality benchmarks (BM) or custom screening values (SV). "No" indicates a water quality benchmark or screening value was available, but not exceeded in any samples. "Yes" indicates the water quality benchmark or custom screening value SV_{LOW} was exceeded in at least one sample. "**YES**" indicates the water quality benchmark was exceeded by a factor of 10 or more or custom screening value SV_{HIGH} was exceeded in at least one sample.

Chemical Class	Chemical Name	SV[a] or BM[b] Exceeded?
Antimicrobial Disinfectants	Phenol	No
	Triclosan	Yes [a]
Detergent Metabolites	4-Nonylphenol	Yes [b]
Fire Retardants	Tris(2-butoxyethyl) phosphate (TBEP)	Yes [a]
Flavors and Fragrances	Hexahydrohexamethyl cyclopentabenzopyran (HHCB)	Yes [a]
Fuels	1-Methylnaphthalene	No
	2-Methylnaphthalene	No
	Isopropylbenzene (cumene)	No
Herbicides	Atrazine	**YES** [b]
	Bromacil	No
	Metalaxyl	No
	Metolachlor	Yes [b]
	Pentachlorophenol	Yes [b]
	Prometon	No
Hormone	Androstene-dione	Yes [a]
	B-sitosterol	Yes [a]
	Estrone	Yes [a]
Insecticides	Carbaryl	Yes [b]
	Chlorpyrifos	No
	Diazinon	No
	Dichlorvos	**YES** [b]
	N, N-diethyl-meta-toluamide (DEET)	Yes [a]
Other	p-Dichlorobenzene	No
	Tribromomethane (bromoform)	No
PAHs	Anthracene	**YES** [b]
	Benzo[a]pyrene	**YES** [b]
	Fluoranthene	**YES** [b]
	Naphthalene	No
	Phenanthrene	Yes [b]
	Pyrene	**YES** [b]
Plastics component	Bis(2-ethylhexyl) phthalate (DEHP)	No
	Bisphenol A	Yes [a]
	Diethyl phthalate	No
PPCP	Carbamazepine	Yes [a]
	Citalopram	Yes [a]
	Diphenhydramine	Yes [a]
	Ibuprofen	**YES** [a]
	Lidocaine	No
	Venlafaxine	**YES** [a]
Solvents	Isophorone	No
	Tetrachloroethene	No

[a] Peer-reviewed scientific literature-derived screening value (Gefell et al. 2019b).

[b] Established water quality benchmark (Baldwin et al. 2016).

Exposure Activity Ratios (EARs)

One of the most promising approaches for associating CECs with their potential biological effects involved comparing the CEC concentrations measured in environmental samples with concentrations at which the chemicals elicit responses in ToxCast high throughput screening assays (Kavlock et al. 2012; Blackwell et al. 2017). ToxCast employs a battery of mostly cell culture-based assays that measure the ability of chemicals to bind to and activate certain proteins, inhibit specific biochemical reactions, alter gene expression, change cell shapes or functions, etc. (Kavlock et al. 2012). These biological activities do not directly equate to clear impacts on survival, growth, or reproduction. However, they can provide a potentially conservative estimate of the relative potency of a chemical to elicit a biological response, which may be mechanistically linked to toxicity that can be expected to occur if the magnitude and duration of exposure are adequate (Appendix E; Krewski et al. 2010).

The ratio of detected chemical concentration in the environment to the concentration at which it elicits activity in a high throughput assay is termed an exposure-to-activity ratio (EAR). While not as definitive as water quality benchmarks or SVs for identifying ecological risks, the data needed to calculate an EAR are available for many more chemicals than those for which benchmarks or SVs based on traditional toxicity testing are. For example, of the 69 chemicals monitored by Baldwin et al. 2016 (Appendix A), water quality benchmarks were available for just 27 (39%) chemicals, while EARs could be calculated for 48 chemicals (70%). Additionally, even when some traditional toxicity data are available, the EAR approach may identify subtle, sublethal impacts that exposure to CECs may cause (e.g., endocrine disruption, potential for behavioral or developmental effects, etc.) that may not be detected in traditional toxicity tests. Furthermore, EARs can be summed for all chemicals detected in a sample that cause activity in the same assay. This provides a means to evaluate cumulative impacts of mixtures. Due to the potential utility of this method, further development and application of the EAR approach was identified as an important goal/focus under Action Plan II.

Weight of Evidence

In some instances, statistical inference approaches were used to prioritize CECs that may have a biological effect when traditional toxicity data or alternative data from programs like ToxCast were not available. Statistical approaches (e.g., partial least squares regression, context likelihood of relatedness) can examine potential co-variation between the concentrations of a chemical detected at a given site and the magnitude of effect on a broad range of biological effect endpoints. These endpoints may be measured in organisms exposed on-site (Appendices B-F) or in laboratory-based assays following exposure to extracts of environmental samples (Appendix E). As part of Action Plan I, multi-variate statistical approaches were applied to identify co-variation between chemical concentrations and the abundance of small molecule metabolites (Appendix E) or messenger RNA (Appendix F) extracted from tissues or fluids from fish exposed on site. Because these approaches identify correlations, rather than causation, they are not definitive for hazard identification or risk assessment. Nonetheless, they were shown to be useful for reducing a long list of detected CECs to a shorter list that could be prioritized for subsequent monitoring and testing (Appendix E; Davis et al. 2016). Additionally, along with complementary approaches like water quality benchmarks, screening values, or EARs, they can provide another line of evidence for use in weight of evidence-based approaches for identifying which CECs may threaten the integrity and health of fish and wildlife populations.

CECs and other Stressors

Some CEC concentrations exceeded available water quality benchmarks and/or screening values. Nonetheless, monitoring of biological responses in either laboratory-reared fish caged in the field or resident fish and wildlife generally did not indicate overt toxicity was occurring. Results to date indicate that individually, CECs are not likely to cause serious declines to current fish and wildlife populations or impair ecosystem function. However, CECs detected in the Great Lakes were associated with subtle biological effects. These effects included changes in gene expression, increased circulating glucose concentrations, altered fish health parameters such as organ weights as a percent of body weight in both resident and laboratory-reared organisms. These subtle effects may have more serious implications when combined with other sources of stress like habitat degradation, changing climate conditions, and competition with invasive species. Ideally, results from this CEC research should be integrated with other monitoring activities conducted as part of the GLRI or on-going management programs to consider a more holistic ecosystem context in which CECs represent one of several stressors to Great Lakes fish and wildlife. It is also noted that biological effects monitoring conducted as part of this effort was not always co-located at the sites where CEC concentrations exceeding water quality benchmarks were detected. Thus, overt impacts are still a possibility at select locations, and these are potential priorities for follow-up investigations.

Weight of Evidence Evaluations

As noted in the introduction (Chapter 1), at the inception of this work on CECs experimental designs, analytical methods, and site selection for different aspects of the research were not coordinated among partners. Consequently, the full spectrum of approaches employed in the research could not necessarily be applied at each site, for each chemical of interest, and/or for all species evaluated. The following examples highlight situations where the weight of evidence collected by the various partners contributed to a broader understanding of key findings. The increased interagency coordination that emerged over the course of GLRI Action Plan I and the associated integration case studies set the stage for a more coordinated cross-agency effort under GLRI Action Plan II

PAHs as a Threat to Great Lakes Fish and Wildlife

Polycyclic aromatic hydrocarbons were one of the classes of chemicals that were evaluated by all partners involved in the CEC research. Often, PAHs are considered legacy contaminants due to the availability of toxicity benchmarks and known inputs from recirculation. However, PAHs were included in this CEC-focused effort due to their pervasiveness and in recognition of the diversity of on-going sources that continue to introduce PAHs into the Great Lakes basin. Results detailed in the Appendices (A-F) provide evidence that PAHs are likely a significant contributor to biological effects detected in several areas and species studied.

Measurable concentrations of PAHs were found in the surface water of 57 different Great Lakes tributaries and at 43% of all sites surveyed (Appendix A). At 20 of these 57 sites, five PAHs (anthracene, benzo[a]pyrene, fluoranthene, phenanthrene and pyrene) frequently exceeded water quality benchmarks, with concentrations predicted to cause biological impacts. Fluoranthene concentrations in Maumee and Detroit rivers were great enough to potentially cause tumors in fish (Appendix F). Likewise, PAHs were among the contaminants frequently detected in sediment samples.

In general, PAHs are rapidly metabolized by vertebrates, consequently, they do not accumulate in fish or birds as do more persistent legacy contaminants. Nonetheless, there was ample evidence for PAH exposure and accumulation among the invertebrates sampled, noting that invertebrates do not metabolize PAHs as readily as vertebrates. For example, PAHs were widely detected in tissues of mussels deployed for monitoring in harbors, tributaries, nearshore, and offshore sites around the Great Lakes Basin (Appendix B). Additionally, across the Great Lakes, emergent aquatic insects ingested by tree swallows were contaminated with PAHs, indicating that animals that eat these invertebrates are subsequently exposed these chemicals (Appendix C). There was enough exposure to PAHs at some sites, as measured in the diet of swallows, to elicit both a physiological response and a reproductive effect in the swallows. Since aquatic insects are part of the diet of many other aquatic and wildlife species, many more animals are likely to ingest PAHs. These observations, along with the exceedance of water quality benchmarks for PAHs at several sites, provide multiple lines of evidence that exposure to and uptake of PAHs is likely widespread in Great Lakes fish and wildlife.

Given the presence of PAHs in water and sediment, accumulation in invertebrates, and probable uptake and exposure in vertebrates suggested by chemical monitoring, we investigated whether there was evidence that PAHs were eliciting a biological response. Several lines of evidence indicate that both fish and birds near Great Lakes tributaries exhibit physiological responses consistent with exposure to PAHs, and that these responses were statistically correlated with presence of PAHs. For example, a common biomarker of exposure to PAHs is expression of the gene cyp1a which codes for a protein that modifies many organic chemicals to make them easier for organisms to excrete. Expression of cyp1a and the activity of the enzyme it codes for (as measured by “EROD activity”) increases in response to halogenated aromatic hydrocarbons and PAHs. In studies of swallows nesting near Great Lakes tributaries, PAHs were found to be a major contributor (22.8%) to increased cyp1a activity as well as enzyme activities commonly triggered when organisms experience oxidative stress which that can damage tissues (total sulfhydryl activity,19.2%; Appendix C). Total PAHs were 16 times greater in the high EROD activity group compared to the normal group.

Increased expression of cyp1a was also found in livers of caged fish exposed at sites in the Maumee and Detroit River AOCs, suggesting exposures to PAHs and/or halogenated aromatic hydrocarbons (Appendix F). Changes in global gene expression in caged fish were also most highly correlated to changes in PAH concentration in surrounding water, and phenanthrene was highly correlated with levels of cyp1a expression. Fluoranthene concentrations were correlated with many changes in gene expression. This was consistent with fluoranthene being at levels exceeding water quality benchmarks (Appendix A, Baldwin et al. 2016) and hazard indices for tumor formation (Appendix A). Resident fish (brown bullhead, white sucker, largemouth and smallmouth bass) in the Ashtabula, Detroit, Genesee, Milwaukee and St. Louis Rivers and Conneaut Creek have also been found to have elevated levels of cyp1a and oxidative stress-related gene expression consistent with exposure to PAHs (Braham et al. 2017). Collectively, these findings provide strong support that PAHs are likely causing biological responses in fish and vertebrate wildlife in the Great Lakes tributaries, even though there is limited accumulation in tissues.

This is cause for concern as PAHs are known to cause a wide range of adverse effects including cardiotoxicity, developmental effects, reduced reproduction, DNA damage, liver and skin tumors, and physical abnormalities in both vertebrates and invertebrates. Several Great Lakes tributaries have been noted to have beneficial use impairments related to fish tumors or other deformities, deformities or reproductive problems in birds or other wildlife, and degraded fish and wildlife populations. Studies of nesting swallows in the Great Lakes (Appendix C) identified a decrease in reproductive success as PAH exposure increased (Custer et al. 2018). However, due to the potential effects of other contaminants (e.g., halogenated aromatic hydrocarbons), as well as ecological variables such as female age and date within season, a more definitive linkage is hard to establish (or reject) without additional study. Studies of sunfish (Appendix D) exposed to Great Lakes tributaries, in cages or as resident fish, suggest that the presence of PAHs and pharmaceuticals influenced blood glucose concentrations, liver anatomy, reproductive organ size and overall maturity levels. Likewise, based on existing adverse outcome pathway (AOP) descriptions (Appendices E, F) linking cyp1a induction and liver damage to potential tumor formation, chronic exposure to PAHs is a plausible contributor to tumors in fish. Investigation of global gene expression in fathead minnows caged for 4 days at sites in the Detroit and Maumee rivers appear to support that association, as gene expression changes related to activation of cyp1a, onset of a fatty liver condition, and activation of genes linked to tumor promotion were all detected (Appendix F). This is consistent with observations of internal and external tumors found in native brown bullhead (*Ameiurus nebulosus*) from the Trenton channel in the lower Detroit river (Appendix D; Blazer et al. 2014, Braham et al. 2017). Overall, there is significant evidence suggesting that PAHs have a strong likelihood to contribute to adverse biological effects and likely represent ongoing threats to fish and wildlife in the Great Lakes. Consequently, PAHs were identified as a priority contaminant class for further investigation under Action Plan II.

Estrogens as a Threat to Great Lakes Fish and Wildlife

Chemicals that can bind to and activate vertebrate estrogen receptors, thereby mimicking effects of this important class of reproductive and developmental hormones, were another group of contaminants for which an integrated analysis was performed. As part of the research under Action Plan I, researchers used four complementary approaches capable of detecting estrogenic chemicals and/or providing insights into their potential effects. The first approach involved monitoring for chemicals that had been previously identified as estrogenic. These included both non-steroidal industrial/commercial chemicals like bisphenol A, alkylphenols and alkylphenol ethoxylates (Appendices A, B; Baldwin et al. 2016), as well as steroidal compounds either naturally excreted by vertebrates, including humans, or administered as therapeutics (Appendix D; Elliott et al. 2017; Jorgenson et al. 2018). Concentrations detected were multiplied by relative potency factors to express the total expected estrogenicity in terms of a concentration of a standard reference chemical, 17β-estradiol, assuming additivity. Summed and normalized concentrations, expressed as 17β-estradiol equivalents (EEQ_{chem}; ng/L), were then compared with benchmark concentrations that would be expected to elicit estrogenic responses in fish. This approach considers only the fraction of estrogenic chemicals that were both detected analytically, and for which relative potency factors have been previously reported in the peer-reviewed scientific literature.

A second approach involved the use of cell culture-based assays to screen water extracts for estrogenic activity (Appendix E; Cavallin et al. 2016; Davis et al. 2016; Li et al. 2017; Jorgenson et al. 2018). Magnitude of response elicited by each sample extract was compared to that evoked by different dilutions of the reference chemical (17β-estradiol) and regression was used to express the activity of the sample in terms of an equivalent concentration (EEQ_{bio}; ng/L). In contrast with the analytical-based approach, the cell-based assay method accounts for all estrogenic or anti-estrogenic chemicals present in the sample extract and does not necessarily assume an additive model. Rather the cells respond to the integrated effect of the mixture, whether that includes additive, antagonistic, or potentially even synergistic effects of multiple chemicals.

A third approach examined biochemical and physiological responses in laboratory-reared fish that were caged in site water for 4 days (Appendix E). For these shorter-term exposures, induction of the egg yolk precursor protein vitellogenin (VTG) in male fish was taken as an indicator of exposure to estrogens. Additionally, gene expression profiling was used, in some instances, to detect estrogenic activity in exposed fish (Appendix F). Unlike cell-based assays that only consider the fraction of chemicals that are extracted from a water sample, the caged-fish approach accounts for all compounds bioavailable in the water column to which the fish are exposed. However, unlike the previous two methods, one cannot easily estimate the total concentration of EEQs from the caged fish response.

Finally, the fourth approach involved monitoring for estrogenic responses in resident fish. In this case, concentrations of VTG in blood plasma of male fish, as well as tissue damage or abnormalities observed through microscopic analysis were examined as signs of estrogenic effects (Appendix D). The resident fish approach, while most difficult to implement from a logistical and statistical perspective, allows one to potentially account for chronic, life-long, exposure to mixtures of estrogenic compounds in the water column and diet. Consequently, the methods employed were complementary, with each having its own advantages and disadvantages.

Within the scope of this investigation, these monitoring approaches were not necessarily brought to bear on the same locations at the same time, which somewhat limits their comparability. Nevertheless, considering the results in an integrated manner provides a more comprehensive picture of the state of estrogenic CEC contamination in Great Lakes tributaries than consideration of any approach alone. For example, based on analytical and cell-based screening of over 250 samples collected in seven tributaries (e.g., St Louis River, Detroit River, Maumee River, Fox River, Milwaukee River, Menominee River, and Kinnickinnic River) distributed across five Great Lakes AOCs in 2010-2013, it was concluded that although estrogenic activity was broadly detected across sites, it was rarely above concentrations expected to impact fish (Appendix E). Additionally, the estrogenic chemicals detected in those samples could reasonably account for the activity observed (Appendix E). However, broader surveillance of over 700 samples from 57 different tributaries (Appendix A) suggested that some of the highest concentrations of EEQ_{chem} were detected at locations, such as the Au Sable, St. Joseph, Rouge, Saginaw, and Raisin Rivers, that were not evaluated using effects-based approaches like testing water extracts in cell-based bioassays or measuring VTG in caged or wild-caught male fish. Considering only non-steroidal estrogens, Baldwin et al. (2016) calculated maximum EEQs ranging from 4-24 ng/L; concentrations that would be of concern biologically. Likewise, a study of 12 tributaries in 2013 and 2014 that considered both steroidal and non-steroidal estrogens calculated maximum EEQ concentrations as high as 28 ng/L, with mean concentrations exceeding 10 ng/L at several sites including the Little Calumet and North Shore Channel (Elliot et al. 2017) and Chicago River (Thomas et al. 2017). Cell-bioassay data were not available for either of these broader surveillance efforts, limiting the ability to determine whether the measured contaminants reasonably account for total biological activity detected at the other sites. Nonetheless, the levels of EEQ_{chem} detected at those sites are a concern relative to benchmarks proposed for total 17β-estradiol equivalents in drinking water or treated wastewaters (see Appendix E for more detail).

Data from biologically-based monitoring of responses in caged or resident fish were challenging to interpret. Given the ubiquitous occurrence of CECs across the Great Lakes basin and variation in environmental variables from one site and season to another, identification of appropriate reference or control populations for statistical comparisons across sites is neither ideal nor clear cut. For example, both Jorgenson et al. (2018) and Thomas et al. (2017) detected differing concentrations of VTG in male fish from different sites. However, without a spatial gradient relative to a source, it was unclear whether the levels detected were just a slight deviation from the normal mean of the population, or whether all sites were being impacted to different degrees. To alleviate this interpretation challenge, Blazer et al. (2018) evaluated their resident fish responses relative to an expected site-independent baseline concentration (10 µg VTG/ml plasma). Using this approach, results suggested estrogenic impacts in a large majority of resident fish sampled across seven different tributaries in 2010-2011 (Blazer et al. 2018). If one assumes this to be an appropriate baseline, results suggest that chronic exposures, even at levels of total EEQ estimated to be non-hazardous based on laboratory studies, may be having long-term endocrine disrupting effects on resident fish.

Overall, there were multiple lines of evidence to suggest that, in some Great Lakes tributary locations, estrogenic CECs may be reaching concentrations that may result in adverse effects in fish. Increased coordination in the application of analytically-based, cell assay-based, and fish-based approaches for monitoring estrogenic contamination, deployed as part of the research effort under Action Plan II, is expected to yield further insight and clarity regarding the following: (1) whether estrogenic CECs currently monitored via analytical methods reasonably account for the majority of estrogenic activity measured in cell-based assays, across a broad range of Great Lakes tributaries; (2) whether EEQ concentrations that exceed certain concentrations reliably and reproducibly induce biological response in caged fish exposed for a defined duration, and could be established as an actionable benchmark for management purposes.

Overarching Management Implications

It is important for natural resource managers to be aware that CECs are ubiquitous throughout the Great Lakes Basin. However, that is not unique to the Great Lakes Basin and CEC prevalence probably exists anywhere there are human influenced landscapes. A better understanding of CEC presence and possible effects on fish and wildlife will lead to improved natural resource management and more successful restoration, conservation, and mitigation efforts. Even where CECs alone may not be causing obvious adverse effects to Great Lakes fish and wildlife, when combined with other stressors (e.g., invasive species, habitat loss or alteration, viruses or pathogens, and/or changing climate conditions), CECs may contribute to overall reductions in population fitness. Understanding what levels of CECs are in the current landscape helps prepare natural resource managers to better understand risks and effects to fish and wildlife populations. CEC presence and effects data can be utilized to further evaluate strategies and inform best management practices in areas where CECs are present.

CECs are Widespread

CECs were detected in all sampled media (water, sediment, tissues) at all Great Lakes tributary stream locations. Although this study focused on the Great Lakes Basin, results could be applicable to nearly any US watershed because of ubiquitous presence of CECs revealed by a recent nation-wide CEC monitoring study (Bradley et al. 2017).

If natural resource managers are concerned that CECs may be impeding management goals and objectives in their areas, screening water, sediment, or organisms for the occurrence of CECs can be a prudent first step to determine if CECs are present at levels known to cause adverse effects. The surveillance data collected under Action Plan I may help identify specific classes of CECs to screen for in different locations. Further, if funding constraints dictate, analysis could be prioritized to first assess the most commonly detected CECs, gradually expanding the analysis to include other suites of CECs as time and resources allow. Monitoring of CECs will help natural resource managers better understand possible risks to natural resources, in relation to other stressors and pressures on fish and wildlife populations (e.g., climate change, habitat loss/alterations, invasive species, etc.), and help prioritize restoration projects.

Effects are Often Subtle and Implications for Ecological Fitness Unclear

In nearly all Great Lakes tributary sites evaluated as part of this effort, CECs were not lethal to fish, mussels, or tree swallows. Biological effects were observed in many cases, but their severity was mild and their relevance to ecological fitness remains uncertain. The results indicate that in many cases, CEC exposure may be a factor contributing to or compounding other stressors, but that CECs are not likely to be the sole contributing factor to population declines, die-offs, or unsuccessful population restoration efforts. Therefore, natural resource managers should not evaluate CECs in isolation from other environmental stressors.

For some CECs, benchmarks established by regulatory authorities or screening values derived from peer-reviewed literature sources are available and can be used for guiding management objectives for remediation or source reduction.

The effects of long-term exposure of CEC on the reproductive health of organisms is still unclear. Our investigations provided evidence of potential CEC-related impacts on tree swallow reproduction. Additionally, there was evidence of physiological stress (e.g., altered glucose levels; signs of oxidative stress) in multiple taxa including mussels, birds, and fish that could influence reproductive fitness. However, more monitoring is needed to further investigate links of CEC exposure to effects on reproductive fitness.

Identifying and Reducing Sources of PAHs Could Reduce Contaminant-Related Stress in a Number of Great Lakes Tributaries

PAHs were consistently detected throughout all the studies, frequently at levels exceeding benchmark standards (Table 2). As a result, monitoring for this chemical class could be a priority for managers looking to better understand sources, transport and fate of CECs in their landscape. Because many of the PAH concentrations exceeded benchmark standards, there is increasing cause for concern regarding their effects on natural resource management objectives. Management strategies could include developing a monitoring schedule for PAHs or looking for evidence of effects on fish and wildlife populations (e.g., reduced survival, reductions or failures in reproduction, rates of tumors and deformities). If benchmarks and/or custom screening values standard values are exceeded, source identification and source reduction strategies actions could take place to reduce PAH levels to, or below, acceptable standards.

Complementary Methods Show Promise for CECs Surveillance and Monitoring

Multiple methods were used to detect CECs and/or their effects on organisms. Natural resource managers looking to implement CEC monitoring and surveillance should choose the method most appropriate to their management objectives. Taking grab samples of water will yield snapshot results of CEC concentrations at the time of sampling. Longer term sampling (e.g., on a monthly, seasonal, or yearly basis) will provide managers with a clearer picture of long-term exposure and CEC prevalence or occurrence. Sampling of sediments from depositional areas can detect persistent contaminants deposited over long periods of time (depending on the depth sampled) but will typically include different contaminants than those found in the water column. Screening organism tissue samples for the occurrence of CECs will help managers better understand what CEC types and concentrations are present in biota. Evaluation of physiological and biological responses to CECs can help natural resource managers determine if risks to fish and wildlife populations exist for specific class(es) of CECs. Members of the CEC research team can assist in selecting the appropriate approaches to address site-specific problems (see https://communities.geoplatform.gov/glri/ for technical contacts).

Implementation of Management Practices is Influenced by Contaminant Source

CECs have many sources (i.e., point, nonpoint) and pathways throughout environmental systems. Wastewater treatment plant (WWTP) effluents, combined sewer overflows (CSOs), illicit discharges, agricultural and urban runoff, and direct inputs can all be possible sources of CECs. Evaluating the landscape for point source and non-point source pollution sources can help managers prioritize project locations and evaluate the potential risks CECs may cause to conservation success. Below we highlight some previous examples where an understanding of CECs and their sources helped to inform management decisions and subsequent actions:

- Sampling conducted under EPA's National Urban Runoff Program initiative and Clean Lakes initiative with the city of Austin, TX, USGS, and Texas State University in 2005 determined coal tar sealant was negatively impacting aquatic communities. The city enacted a city-wide ban on coal tar-based sealants upon the determination that PAHs from the sealants were causing harm to wildlife, including the federally endangered Barton Springs salamander. Follow up studies conducted by USGS indicate that, as of 2014, PAH concentrations have declined by as much as 58% in some locations (City of Austin, 2005).
- Limiting jeopardy of endangered species by informing federal actions was illustrated previously in the use of lampricides in areas of endangered species occurrence. To stop sea lamprey damage, application of the lampricide 3-triflouromethyl-4-nitrophenol (TFM) was necessary in Great Lakes streams which also contain the endangered freshwater mussel snuffbox (*Epioblasma triquetra*), and their host fish log perch (*Percina caprodes*), as well as feeding and nesting habitat of the endangered shorebird piping plover (*Charadrius melodus*). Data similar to those generated by this CEC project was used to assess the potential impact TFM may have on these endangered species (Boogard et al. 2013, 2015). This analysis led to recommendations by USFWS that allowed best management action plan for TFM application timing and concentration that was protective of the species of concern as well as ensuring eradication of the sea lamprey.
- State/federal coordination of herbicide permitting to eliminate aquatic nuisance plants from lakes and streams illustrates another application for CEC data. When permits were requested by homeowners to apply herbicides along a chain of lakes in Michigan that historically hosted the endangered snuffbox mussel, the state with USFWS guidance developed a recommended herbicide guide utilizing data and principles similar to those generated by the CEC project. This living guidance document enables state permitting officers to allow permits based on science for best management practices for the types, rates, and timing of herbicides to limit jeopardy of snuffbox populations and other aquatic species.
- Working with hatcheries and streamside rearing facilities to determine placement of new facilities is another application of CEC data. Streamside rearing facilities and traditional hatcheries pump water from adjacent streams for use in the rearing tanks. Some facilities are located near urban centers or in waterways known to contain CECs that can be detrimental during the vulnerable life stage of rearing from egg to juvenile (typically May – Oct.). Monitoring for CECs and prioritizing locations for new facilities with lower CEC occurrence and concentrations when all other site aspects are equal is a possible solution to decrease CEC exposure.
- Plasticizers, antioxidants, detergent metabolites, flame retardants, nonprescription drugs, flavors/fragrances, dyes/pigments, and human-associated bacteria were all greatest in watersheds with the most urban influence. Many of these CECs have been shown to originate from imperfect sanitary conveyance systems that can be rapidly repaired. For example, in 2007, samples from an outfall to Honey Creek near Miller Park in Milwaukee, Wisconsin were positive for human-associated bacteria markers. Follow-up dye testing confirmed a misconnection of a sewage line from Miller Park that was remedied to improve water quality in Honey Creek. Enhancing illicit discharge detection and elimination programs (IDDE) would enable identification and remedy of misconnections and faulty sewer pipes

around the Great Lakes similar to this incident in the Honey Creek watershed (Behm 2007).

- Other CECs that are commonly particle associated such as PAHs were also most prevalent in urban areas. Multiple studies have demonstrated that PAHs in runoff can be removed in common urban stormwater using green infrastructure practices that focus on settling or filtration of particulate matter. One example at the University of Maryland campus in 2006-2007 demonstrated that a bioretention cell removed 87% of the PAHs from urban stormwater that were retained in surficial deposits, allowing for maintenance efforts to remove and dispose of these contaminants (DiBlasi et al. 2009). Installation of urban runoff and green infrastructure may similarly reduce PAH loadings to the Great Lakes and its tributaries.

Data Availability

To assist in natural resource manager decision-making, all data from this investigation are publicly available in databases, in peer-reviewed open source publications, or by contacting the corresponding author of the research project summaries (Appendices A-F) or the technical contacts listed at https://communities.geoplatform.gov/glri/.

- Tree swallow data is available through the Environmental Conservation Online System (ECOS, https://ecos.fws.gov/ecp/), which can be accessed using the Wildlife & Environmental Contaminants mapper.

- Data collected by NOAA can be accessed using the DIVER Explorer Application tool (https://www.diver.orr.noaa.gov/).

- Data collected by USGS with USFWS can be found in the USGS National Water Information System (https://waterdata.usgs.gov/nwis?) and the USGS Science based-catalog online system (https://www.sciencebase.gov/catalog/).

- Data from EPA is made publicly accessible via ScienceHub and can be accessed via the EPA Environmental Dataset Gateway (edg.epa.gov) or at Data.gov

- Transcriptomics data from ACOE is publicly accessible via the Gene Expression Ominbus site (GEO; https://www.ncbi.nlm.nih.gov/geo/).

Links and/or direct database access to these data sources will also be made available through https://communities.geoplatform.gov/glri/ as soon as feasible.

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Appendix A

Organic Contaminants, Microplastics, Waterborne Pathogens, and Host-Associated Bacteria Surveillance and Potential Biological Effects in Great Lakes Tributaries

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A.1. Introduction

Anthropogenic activities related to industrial, agricultural, domestic, and urban water uses introduce an untold number of contaminants into the Great Lakes and their tributaries on a daily basis (Bennie et al. 1997; Blair et al. 2013; Venier et al. 2014). Flame retardants, drugs, herbicides, plasticizers, polycyclic aromatic hydrocarbons (PAHs), and other organic compounds (collectively referred to as organic waste compounds or OWCs) as well as plastic debris, and microbiological organisms enter waterways through wastewater treatment plant (WWTP) discharges, combined sewer overflows, leaking septic and municipal sewer systems, urban and agricultural runoff, industrial discharges, and atmospheric deposition, among others (Barber et al. 2015; Kolpin et al. 2002).

A surveillance program was conducted in Great Lakes tributaries from 2010-2014 to: 1). define general occurrence and magnitude of multiple classes of OWCs, plastic debris, and microbiological organisms, 2). to prioritize OWCs based on prevalence and potential for effects on ecological species, 3). to define the frequency of occurrence and magnitude of microplastics contamination, and 4). to use microbiological genetic markers to define contamination from sewage and cattle manure sources, and 5). to define the various conditions under which each of these contaminants are most prevalent.

A.2. Organic Contaminants

Tributaries of the Great Lakes are impacted by a diverse set of influences that can introduce numerous contaminants into waterways. Exposure to many of these compounds can result in adverse ecological effects, with some that can be serious enough to lead towards a decline or collapse in populations (Collier et al. 2013; Ingersoll et al. 2002; Johnson et al. 2013). The ability of some of these compounds to bioaccumulate (Ismail et al. 2014; Jenkins et al. 2014) creates a risk to organisms higher up the food chain including mink, river otter, bald eagles, osprey, and humans (Hinck et al. 2009; Jenkins et al. 2014). Most drinking water treatment plants do not fully remove many of these compounds from the water supply, creating another exposure route for humans (Kingsbury et al. 2008; Stackelberg et al. 2004).

A number of factors may influence the occurrence of contaminants in environmental waters including land use, hydrologic condition and season. As a result, there is a need for management strategies that consider many different factors including a multitude of chemical contaminants from different sources to establish priorities for resource allocation.

A.2.1 Objectives

The overall objective of the study was to assess the occurrence and possible adverse biological effects of organic contaminants in Great Lakes tributaries. Specific objectives were as follows: 1) identify occurrence and magnitude of monitored compounds, 2) define how presence and magnitude of these compounds vary by land cover, flow regime, and season, 3) prioritize compounds based on screening techniques that estimate potential for biological effects, 4) prioritize tributaries with respect to potential biological effects, and 5) develop techniques for use of high-throughput screening data (ToxCast: https://www.epa.gov/chemical-research/toxicity-forecasting) to expand capabilities for assessing potential biological effects from chemical concentrations and 6) develop techniques to link the chemical exposures to potential adverse outcomes for ecological species using the adverse outcome pathway wiki (AOP-Wiki; aopwiki.org). In this first phase of GLRI, objectives 1 and 2 were completed, a traditional method of achieving objectives 3 and 4 was completed, and the groundwork was established for achieving objectives 3-6 using the ToxCast database and the AOP-Wiki. The current section on organic contaminants in Great Lakes tributaries provides a summary of findings from the first phase of GLRI; additional detail and supporting data are published elsewhere (Baldwin et al. 2016a).

A.2.2 Methods

Study sites included 57 Great Lakes tributaries (Figure A.1). Flow from these sampling sites accounted for approximately 41% of the total tributary inflow to the Great Lakes. Watershed drainage areas ranged from 101 – 16,400 square kilometers (km^2), with mean annual flows from 2.58 – 219 cubic meters per second (2010- 2013). Watershed land cover varied from dominantly urban (up to 92% of watershed) to agricultural (84%) to forest and wetland (93%). Watershed population densities ranged from 3.3 - 2,498 people/mi^2. The portion of river flow from WWTP effluent ranged from less than 1% - 47%.

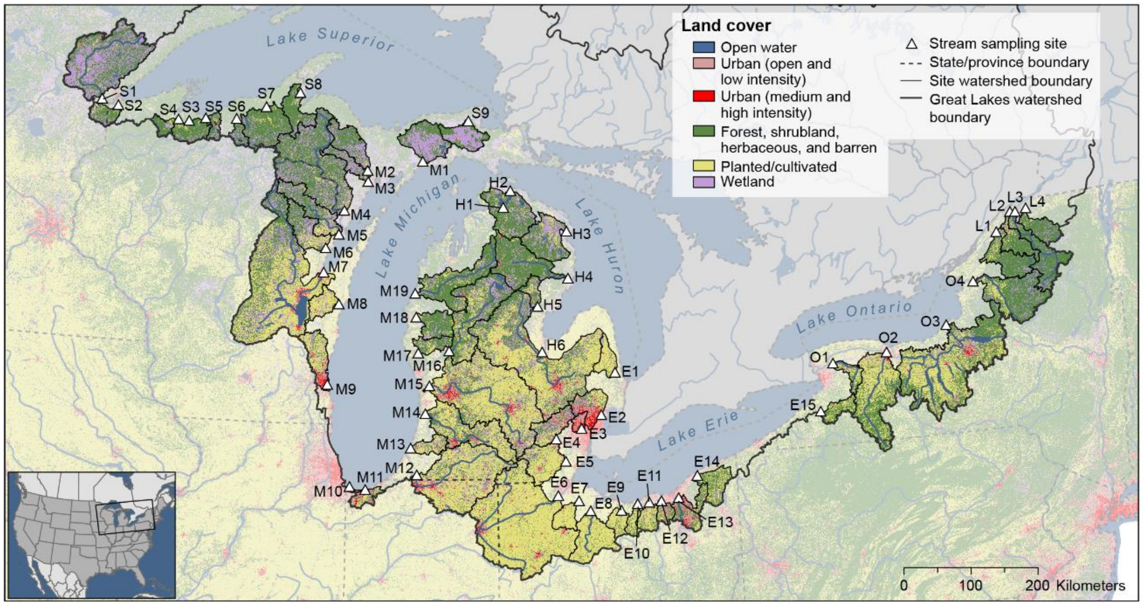

Figure. A.1. Sampling locations, watershed boundaries, and watershed land-uses. Map IDs are defined in Table A.1. (Instituto Nacional de Estadística Geografía e Informatica, 2006a, Instituto Nacional de Estadística Geografía e Informatica, 2006b; National Atlas of the United States, 2005, U.S. Department of Agriculture-Natural Resources Conservation Service, 2009).

Of the 709 water samples collected between September 2010 and September 2013, thirty-eight sites were sampled 1–2 times each, generally during low-flow and medium-flow periods, and the remaining 19 sites were sampled more frequently, with 7– 64 samples each, during runoff and low-flow conditions and throughout different seasons. Flow composite samples were collected by permanently stationed automatic samplers at eight of the 19 frequently-sampled sites (Table A.1). Whole water samples were analyzed for 69 organic waste compounds (OWCs) (USGS National Water Quality Laboratory schedule 4433; (Zaugg et al. 2006)).

OWCs were aggregated into 15 classes: antioxidants, dyes and pigments, fire retardants, PAHs, plasticizers, fuels, solvents, herbicides, insecticides, antimicrobial disinfectants, detergent metabolites, flavors and fragrances, nonprescription drugs, sterols, and miscellaneous (Baldwin et al. 2016a).

Mean contaminant concentrations by site were analyzed for relationships with land cover attributes, streamflow condition (low-flow versus runoff), and season. Sample results were also analyzed for potential adverse biological impact by comparison with established water quality benchmarks from institutions such as U.S. Environmental Protection Agency and Canadian Council of Ministers of the Environment (27 compounds) and by using 17β-estradiol equivalency factors (8 compounds). Toxicity quotients (TQs) were computed for each site by dividing the greatest measured concentration of a compound at a particular site by the lowest known water quality benchmark for that compound. EEQ's were computed by multiplying measured concentrations of eight nonsteroidal compounds (bisphenol A, p-dichlorobenzene, 4-nonylphenol, 4-nonylphenol monoethoxylate, 4-nonylphenol diethoxylate, 4-tert-octylphenol, 4-tert-octylphenol monoethoxylate, and 4-tert-octylphenol diethoxylate) by their respective estradiol equivalency factors (EEF) and summing for each sample (Vajda et al. 2008).

Table A.1. Site characteristics and types of samples collected, 2010-2013. Drainage area from NWIS unless unavailable, then GIS computed. [A, water samples collected using an autosampler; n, number of samples; ID, identification, AgMix, agricultural mix of pasture/hay and crops].

Site Name	Map ID	Dominant land cover	n	Site Name	Map ID	Dominant land cover	n
StLouis, MN	S1	Wetland	31	Cheboygan, MI	H2	Wetland	2
Nemadji, WI	S2	Wetland	1	Thunder Bay, MI	H3	Wetland	2
Bad, WI	S3	Forest	1	AuSable, MI	H4	Forest	26
White, WI	S4	Forest	1	Rifle, MI	H5	Forest	2
Montreal, WI	S5	Wetland	1	Saginaw, MI	H6	Crops	31
Presque Isle, MI	S6	Wetland	1	Black, MI	E1	Crops	2
Ontonagon, MI	S7	Forest	30	Clinton, MI [A]	E2	Urban	43
Sturgeon, MI	S8	Forest	1	Rouge, MI [A]	E3	Urban	43
Tahquamenon, MI	S9	Wetland	1	Huron, MI	E4	Urban	2
Manistique, MI	M1	Wetland	1	Raisin, MI [A]	E5	AgMix	44
Escanaba, MI	M2	Wetland	2	Maumee, OH [A]	E6	Crops	64
Ford, MI	M3	Wetland	2	Portage, OH [A]	E7	Crops	64
Menominee, WI [A]	M4	Wetland	40	Sandusky, OH	E8	Crops	2
Peshtigo, WI	M5	Wetland	1	Huron, OH	E9	Crops	2
Oconto, WI	M6	Crops	1	Vermilion, OH	E10	Crops	2
Fox, WI	M7	Crops	7	Black, OH	E11	AgMix	2
Manitowoc, WI [A]	M8	AgMix	43	Rocky, OH	E12	Urban	2
Milwaukee, WI [A]	M9	Urban	45	Cuyahoga, OH	E13	Urban	28
IndianaHC, IN	M10	Urban	2	Grand, OH	E14	Crops	2
Burns, IN	M11	Urban	31	Cattaraugus, NY	E15	AgMix	1
StJoseph, MI	M12	Crops	25	Tonawanda, NY	O1	AgMix	1
Paw Paw, MI	M13	Crops	1	Genesee, NY	O2	AgMix	14
Kalamazoo, MI	M14	AgMix	1	Oswego, NY	O3	AgMix	26
Grand, MI	M15	AgMix	2	Black, NY	O4	Forest	1
Muskegon, MI	M16	Forest	2	Oswegatchie, NY	L1	Forest	1
White, MI	M17	Crops	2	Grass, NY	L2	Forest	1
Pere Marquette, MI	M18	Forest	2	Raquette, NY	L3	Forest	1
Manistee, MI	M19	Forest	2	StRegis, NY	L4	Forest	16
Indian, MI	H1	Forest	2				

A.2.3 Key findings

One or more compounds were detected in 92.5% of the 709 samples. Six sites were absent of detections, including White (S4), Tahquamenon (S9), Manistique (M1), Black (O4), Oswegatchie (L1), and Grass (L2), all of which had only one sample collected (Table A.1). Mixtures of 10 or more compounds were detected at 35% of sites, with a maximum of 53 compounds detected in a single sample. The most frequently detected class of compounds was the insecticides, with an overall occurrence rate of 60%. The majority of the insecticide class detections were for two compounds: DEET and carbazole. Other frequently detected classes include the PAHs (43%), herbicides (37%, primarily metolachlor and atrazine), and flavors/fragrances (31%, primarily HHCB and benzophenone). All other compound classes were detected in less than 25% of samples. The solvents, miscellaneous, and antimicrobial disinfectant classes were the least frequently detected, with occurrence rates of less than 5%.

Watershed land cover was related to occurrence and concentration for many of the compound classes. A pattern of relatively low concentrations in forest- and wetland-dominated watersheds, moderate concentrations in agriculture-dominated watersheds, and higher concentrations in urban-dominated watersheds was observed for the classes insecticides, PAHs, plasticizers, antioxidants, detergent metabolites, fire retardants, nonprescription drugs, sterols, flavors/fragrances, and dyes/pigments (Figure A.2). The only class with frequent detections which did not follow this pattern was herbicides, with concentrations in agriculture-dominated watersheds comparable to or greater than those in urban-dominated watersheds.

Seasonal differences in compound concentrations were observed for four compounds, all of which had relatively high detection frequencies. Compounds that had significantly greater concentrations in warm weather months included Metolachlor, Atrazine, and DEET, consistent with common use patterns of these chemicals. HHCB concentrations were significantly greater in winter. Seasonal differences varied by site. For example, a clear seasonal pattern was observed for atrazine and metolachlor in samples from the highly agricultural Maumee and Portage Rivers in Ohio, with summertime concentrations 1-2 orders of magnitude greater than wintertime concentrations.

One or more water quality benchmarks were exceeded in samples from 20 sites (Figure A.3; (Baldwin et al. 2016a)). Many of the sites with regular exceedances were those dominated by urban land cover. Water quality benchmarks were exceeded by a factor of 10 (TQ >10) at seven sites: Rouge, Indiana Harbor Canal, Clinton, Cuyahoga, Milwaukee, St. Joseph, and Portage. The Clinton River had the most compounds with exceedances (9), followed by the Rouge (8), St. Joseph (7), and Milwaukee (6) rivers. Compounds with the most frequent water quality benchmark exceedances were the PAHs benzo[a]pyrene, pyrene, fluoranthene, and anthracene, the detergent metabolite 4-nonylphenol, and the herbicide atrazine. Water quality benchmarks were exceeded by a factor of 10 or more (up to a factor of 117) for six compounds: pyrene, benzo[a]pyrene, fluoranthene, dichlorvos, atrazine, and anthracene.

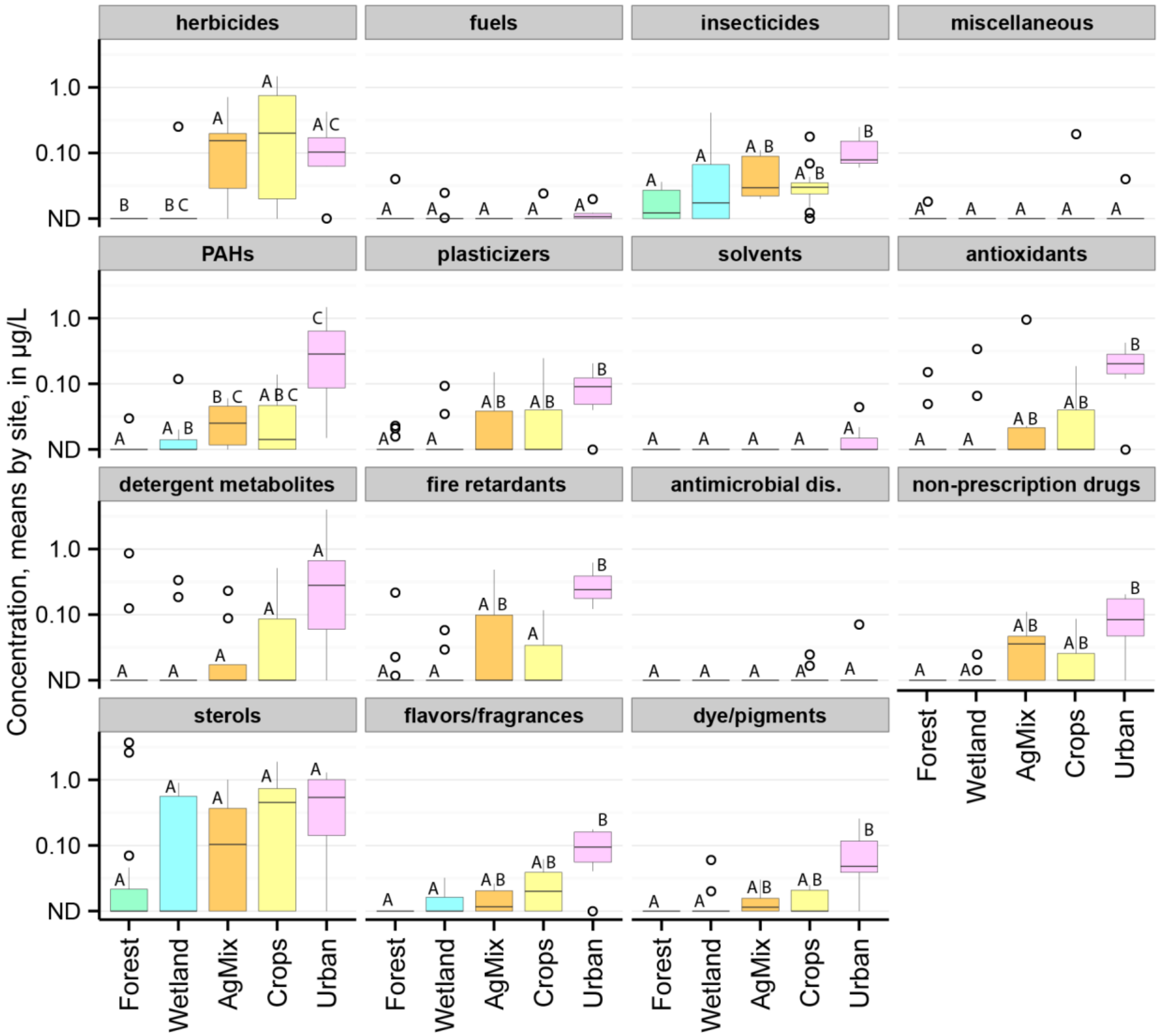

Figure A.2. Dominant land cover and site mean concentrations of compound classes. Number of sites per land use category: forest, 15; wetland, 12; AgMix, 9; Crops, 13; Urban, 8. Boxplot labels A, B, and C indicate which groups of samples are statistically similar (those sharing a common letter) and statistically different (those not sharing a common letter) using the Kruskal-Wallis multiple comparisons test (p-values < 0.05). [ND, not detected; µg/L, micrograms per liter; antimicrobial dis. antimicrobial disinfectants; AgMix, agricultural mix of pasture/hay and crops].

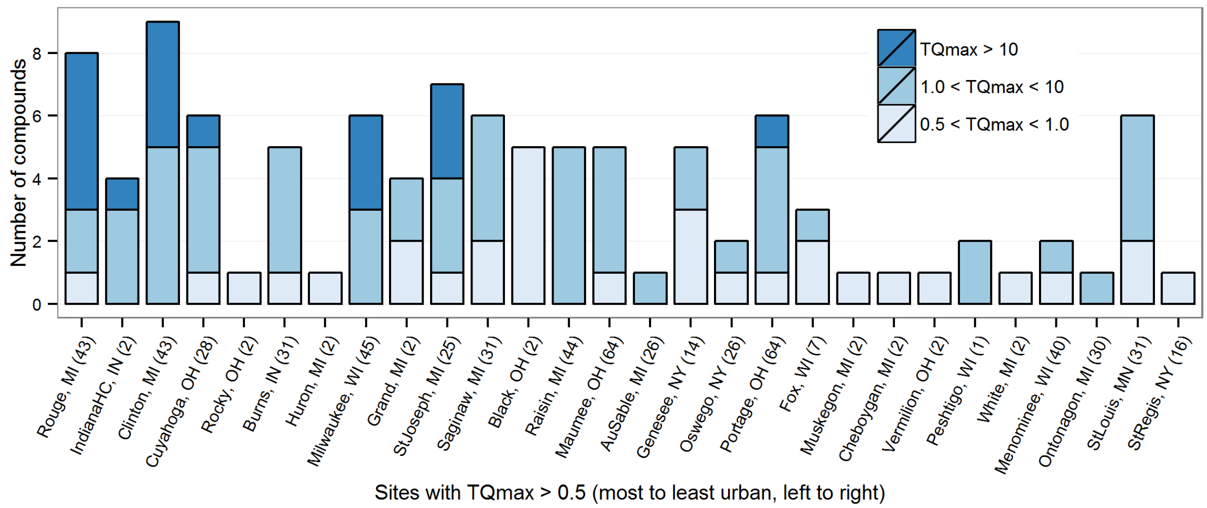

Figure A.3. Sites with compound concentrations approaching (0.5 < TQmax < 1.0) or exceeding (TQmax > 1.0) water quality benchmarks. Total number of samples at each site shown in parentheses. [TQmax, maximum toxicity quotient].

Sixty-eight percent of sites had detections of nonsteroidal endocrine-disrupting compounds (EDCs; (The Endocrine Disruption Exchange, Inc. 2012). Mixtures of EDCs (detectable concentrations of two or more) were observed in samples from 61% of sites--highlighting the importance of chemical mixtures. Twenty-three percent of sites had ten or more EDCs (maximum = 21 at River Rouge) in a single sample. Computed EEQs indicated medium to high risk (greater than the no observable effect concentration or lowest observable effect concentration, respectively) of estrogenic effects for intersex or vitellogenin induction at 10 sites. Steroidal estrogens were not measured, and therefore, these estrogenic effects are likely considerably underestimated.

This study highlights the complexity of compound mixtures in streams, especially streams with urban influences. There was an approximately four-fold difference in the mean number of detected compounds per sample and the mean total sample concentration between sites with greater than 15% urban land cover and those with less than 15% urban land cover. Along with other urban-associated factors such as increased stream flashiness, OWCs have potential to stress stream ecosystems and contribute to degraded populations of fish, invertebrates, and other organisms (Bell et al. 2012).

A.3. Microplastics

Concern surrounding plastics, and especially microplastics particles (less than 5 mm in diameter), in aquatic environments has been growing in recent years. Microplastics are introduced into aquatic environments from a variety of sources: spillage of production materials; atmospheric deposition; wastewater treatment plant (WWTP) effluent and sludge; and degradation of larger items, such as Styrofoam, plastic bags, bottles, wrappers, cigarette butts, and tires.

The ecological consequences of microplastic contamination is an active area of research and includes impacts at multiple trophic levels, uptake, accumulation, associated adverse impacts on reproduction, metabolism, liver physiology, and other effects (Anbumani et al. 2018). In addition, ingested microplastics can also serve as a vector for exposure to harmful chemicals including components of plastic formulations, chemicals sorbed to plastic particles and pathogens (Anbumani et al. 2018; McCormick et al. 2014).

In the Great Lakes, microplastics concentrations as high as 466,000 particles/km^2 have been observed (Eriksen et al. 2013). Although tributaries were assumed to be the major source of microplastics, published characterization of microplastics in rivers was scarce. The available studies provided a valuable foundation but lacked sufficient scope and scale to provide insight into the influence of key watershed attributes and hydrology (Wagner et al. 2014).

A.3.1 Objectives

The objectives of this study were to (1) determine occurrence and concentrations of microplastics in Great Lakes tributaries, (2) determine relations between microplastics and watershed attributes such as land cover, population density, and wastewater effluent contribution, and (3) explore the role of hydrology on microplastic occurrence. A summary of study findings is provided here; additional detail and supporting data are published elsewhere (Baldwin et al. 2016c, 2016d).

A.3.2 Methods

Study sites included 29 Great Lakes tributaries in 6 states (Figure A.4). Watershed drainage areas of the tributaries varied from 101 – 16,400 square kilometers (km^2), with 2.9 – 92% urban land cover, and 0 – 44% wastewater effluent as a percentage of streamflow.

A total of 107 samples were collected from April 2014 to April 2015. Each tributary was sampled three or four times, capturing low-flow and runoff-event conditions. Samples were collected using a 1.5 m long, 333 µm neuston net with an opening 100 cm wide x 40 cm high (Sea-Gear Corp. Miami, Florida, USA). The net skimmed the surface and upper 20-35 cm of the water column, keeping a portion of the net opening above water. Samples were collected by boat, from a bridge, or by wading (Figure A.5A-C).

Samples were processed and sieved into 3 size classifications (Baldwin et al. 2016b). The sample from each sieve class was visually observed using a dissection microscope, and microplastic particles were thereby enumerated and categorized according to morphology as: fragments (broken down pieces of larger debris such as plastic bottles), pellets/beads (preproduction pellets, microbeads from personal care products and bead blasting, and other spheroids), lines/fibers (particles of fishing line and nets, and fibers from synthetic textiles), films (plastic bags and wrappers), or foams (foam cups, take-out containers, packaging) (Figure A.5D,E). Plastic particle concentrations were reported in particles per cubic meter (p/m^3).

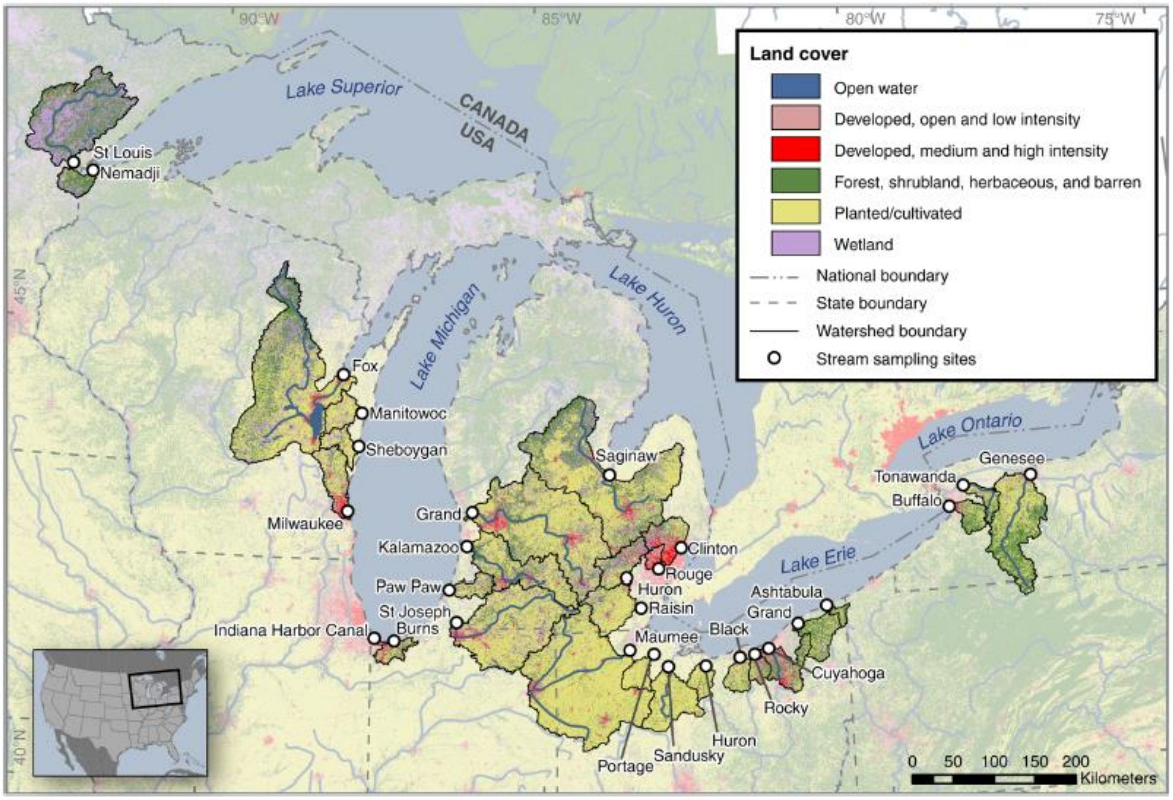

Figure A.4. Sampling locations, watershed boundaries, and land cover for 29 tributaries of the Great Lakes. Map comprised of various spatial datasets (Instituto Nacional de Estadística Geografía e Informática et al. 2006a, 2006b; National Atlas of the United States, 2005; U.S. Department of Agriculture-Natural Resources Conservation Service et al. 2009).

Concentration differences between nonurban low-flow, nonurban runoff-event, urban low-flow, and urban runoff-event samples were evaluated, with urban samples defined as those from watersheds with greater than 15% urban land cover.

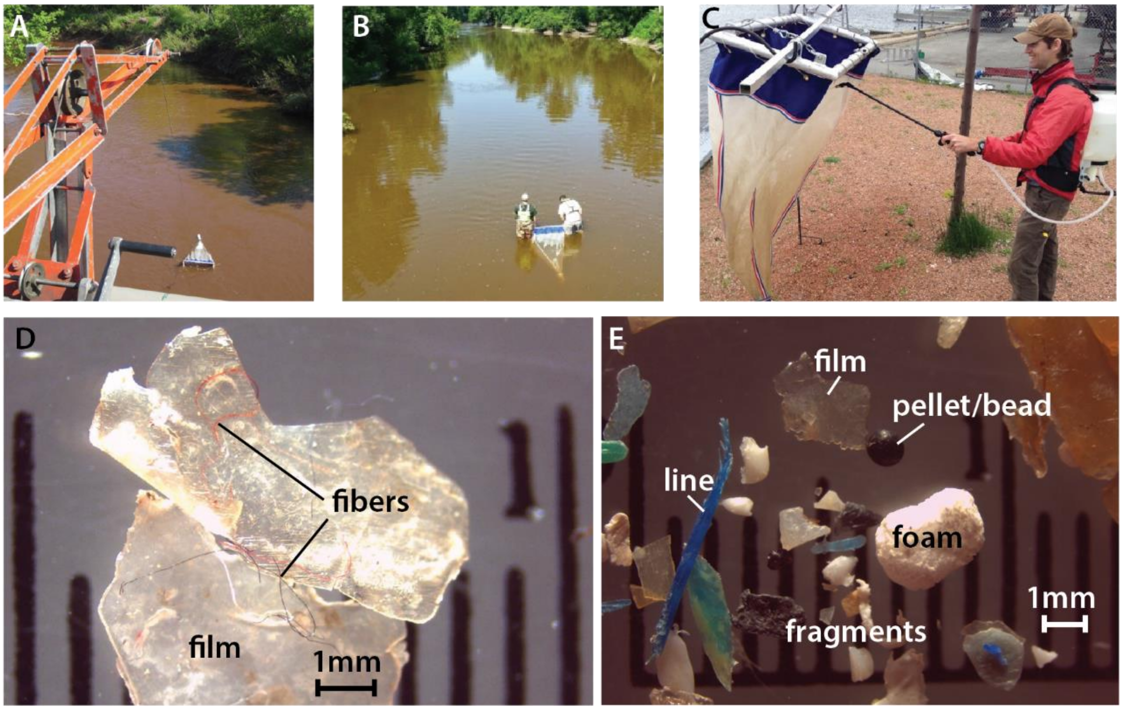

Figure A.5. Sample collection using (A) a bridge crane and (B) by wading; (C), washing particles from the net into the cod end using a backpack sprayer; (D, E), microscopic images of assorted microplastic particles.

A.3.3 Key findings

Plastic particles were found in all 107 samples analyzed (complete sample results published previously, Baldwin et al. 2016c). Sample concentrations ranged from 0.05 - 32 p/m^3 (median 1.9 p/m^3, mean 4.2 p/m^3). Seventy-two percent of particles were in the smallest size range sampled (0.355-0.99 mm), 26% were in the 1.0-4.75 mm size range, and 2% were > 4.75 mm. Seventy-one percent of plastic particle types were lines/fibers (mostly fibers), 17% were fragments, and the remaining particles were foams, films, and pellets/beads, accounting for 8%, 3%, and 2% of all particles, respectively.

Concentrations of fragments, pellets/beads, films, and foams were positively correlated with watershed attributes related to urban development, including total urban land cover (Figure A.6), population density, and (films excepted) percent impervious cover. Hydrology also appeared to influence concentrations of these particle types: in urban and nonurban watersheds, concentrations of fragments, films, and foams were greater during runoff-events than during low-flow conditions when normalized by mean concentration for the sampling site (Figure A.7). These litter-related plastics can be transported efficiently in urban conveyance systems from impervious areas to receiving waters.

Fibers/lines were ubiquitous across all land use types (Figure A.6); concentrations were not correlated with any of the tested watershed attributes, nor were they affected by hydrology (Figure A.7). Recent research indicates that atmospheric deposition may be one important source of fibers (Dris et al. 2016) that could subsequently enter streams via direct deposition or washoff from surfaces throughout the watershed. None of the plastic types were significantly correlated with the contribution of wastewater effluent to streamflow; however, land application of WWTP sludge may be a significant source of fibers in agricultural areas (Kang et al. 2018). Further work is necessary to comprehensively evaluate sources of fibers to streams.

Given the abundance of microplastics of less than 333 µm reported previously (Dris et al. 2015; OSPAR, 2009), the current study likely underrepresents true microplastic concentrations. This can be ecologically significant since such particles can be taken up into cells and can translocate from the gut into the circulatory system (Browne et al. 2008), and their larger surface area to volume ratio enhances potential as vectors for sorbed contaminants.

The relative proportion of particle types in the current tributary study differs greatly from previous findings in the Great Lakes themselves. Most notably, the difference in the proportion of fibers/lines in the current study (71%) is much greater than the proportion of fibers in surface samples from the lakes (up to 14%). Hydraulics within the river systems as compared to the Great Lakes together with the physical properties of the plastics may explain this difference in abundance of fibers. Negatively-buoyant fibers made of polymers such as polyester, rayon, nylon, and cellulose acetate may remain in suspension in the turbulent flow of a river (allowing them to be captured by surface sampling), but likely settle out upon reaching the more quiescent lakes. Accumulations in sediment may have important effects on benthic organisms, as well as higher trophic level organisms reliant on these benthic organisms.

This study provides an important baseline for future studies. The number and diversity of sampling locations, the regional scale, and the incorporation of varying hydrologic conditions provided a multifaceted approach that allowed for the exploration of many factors potentially influencing the prevalence of plastic debris in rivers. The results have advanced our currently limited understanding of the sources, transport, and fate of plastics in fluvial systems.

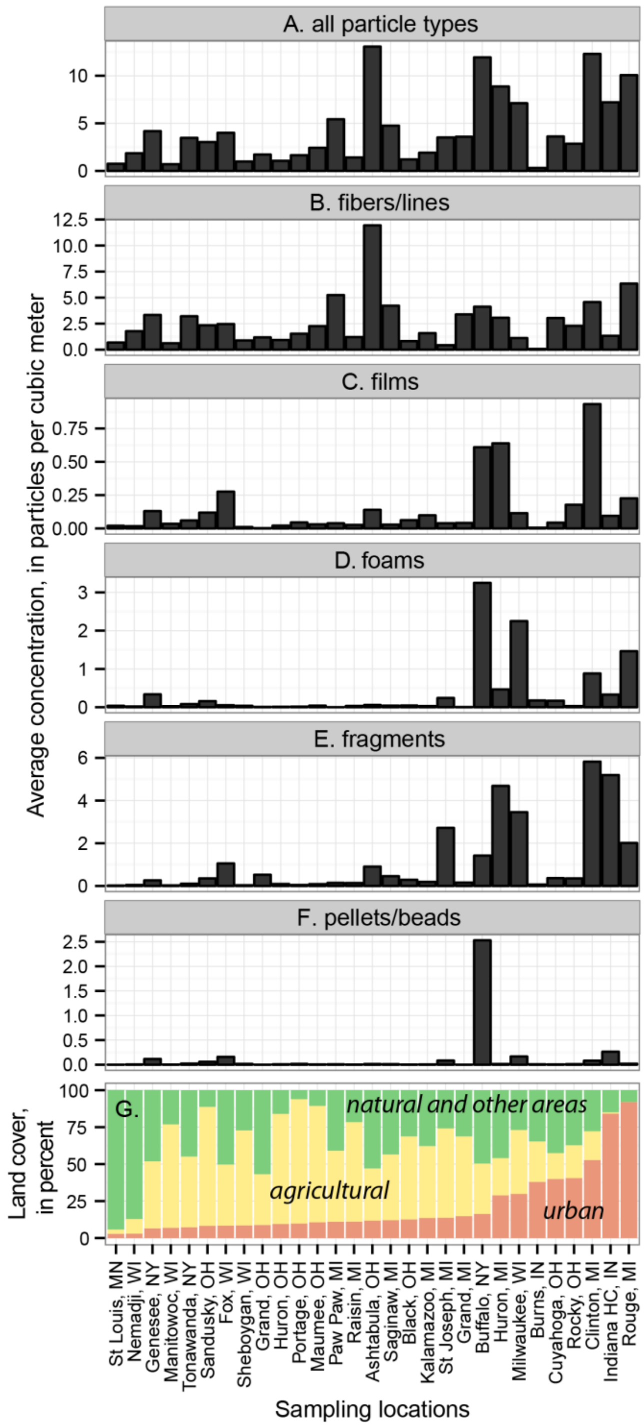

Figure A.6. Average concentrations of plastic particles (A-F) and watershed land cover (G) at sampled Great Lakes tributaries, 2014-15.

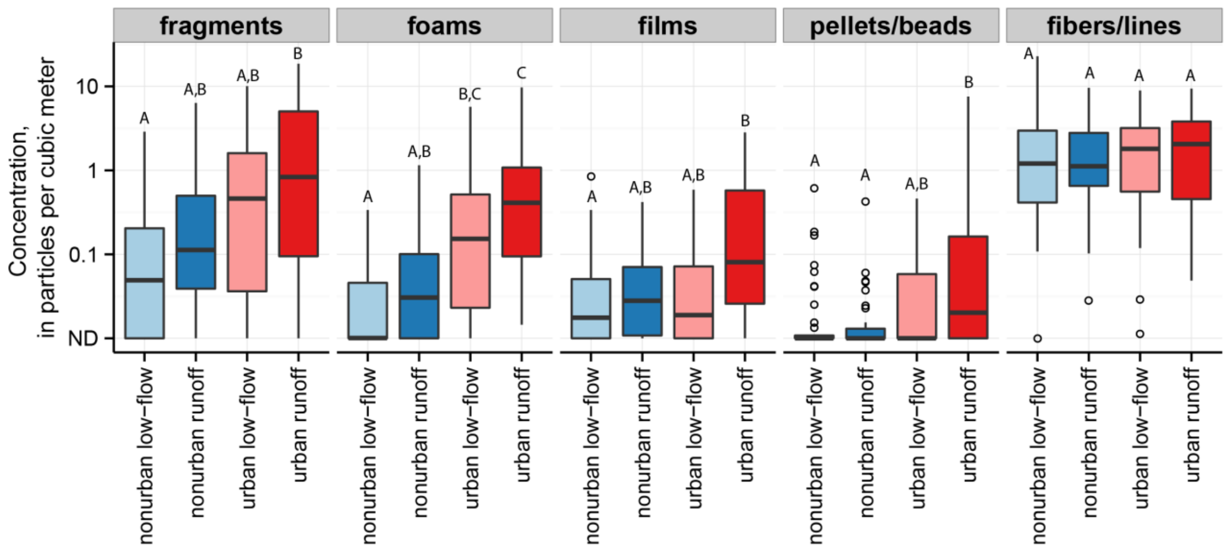

Figure A.7. Plastic concentrations in nonurban low-flow (n = 40), nonurban runoff (n = 35), urban low-flow (n = 17), and urban runoff (n = 15) samples. Urban watersheds are those with greater than 15% urban land cover. Boxplot labels A, B, and C indicate which groups of samples are statistically similar (those sharing a common letter) and statistically different (those not sharing a common letter) using the Kruskal-Wallis multiple comparisons test (p-values < 0.05). [boxes, 25th to 75th percentiles; dark line, median; whiskers, 1.5 x the interquartile range (IQR); circles, values outside 1.5 x the IQR; ND, not detected].

A.4. Microorganisms

Human and livestock waste are two substantial sources of contamination that enter Great Lakes waterways through a variety of pathways. Human waste sources include degraded public and private sanitary sewer lines and improper connections, sanitary and combined sewer overflows, treated wastewater effluent, properly functioning and defective septic systems, and land application of waste effluent. Livestock waste enter waterways through direct access to streams, overland flow from barnyards, pastures, and manure application, and through subsurface drain tiles. These waste streams serve as substantial sources of waterborne pathogens and chemical contaminants to waterways where they can pose risks to human and/or ecological health. However, since their occurrence in wastes vary hydrologically, temporally, and seasonally (for example, human viruses are present only when the host population is infected and varies seasonally and temporally), these contaminants may not always be detectable when human and agricultural wastewater contamination is present. Therefore, testing for non-pathogenic, host-associated indicators can provide considerable value since they are abundantly present in the original fecal sources and remain at detectable levels even after substantial dilution in receiving waters (Lenaker et al. 2018).

A.4.1 Objectives

Building upon the investigation of organic waste compounds reported in section 3.2, the overall objective for the current study was to characterize variability of microbiological contaminants and potential hazard from waterborne pathogens in tributaries of the Great Lakes. Specific objectives were to 1) provide information on the prevalence of human and livestock waste contamination in tributaries of the Great Lakes using host-associated indicators, 2) investigate the occurrence of waterborne pathogens as a result of human and livestock contamination, and 3) characterize variability of host-associated indicators by hydrology (low-flow and periods of increased runoff), season, and watershed attributes. The current section on microorganisms provides a summary of findings from the first phase of GLRI; additional detail and supporting data are published elsewhere (Corsi et al. 2018; Dila et al. 2018; Lenaker et al. 2017).

A.4.2 Methods

Study sites included eight Great Lakes tributaries selected to represent a gradient of urban and agricultural land covers (Figure A.8). Flow-weighted composite water samples were collected during low-flow and runoff event periods from February 2011 to June 2013 and analyzed for waterborne pathogens (290 samples) and host-associated bacteria (214 samples). Host-associated bacteria analyses included human *Bacteroides* (HB), *Lachnospiraceae* (Lachno2, human associated) and ruminant *Bacteroides* (BacR). Waterborne pathogen analyses included eight human viruses, eight bovine viruses, two protozoa, and four bacteria. Detailed sampling, analytical methods, resulting data, and data analysis methods have been previously described (Corsi et al. 2014; Dila et al. 2018; Lenaker et al. 2017).

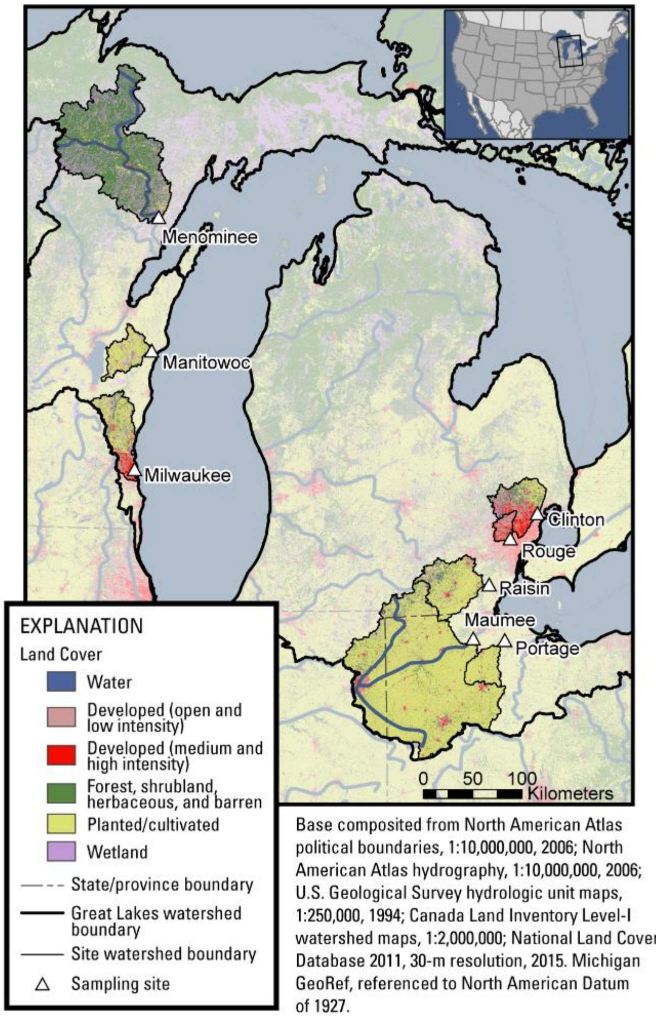

Figure A.8. Sampling locations and land cover in eight tributaries of the Great Lakes. Map comprised of various spatial datasets.

A.4.3 Key findings

Overall, five of the eight human viruses and four of the eight bovine viruses analyzed were detected at least once. Human viruses (n=290) were present in 16% of samples, and the human bacterial markers HB and Lachno2 (n=219) were present in 94% and 87% of samples respectively. Bovine viruses and pathogenic bacteria were present in 14% and 1.4% of samples (n=290), respectively, and the ruminant marker, BacR, was present in 47% of samples (n=219). Protozoa were not detected during the study period.

Evidence of human and bovine fecal pollution was present in all eight watersheds (Figure A.9). Occurrence of human markers was generally higher in watersheds with the most urban influence. Likewise, occurrence of bovine markers was generally highest in watersheds with higher densities of cattle and pasture land. The Portage River was the most notable exception to human and bovine bacterial marker trends; the exact cause of these differences is unknown but is thought to be attributable to a variety of factors including those that have efficient conduits directly to the stream such as combined sewer overflows or drain tiles. The comparatively lower bovine and human virus occurrences at this site highlights the utility of using multiple parameters in evaluating waste contamination.

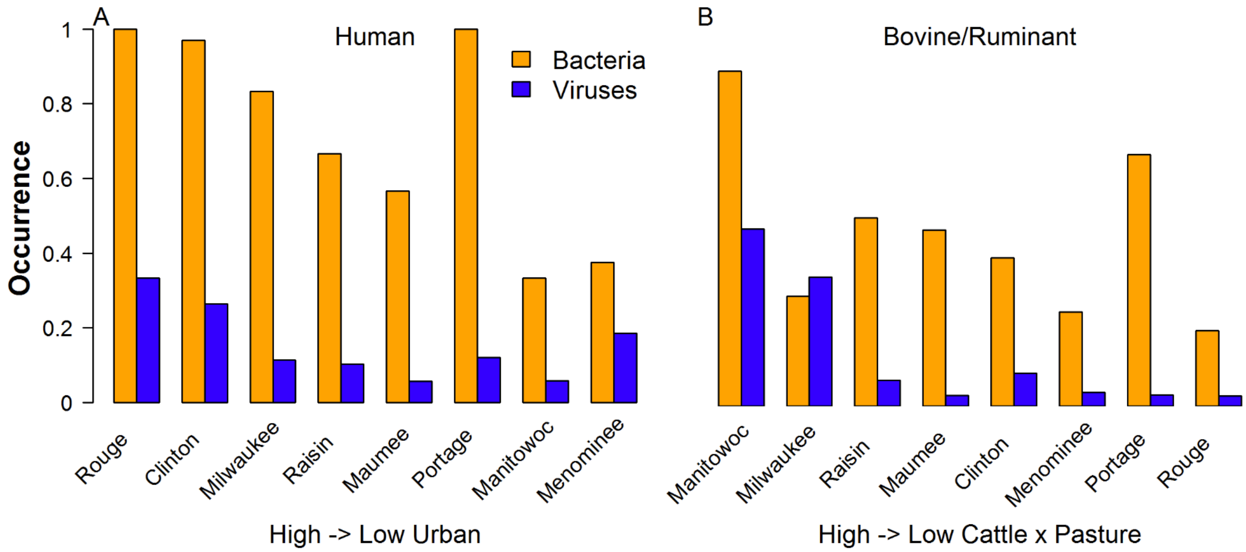

Figure A.9. Occurrence of microbiological indicators of human and cattle waste in samples collected at eight Great Lakes tributaries, 2011-2013. The sum of human bacteria markers and the sum of human viruses are used as indicators of human waste (A), and ruminant *Bacteroides* and the sum of bovine viruses are used as indicators of cattle waste (B).

Mean concentrations of human bacterial markers in urban and mixed land use watersheds (Clinton River, Rouge River, Milwaukee River) were ~10 to 30-fold greater during runoff-events compared with low-flow periods; however, mean concentrations in agricultural watersheds did not differ with hydrologic condition (Figure A.10A). In contrast, mean concentrations of BacR were greater during runoff-events (compared with low-flow periods) across all sites (Figure A.10B); highest mean concentrations were observed in runoff-event samples from the watershed with the highest cattle density (Manitowoc River). Mean concentrations of human and bovine viruses were not significantly different with respect to flow conditions at individual sampling locations nor when data were grouped by land cover or cattle density characteristics (Figures A.10C and D).

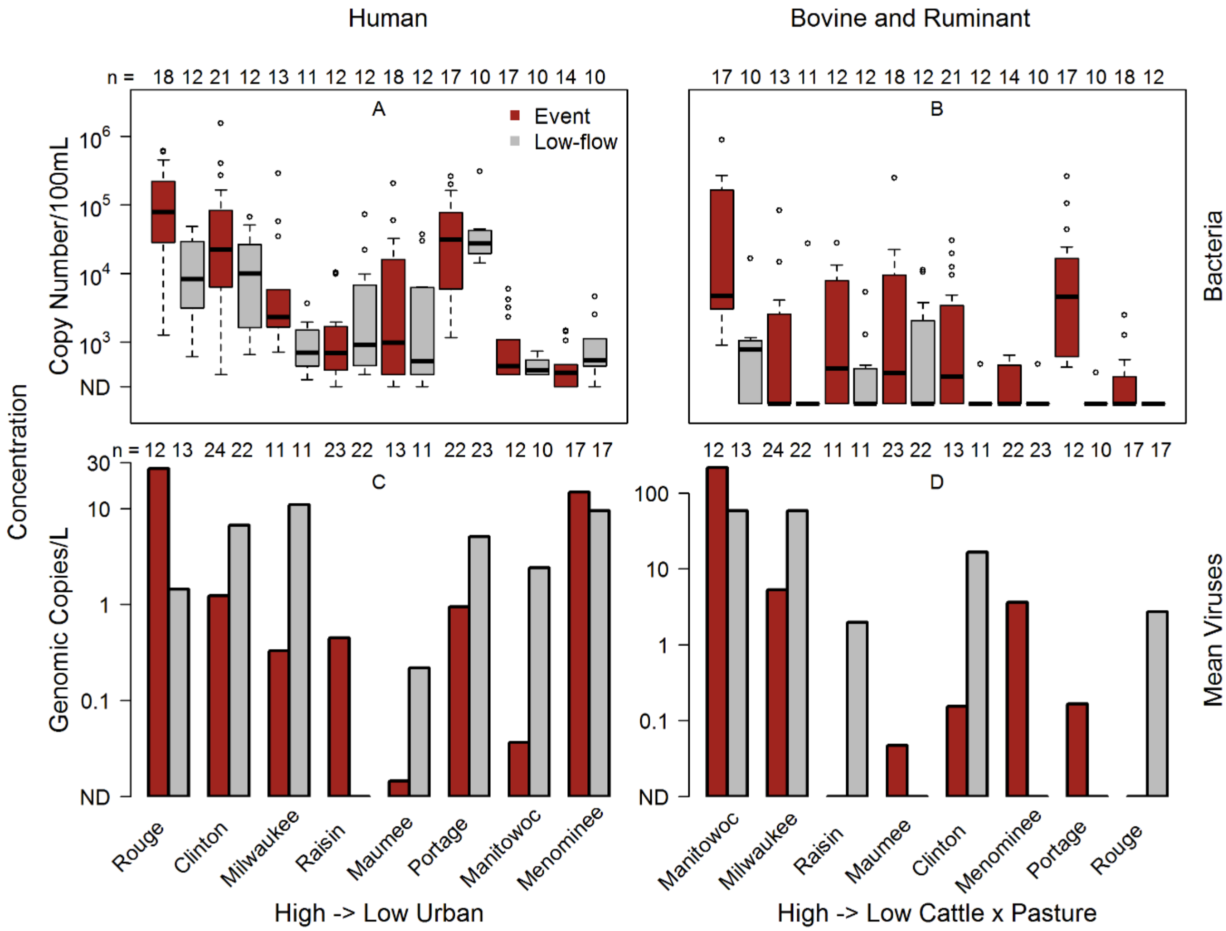

Figure A.10. Boxplots of host-associated bacteria and bar plots of mean virus concentrations at eight Great Lakes watersheds from 2011-2013, categorized by hydrologic condition. Host-associated bacteria include the sum of human *Bacteroides* and *Lachnospiraceae* (A) and ruminant Bacteroides (B). Viruses include mean of the sum of human viruses (C) and mean of the sum of bovine viruses (D).

Human- and cattle-associated bacterial markers and viruses were all present to varying degrees in all seasons, with greater concentrations observed during the cooler months of December through April than other months (Figure A.11). This same pattern was also true when considering runoff-event periods and low-flow periods separately with only one exception: the mean sum of human viruses had no significant difference between the two seasonal periods during runoff-events. This seasonal difference could be influenced by multiple possible factors including: increased survival in cold weather resulting from decreased sun exposure due to ice cover and shorter daylight periods more efficient transport during periods with saturated soils, winter spreading of manure on frozen ground, and less disinfection of treated wastewater compared to warm weather periods.

Multiple regression analysis was used to explore factors that explain variability in host-associated marker flux (marker quantity per unit time per unit watershed drainage area) for samples collected during rainfall periods. For each of the three host-associated markers investigated, one regression equation representing all eight sites was effective at describing variability of flux using four predictor variables: population density (human for HB and Lachno2, and cattle for BacR) as an indicator of source, and season, rainfall depth, and percent drain tile coverage which all influence hydrology (Dila et al. 2018). Coefficients for the seasonal variables reflected a peak seasonal contribution in late winter and early spring for human and ruminant indicators. This is consistent with direct analysis of seasonal data described above. These cooler months are typically the time of year in the Great Lakes region when the ground is saturated or frozen and low-flow levels in streams are greatest, leading to efficient runoff mechanisms. Tile drainage also increases efficiency of watershed hydraulics, which leads to efficiency in contaminant transport, including microorganisms (Wang et al. 2010).

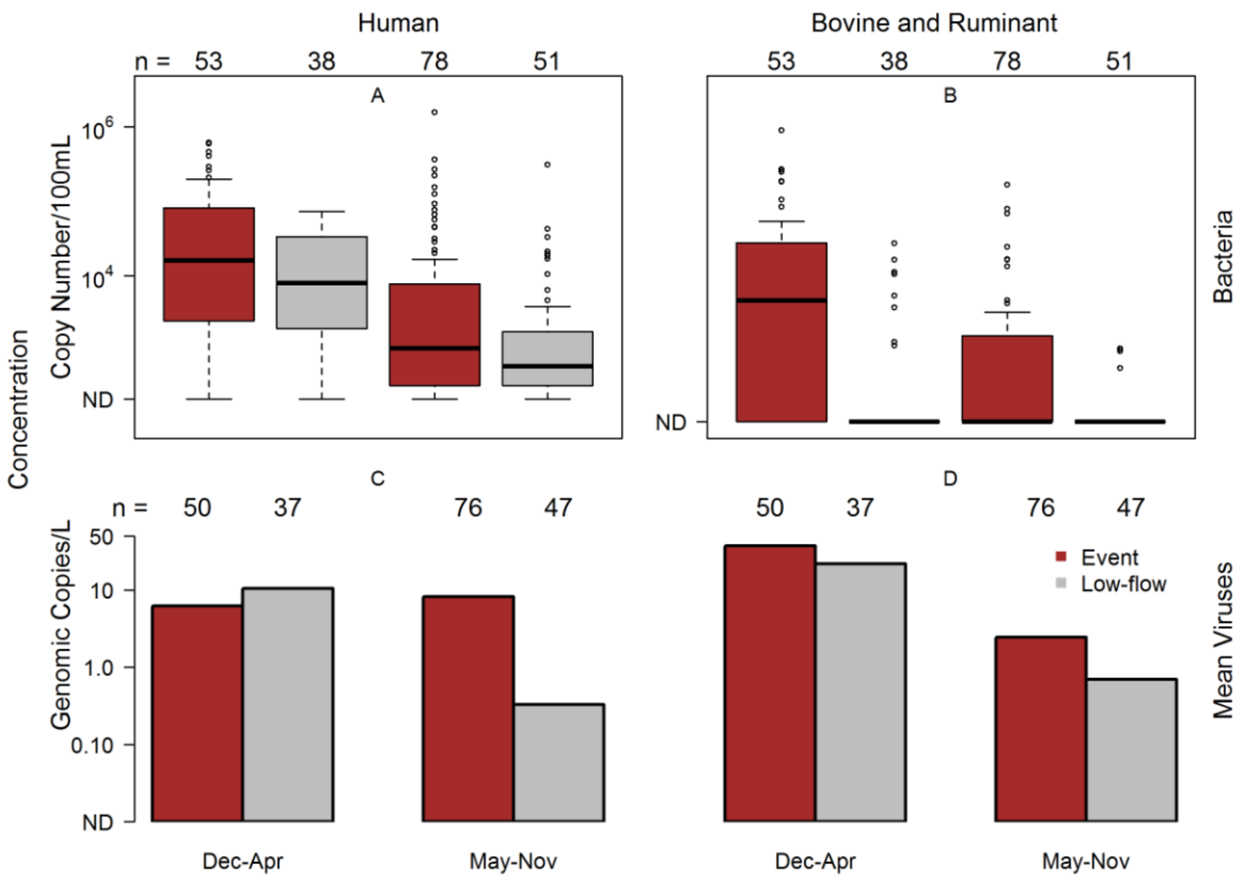

Figure A.11. Boxplots of host-associated bacteria and bar plots of mean virus concentrations at eight Great Lakes watersheds from 2011-2013, categorized by Seasonal grouping and hydrologic condition. Host-associated bacteria include the sum of human bacteria markers (A) and ruminant Bacteroides (B). Viruses include mean of the sum of human viruses (C) and mean of the sum of bovine viruses (D).

Collectively, three different classes of parameters were measured in the first phase of GLRI that indicate presence of human waste: human-associated bacteria markers (Dila et al. 2018), human viruses (Lenaker et al. 2017), and chemicals associated with human waste, including 3-non-prescription drugs and 10-flavors and fragrances (Chapter 3, section 3.2; Baldwin et al. 2016). The prevalence of each of these three classes of human waste indicators is dependent on multiple factors that rely on source concentration as well as fate and transport properties. Even with the inherent differences in these factors, occurrence and concentration of human viruses and human-waste chemicals both increased with increasing human bacterial marker concentration (Figure A.12). Together, these results indicate that the human-associated bacteria markers can be used as indicators of a potential health hazard from waterborne pathogens and presence of toxic chemicals associated with human and cattle waste. Given the relatively low cost and ubiquitous presence in the host, host-associated bacterial markers are a reasonable choice for screening for the presence and magnitude of human and cattle waste in surface water.

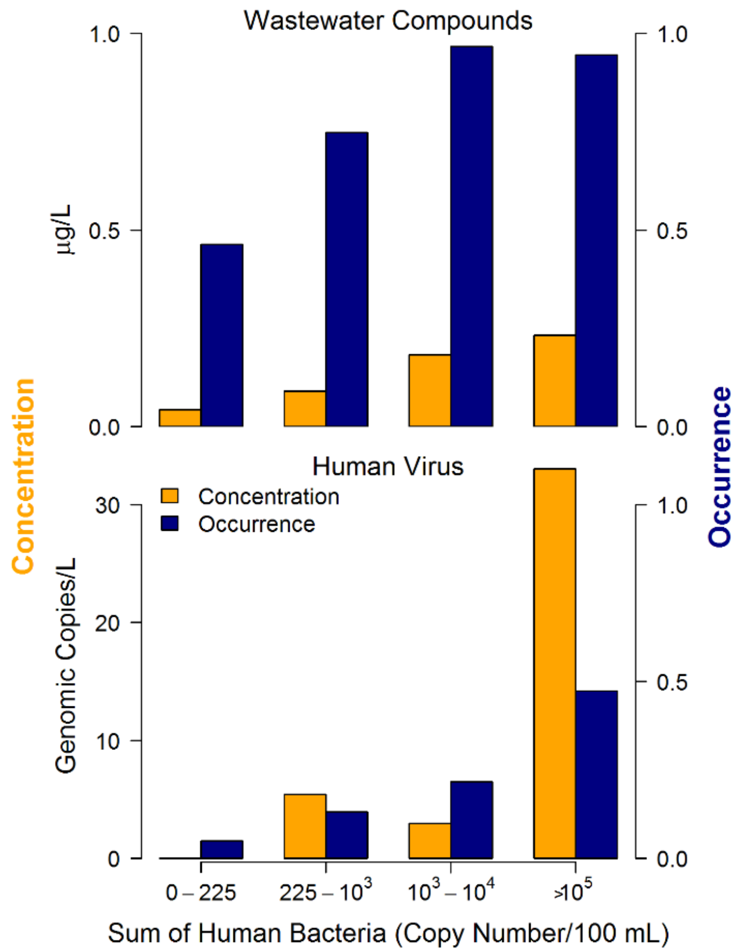

Figure A.12. Comparison of human-associated bacteria markers with the concentration and occurrence frequency of human wastewater associated compounds and human virus samples collected from eight Great Lakes Tributaries, 2011-2013.

A.5. Knowledge gaps

- To fully understand potential for biological effects of CECs, there is a need to expand this assessment from the limited number of compounds in the current evaluation to a broader suite of compounds in specific chemical classes including parent and degradation products.

- Results indicated that a number of compounds at numerous sites occur at concentrations exceeding water quality benchmarks and exceeding levels at which EEQs would indicate potential for reproductive issues. Even so, water quality benchmarks and estradiol equivalency information are available for fewer than half of the sampled compounds. There is a need to expand the evaluation of potential biological effects to include a larger proportion of the monitored compounds. Evaluation that leverages the growing database of chemical-specific high throughput in vitro biological activity data being generated via the ToxCast program (https://www.epa.gov/chemical-research/toxicity-forecasting) has been initiated for this purpose (see Appendix E).

- There is a need to expand the evaluation of potential biological effects to include consideration of monitoring results with chemical mixtures. Use of information from the ToxCast database to estimate cumulative biological activity for chemicals that influence common biological pathways has been initiated for this purpose (see Appendix E).

- Comparison of chemical monitoring results to water quality benchmarks provides information on potential for adverse effects but does not indicate hazard for specific biological functions. To give resource managers confidence in monitoring-based evaluation results, the findings must be validated. To provide enough information to design experiments for validating evaluations based on chemical monitoring, it is important to identify the specific biological pathways of concern. One way to achieve this would be to link results from a ToxCast-based evaluation with information captured in the adverse outcome pathway wiki (Society for the Advancement of Adverse Outcome Pathways, 2018).

- Objectives of the continued research on this program are to take advantage of information in ToxCast to prioritize chemicals, identify sites at which these chemicals occur, and screen for potential adverse biological effects that may be associated with those chemicals. To achieve these objectives, software tools are needed to efficiently facilitate this type of evaluation of complex multidimensional data sets containing multiple samples per site and numerous chemicals per sample at many sites.

- Short-term variability of CECs may play an important role in acute effects on biological health, however most efforts for evaluation of CECs rely on a limited number of observations at a low collection frequency. Additional data is needed to define sub-daily variability in exposure concentrations (due to hydrologic condition, etc.).

- The fate of plastic fibers once delivered from tributaries to the Great Lakes is in need of investigation. From the current study, it was determined that the distribution of different microplastic particle morphologies was different in tributaries compared to the Great Lakes due to the large proportion of fibers/lines in tributary samples. To investigate whether deposition of negatively-buoyant fibers contributes to this result, a study of bed sediment samples from Lakes Michigan and Erie has been undertaken.

- Previous water sampling methods capture only particles on the surface. Additional work is needed to quantify the abundance and morphologies of plastics vertically throughout the water column to better characterize microplastics in the aquatic environment.

- The size range of particles captured is dependent on the mesh size of sampling nets. Additional work is needed to define particles in smaller size ranges that have greater likelihood of uptake and potential adverse impact on organisms in the lower trophic levels.

- Further work is necessary to thoroughly evaluate sources of microplastics to receiving waters and potential for management options.

- A relation with human-associated bacteria markers and wastewater-associated chemicals was established. Current methods to evaluate the many wastewater compounds with potential adverse ecological effects are expensive and time consuming. There is a need for an efficient and cost-effective method to screen for potential biological effects for which host-specific markers may play an important role.

- There is a critical need for a more comprehensive characterization of the fate and transport characteristics of contaminants and pathogens moving from tributaries into the nearshore zone of the Great Lakes, in order to more accurately evaluate their potential ecological and human health consequences from exposure during recreational activities.

A.6. Management Implications

Resulting information concerning OWCs, microplastics, and microorganisms provide characterization of these contaminants that can be used to help identify the most likely sources and scenarios for which they are most prevalent. Land use, hydrologic, and seasonal associations with specific contaminant classes can be used to evaluate the practicality of designing management scenarios for controlling specific contaminants that have been identified to be of greatest potential concern:

Urban land use:

- The greatest concentrations of PAHs were present in urban watersheds and did not vary with season. PAH contamination can be introduced from individual point sources such as coal-fired power plants or other industrial activities or from more diffuse non-point sources such as coal-tar pavement sealcoat or vehicle exhaust. Remedies for individual sources must be considered on a case-by-case basis, but control of diffuse sources would require community-level considerations or by implementation of urban stormwater runoff management and green infrastructure practices.

- Human-associated bacterial marker concentrations and flux increased as urban influence in the watershed increased. They were also greater during cool weather months and during periods of increased runoff. Illicit discharge detection and elimination programs (IDDE) in the Great Lakes region are likely to underestimate the severity of sewage contamination because the most common activity periods for IDDE programs include dry weather periods during the ice-free months. Shifting a portion of IDDE efforts to cool weather months and during runoff periods would increase the likelihood of capturing the periods of greatest contamination.

- Insecticides, plasticizers, antioxidants, detergent metabolites, fire retardants, nonprescription drugs, sterols, flavors/fragrances, and dyes/pigments were all greatest in watersheds with the most urban influence. Control options for these contaminants include IDDE programs for those originating from sewage, or urban stormwater runoff management practices for other sources. Urban stormwater runoff practices are variably effective for these classes of contaminants given that many of them are in solution, and many management practices rely on particulate deposition.

Agricultural land use:

- Herbicides (Atrazine and metolachlor) were identified as a high priority with the greatest concentrations coinciding with application periods during late spring and early summer. Strategies to reduce the potential biological impact of pesticides would be most effective if designed to reduce pesticide runoff during application with consideration of the variable hydrologic and vegetation cover conditions during this elevated concentration period.

- Concentrations and flux of cattle-associated microorganisms increased with cattle density and percent pasture in the watershed. They were also greatest during winter and early spring, consistent with other common agricultural pollutants such as nutrients and sediment. Multiple options for runoff management are available for these land uses, and study results indicated that there is potential for substantial reductions if chosen agricultural management practices are implemented to be effective during these cool weather months.

Microplastics:

- Concentrations of most types of microplastics increased with increasing urban influence in the watershed but were present in nonurban watersheds as well.

Contamination of several morphologies of microplastics were more prevalent during runoff periods in urban and nonurban watersheds. This indicates that agricultural and urban runoff control measures that rely on filtration or infiltration would likely be effective for removal of many microplastics, but microplastics with positive buoyancy may not be captured in control measures that rely on particle deposition.

- Microplastics have not been studied sufficiently within watersheds to understand sources well. Control measures would have a greater likelihood of success if watershed management efforts first focused on source area identification efforts and developed estimations of the relative quantity of microplastics originating from specific sources.

Modeling results for host-associated bacterial markers can enhance watershed management activities: One regression model per host-associated bacterial marker was effective at describing variability in all eight watersheds. This result indicates that there is potential for transferability of this model to additional watersheds to evaluate human and cattle waste contamination for watershed management activities such as estimation of total maximum daily loads.

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A.8. Products

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Appendix B

Monitoring of Contaminants of Emerging Concern by Great Lakes Mussel Watch

Lead Organization: National Oceanic and Atmospheric Administration, National Centers for Coastal Ocean Science, Silver Spring, MD, USA

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B.1 Introduction

NOAA's Mussel Watch Program (MWP), a longstanding national contaminant monitoring program that utilizes resident bivalves as bioindicators of water quality, launched its monitoring activities in the Great Lakes in 1992. The then recently established non-indigenous population of Ponto-Caspian dreissenid mussels in the Great Lakes (except Lake Superior) with attributes such as high filtering capacity, ability to bioaccumulate chemical contaminants with limited ability to metabolize them, sedentary habits and widespread distribution on hard substrates was identified as a prospective tool for contaminant monitoring. The slew of research on dreissenid mussels, following their introduction in the Great Lakes, revealed their role in contaminant cycling via trophic transfer from the base of the food web to top predators, thus bolstering the value of using dreissenid mussels as a bioindicator by MWP. The program established 23 long-term monitoring sites within the Great Lakes region at near shore sites away from known outfalls and hotspots mirroring the national program directive to sample from areas intended to represent general conditions of broad coastal areas for water quality assessment. MWP monitored approximately 150 chemicals, including trace metals and persistent, bioaccumulative and toxic chemicals, the so-called "legacy contaminants" that were prevalent during the industrial revolution and have been banned since the 1970s following the implementation of many environmental regulations.

With increased attention on the much larger group of chemicals that remain unregulated and unmonitored in the aquatic environment known as the contaminants of emerging concern (CEC), MWP began assessing how best to incorporate these new classes of chemicals in its monitoring protocol in the early 2000s. Polybrominated diphenyl ethers (PBDE), a group of flame retardant chemicals, was the first CEC to be added to the monitoring list in 2004. MWP conducted pilot CEC studies in the Chesapeake Bay, Biscayne Bay, and the Gulf of Farallones in 2006 and in the Southern California Bight led by the Southern California Coastal Water Research Project in 2011. Concurrently, under the Phase 1 Action Plan of the Great Lakes Restoration Initiative, MWP expanded, adding sites in Areas of Concern (AOC) in 2009/2010 (Kimbrough et al., 2014) and conducting place-based contamination assessments by adopting new approaches and techniques including use of caged mussels and effects-based tools. Mussels can be caged and strategically relocated for precise place-based assessments (monitoring along a pollution gradient, pre and post remediation/restoration assessments and/or contaminant source tracking) for both legacy and CEC monitoring.

Though the focus of Phase 1 monitoring was to provide data for historic contamination, MWP obtained limited tissue data for CECs by coupling field efforts with retrospective analyses of tissue samples. The main objectives of this opportunistic CEC monitoring during Phase1 was to determine the feasibility of using dreissenid mussels for CEC monitoring and explore the use of effects-based monitoring tools in mussels. MWP intended this Phase 1 subsidiary activity to be a testing ground before committing to full-fledged CEC work in Phase 2 and to answer two fundamental questions: 1) What is the occurrence, frequency and spatial distribution of CECs in dreissenid mussel tissue? 2) Can we identify mussel health metrics to link exposure to biological effects through collaborative partnership with academia and federal partners?

B.2 Methods

Dreissenid mussel samples from basin-wide monitoring (2009/2010), place-based contamination assessments in specific AOCs (2011-2014), and offshore sampling (obtained in partnership with EPA's Great Lakes Fish Monitoring and Surveillance Program; 2012-2014) were chosen for CEC analysis. Given the natural variability and heterogeneity in chemical contamination sources and processes in different zones of the lake, mussel sampling sites were categorized as 1) Harbor-River-Tributary sites 2) Nearshore sites and 3) Offshore sites (Figure B.1A). Samples are a combination of in situ mussels collected from hard substrates and caged mussels deployed in rivers and tributaries. The CECs included in this report are polycyclic aromatic hydrocarbons (PAH), polybrominated diphenyl ethers (PBDE), pharmaceutical and personal care products (PPCPs) and alkylphenols (AP). A comprehensive report of CECs in mussels from the Great Lakes collected between 2013- 2016 can be found in Kimbrough et al. 2018. To increase the likelihood of finding CECs, samples from rivers and harbors (mainly Milwaukee Estuary AOC and Niagara River AOC (Figure B.1B-C)) were selected preferentially over samples collected from relatively less polluted nearshore and offshore sites. The methods of in situ mussel collection, caged mussel deployment and collection, and analytical methods for CECs and effects-based tools can be found in detail in the Quality Assurance Project Plan (QAPP available at https://coastalscience.noaa.gov/project/great-lakes-mussel-watch-supports-presidents-great-lakes-restoration-initiative/).

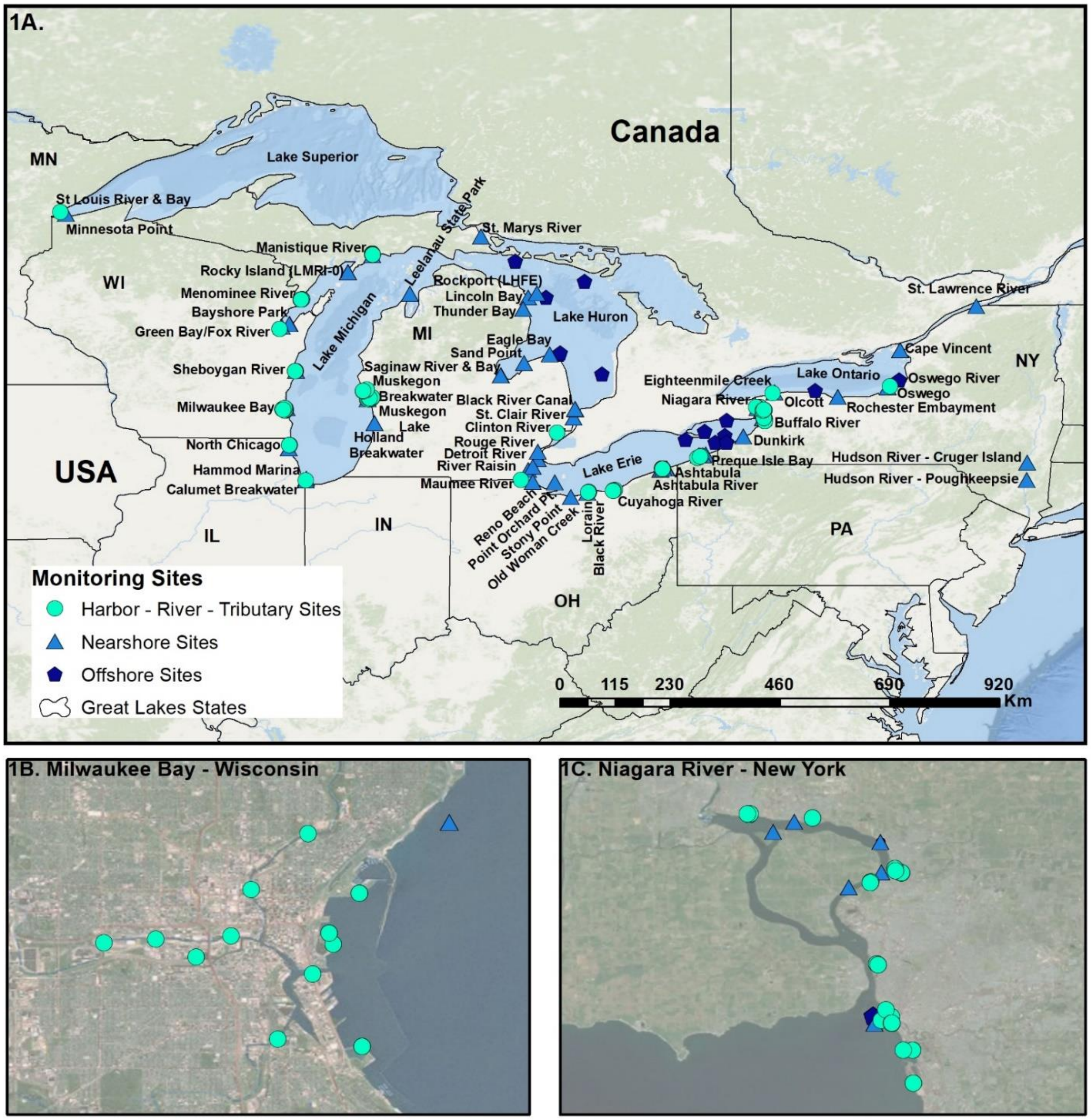

Figure B.1: The general location of mussel sampling sites in the Great Lakes for characterization of CECs from 2009- 2014. Most locations have 1-3 sites with the exception of Milwaukee Estuary (13 sites; Fig 1B) and Niagara River AOC (28 sites; Fig 1C). PAHs were monitored at all sites and PBDEs at a smaller subset of sites between 2009- 2014. PPCPs were retrospectively analyzed in mussel tissue samples collected from Milwaukee Estuary in 2013, Niagara River, Presque Isle Bay, Ashtabula River, Cuyahoga River and Black River AOCs along eastern shore of Lake Erie, and Thunder Bay National Marine Sanctuary in Lake Huron in 2014.

All chemical concentrations in mussel tissue were blank corrected and values below detection limit were assigned zero. PAH concentration is reported as the sum of 54 compounds, PBDE as the sum of 52 congeners and both PAH and PBDE sums were expressed in ng/g dry weight. PPCP concentrations are expressed in ng/g wet wt. Hierarchical Ward's cluster analysis was used to cluster the PAH and PBDE concentrations into high, medium and low concentrations. Non-parametric Wilcoxon text was used to test significant differences in concentrations across zone types.

B.3 Results and discussion

B.3.1. Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs have been monitored by MWP from the inception of the program in 1992 and its national status and trends have been summarized for the nation (Kimbrough et al. 2008). As PAH compounds have current and ongoing sources and many compounds are toxic, it is necessary to dissociate the negative impacts of PAH from those of hydrophilic, less persistent CECs and hence are included in the CEC analyses.

Two hundred and twenty-one dreissenid tissue samples collected from around the Great Lakes were analyzed for PAHs from 2009-2014. PAHs were ubiquitous in distribution and the total concentration (sum of 54 compounds) ranged from 15.8-112638.1 ng/g dry wt in mussels from 2009/2010 basin-wide monitoring (Figure B.2).

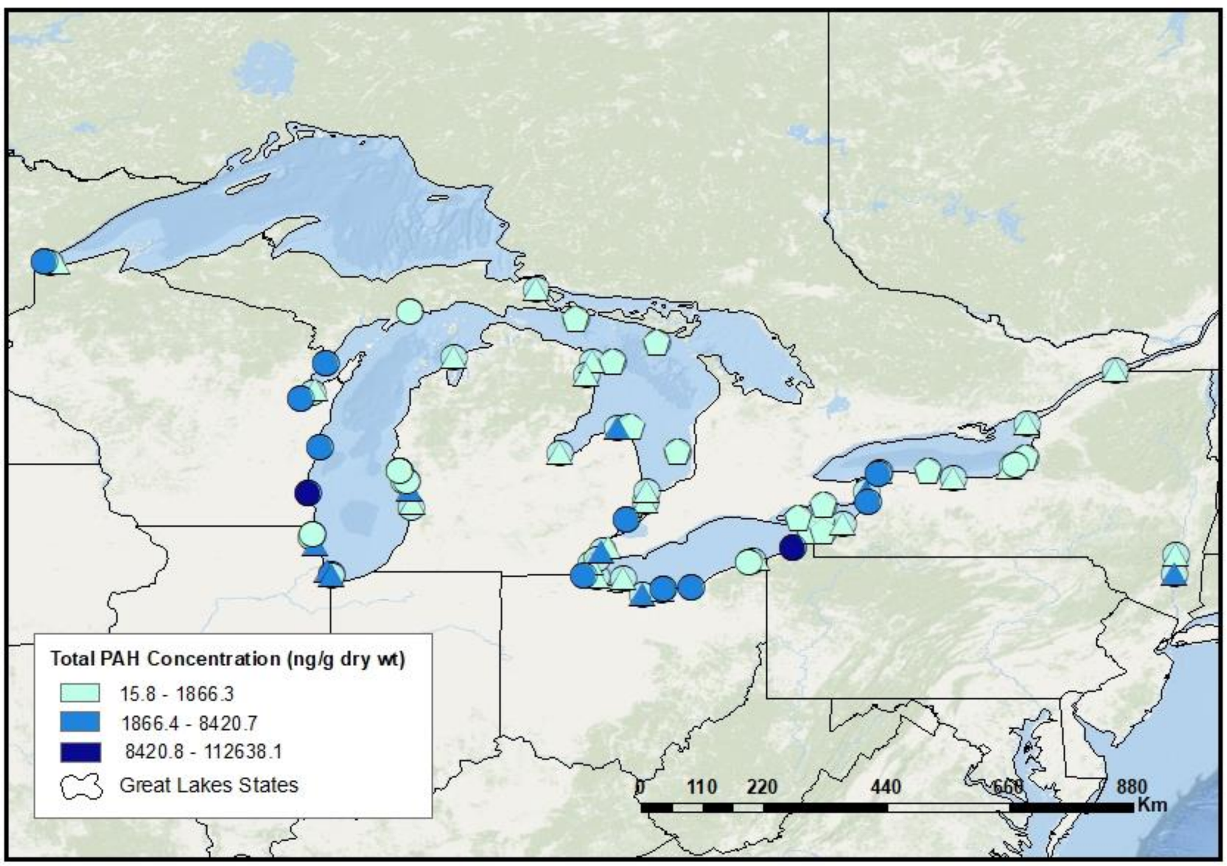

Figure B.2: The total PAH concentration (sum of 54 compounds; ng/g dry wt) in mussel tissue from harbor-river-tributaries (❍) and nearshore sites (△) collected in 2009/2010 and from offshore sites (⬠) sampled from 2010-2014 in partnership with EPA Great Lakes Fish Monitoring and Surveillance Program.

Following this basin-wide monitoring effort, MWP conducted place-based contamination assessments at several AOCs using both caged and in situ mussels. For example, the PAH distribution in the main stem of the Niagara River and seven of its tributaries were assessed to identify the contamination hotspots (Figure B.3) within the AOC boundary.

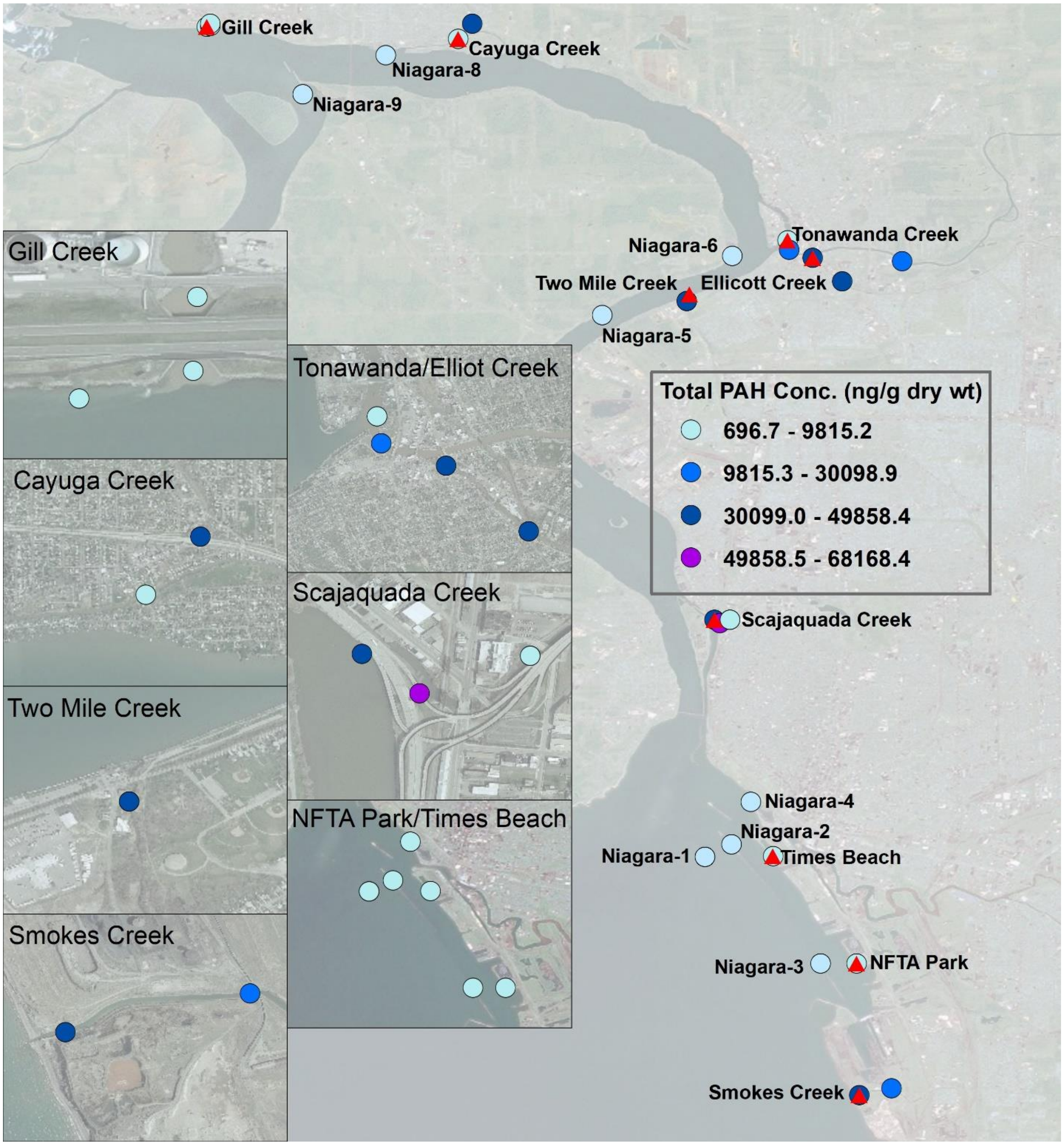

Figure B.3: PAH characterization using caged and in situ mussels at Niagara River AOC conducted in 2014.

When all the PAH data were pooled and analyzed, we found that the total PAH concentration from harbor-river-tributary sites were significantly higher than mussels from offshore and nearshore sites (Wilcoxon test, $p < 0.05$; Figure B.4 excluding extreme outliers). The harbor-river-tributary site samples were mainly from Milwaukee Estuary and Niagara River AOCs. The large number of samples from different zone types in the Great Lakes across years allows MWP to examine the pattern of PAH composition and distribution in mussels (Kimbrough et al., In Preparation). Unlike vertebrates, mussels possess only limited ability to metabolize PAHs and hence are historically regarded as a good indicator for PAH monitoring.

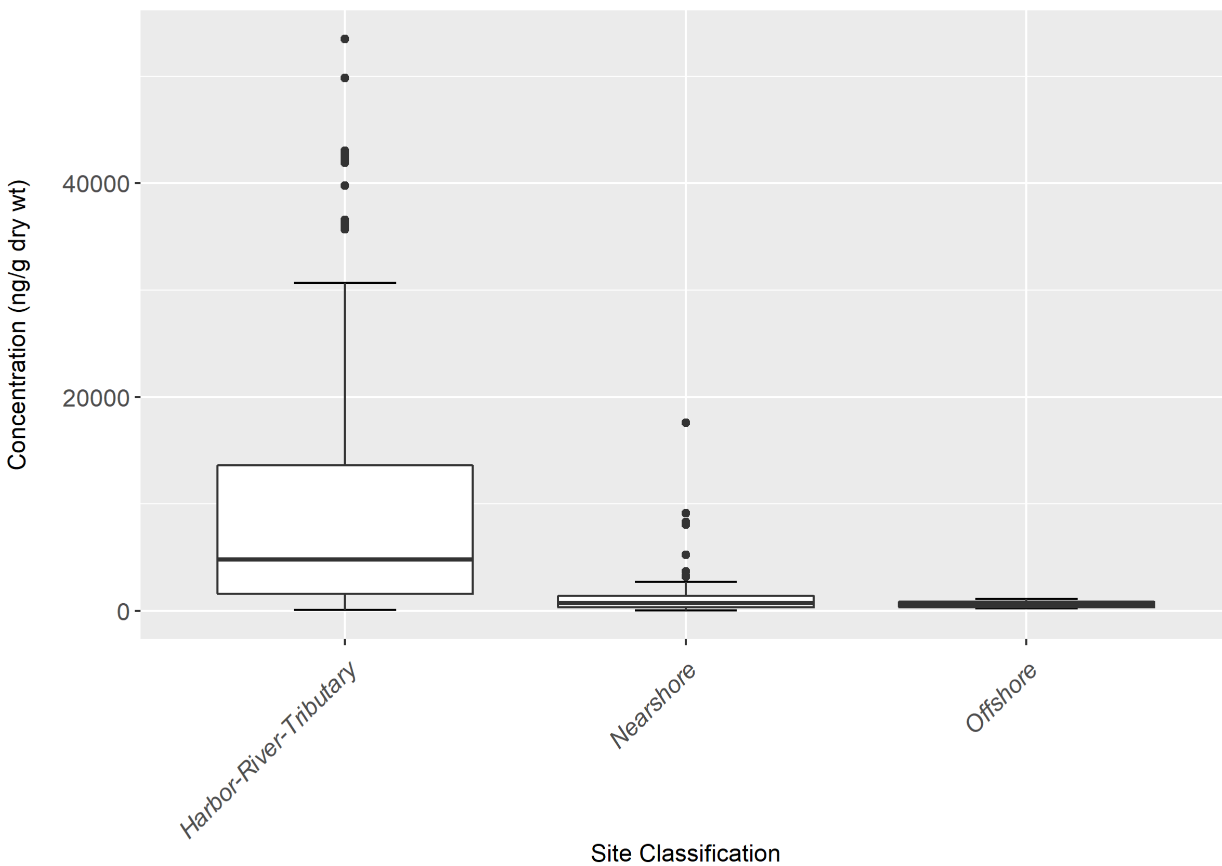

Figure B.4: Whisker plot for total PAH mussel tissue concentrations obtained from 2009-2014 across three zone types in the Great Lakes.

B.3.2. Polybrominated Biphenyl Diethers (PBDEs)

A national summary of Mussel Watch bivalve and sediment samples collected from 2004 through 2007 were summarized for PBDEs (Kimbrough et al. 2009) and was followed by a regional Great Lakes assessment. PBDEs were present in all mussel samples collected during basin-wide monitoring in 2009-2011 and the concentration ranged from 3.2-126.2 ng/g dry wt (Figure B.5).

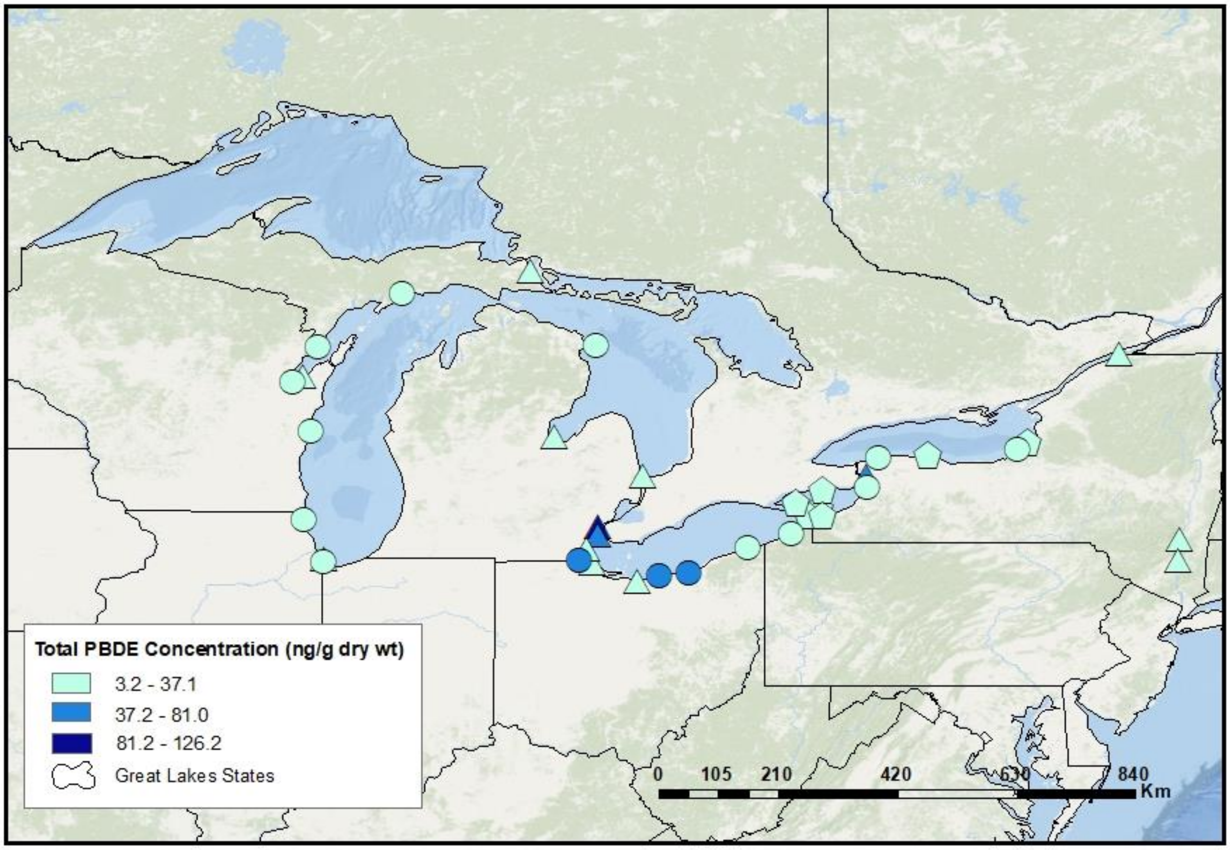

Figure B.5: The total PBDE concentration (sum of 51 congeners) in mussels from Harbor-river- tributaries (❍) and nearshore sites (△) collected from 2009-2011 and from offshore sites (⬠) sampled from 2010-2014 in partnership with EPA Great Lakes Fish Monitoring and Surveillance Program.

Additional samples analyzed over the years (2009-2014) show that PBDEs in offshore and nearshore mussel sites were significantly lower than mussels from harbor-river-tributary sites (Wilcoxon test, $p < 0.05$; Figure B.6).

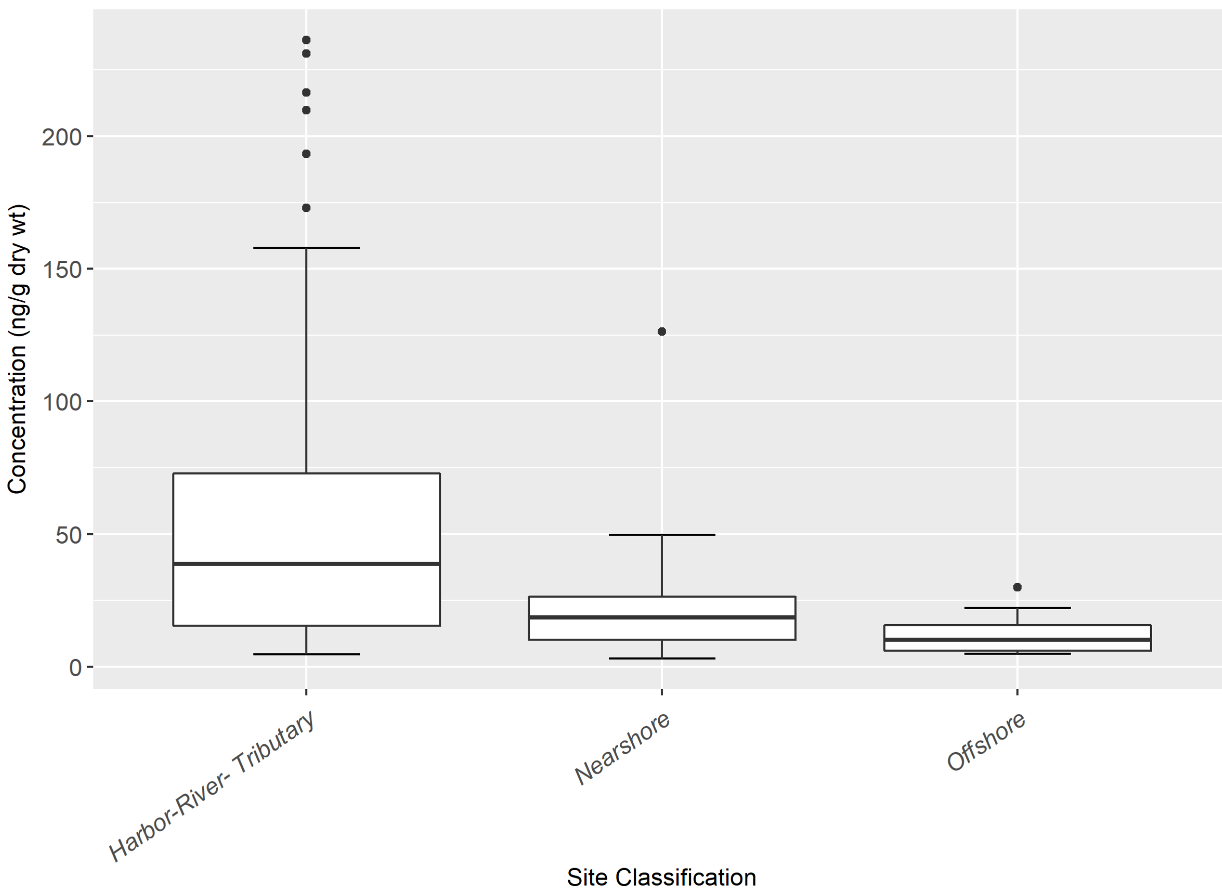

Figure B.6: Whisker plot for total PBDE mussel tissue concentrations obtained from 2009-2014 across three zone types in the Great Lakes.

The harbor-river-tributary site samples were mainly from Milwaukee Estuary and Niagara River AOCs. The Niagara tributary sites had the highest concentration of PBDEs (Figure B.7). Of the 51 congeners analyzed, only 16 congeners were detected in more than 20 percent of the samples. PBDE 47, 99, 154 and 206 were the most dominant congeners with more than 75% detection.

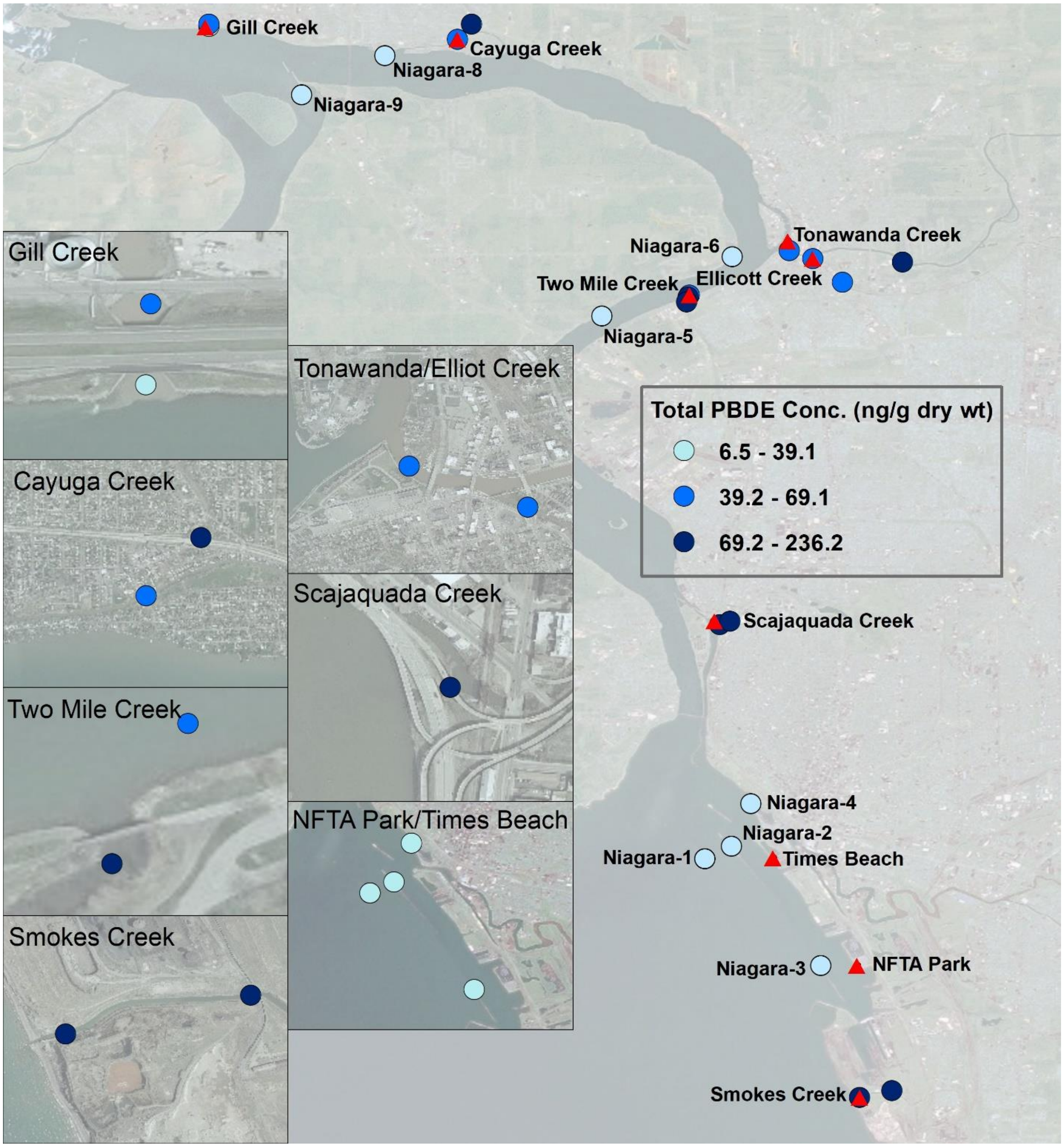

Figure B.7: PBDE characterization using caged and in situ mussels at Niagara River AOC conducted in 2014.

B.3.3. Pharmaceuticals and Personal Care Products (PPCPs)

PPCPs analyzed included over-the-counter, elicit and prescription drugs, synthetic musks, antimicrobials, antibiotics and insect repellents (Kimbrough et al., 2018). 141 PPCP compounds were analyzed in 41 tissue samples, of which only 40 compounds were detected. Amitryptyline (anti-depressant), DEET (insect repellant) and Sertraline (anti-depressant) were the most commonly detected PPCPs in mussel samples (Figure B.8).

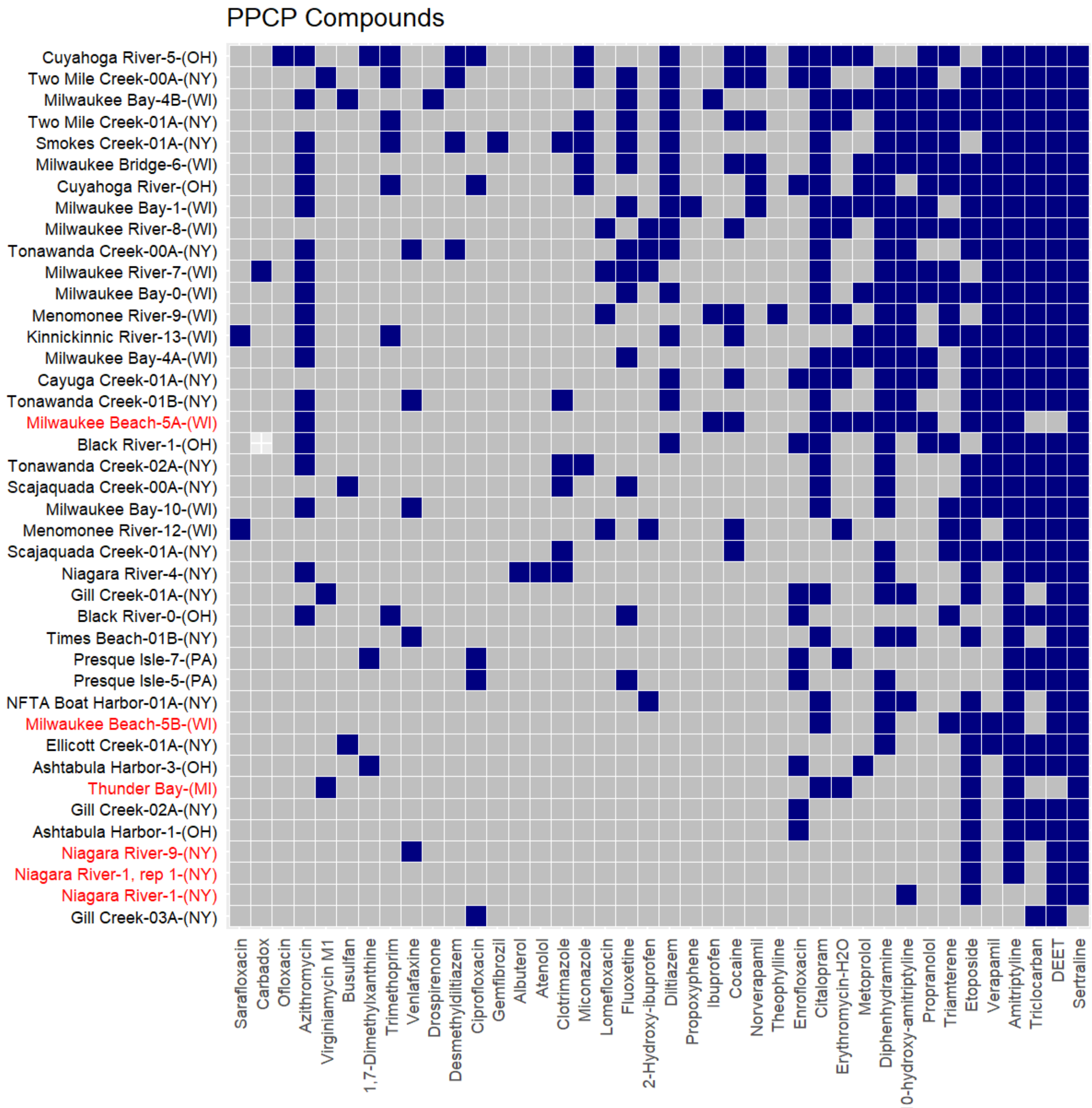

Figure B.8: Presence (■) and absence (■) of pharmaceutical and personal care products found in mussel tissue at various locations. The locations in red are nearshore sites and the rest are harbor-river-tributaries sites.

At least 10 compounds were detected in nearshore sites (situated away from known outfalls) that can be considered as reference sites for mussels. This finding underscores the need for spatially robust monitoring of CECs. Figure B.9 provides perspective on the relative concentration of the PPCPs that were above three times the detection limits and occurred at least at five sites.

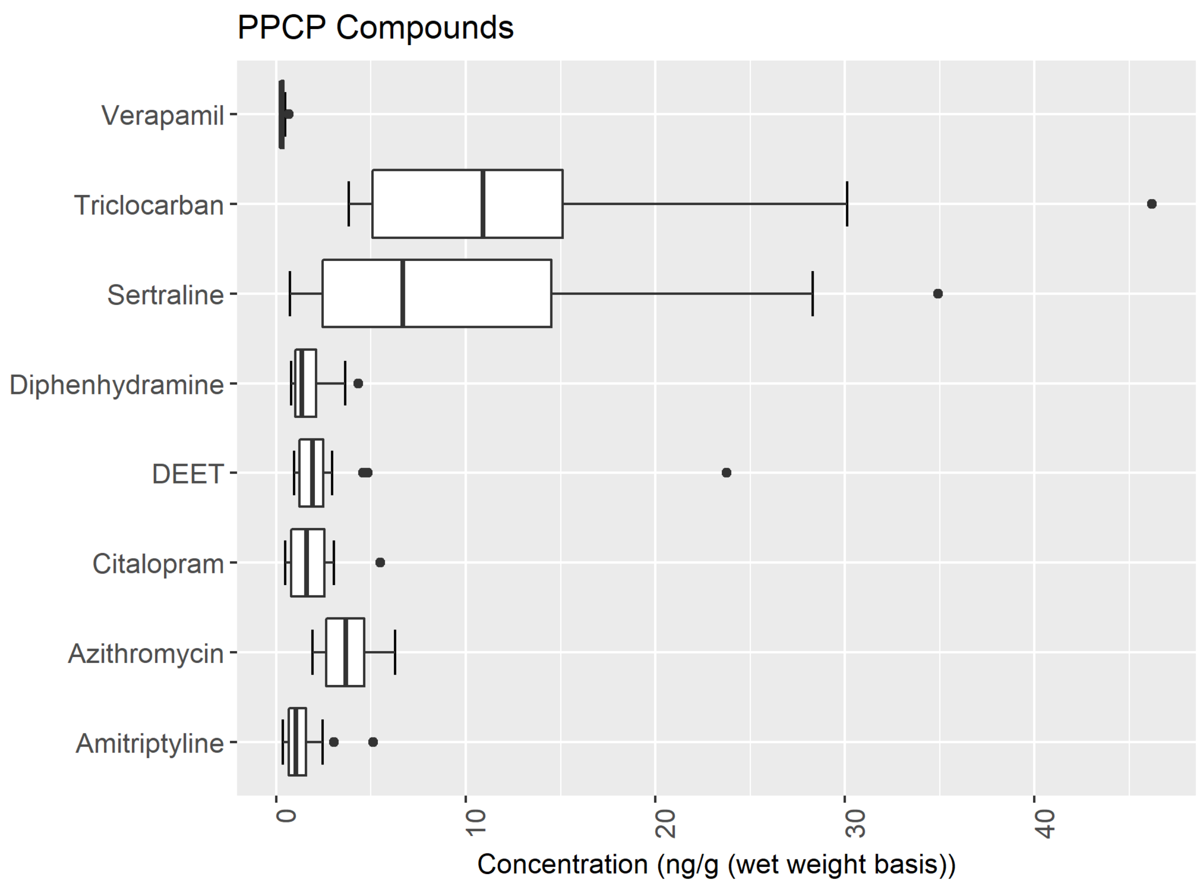

Figure B.9. Concentrations of selected pharmaceutical and personal care products. Only chemical compounds found at more than five sites that had concentrations above 3 x detection limit are included. This plot provides perspective to the relative concentrations of the most commonly found PPCPs in dreissenid mussel tissue.

B.3.4. Alkylphenols and Alkyl Ethoxylates

Alkylphenols are used to make alkylphenol ethoxylates (APEO), which are widely used as industrial surfactants. Two AP compounds- 4-nonylphenol (4-NP), 4-n-octylphenol (4n-OP) and two APEO compounds- 4-nonylphenol monoethoxylate (NP1EO) and 4-nonylphenyl diethoxylate (NP2EO) were analyzed in 32 tissue samples. All four compounds were detected in dreissenid mussels and three of the four compounds were detected at all sites including nearshore sites that can be considered as reference sites (Figure B.10).

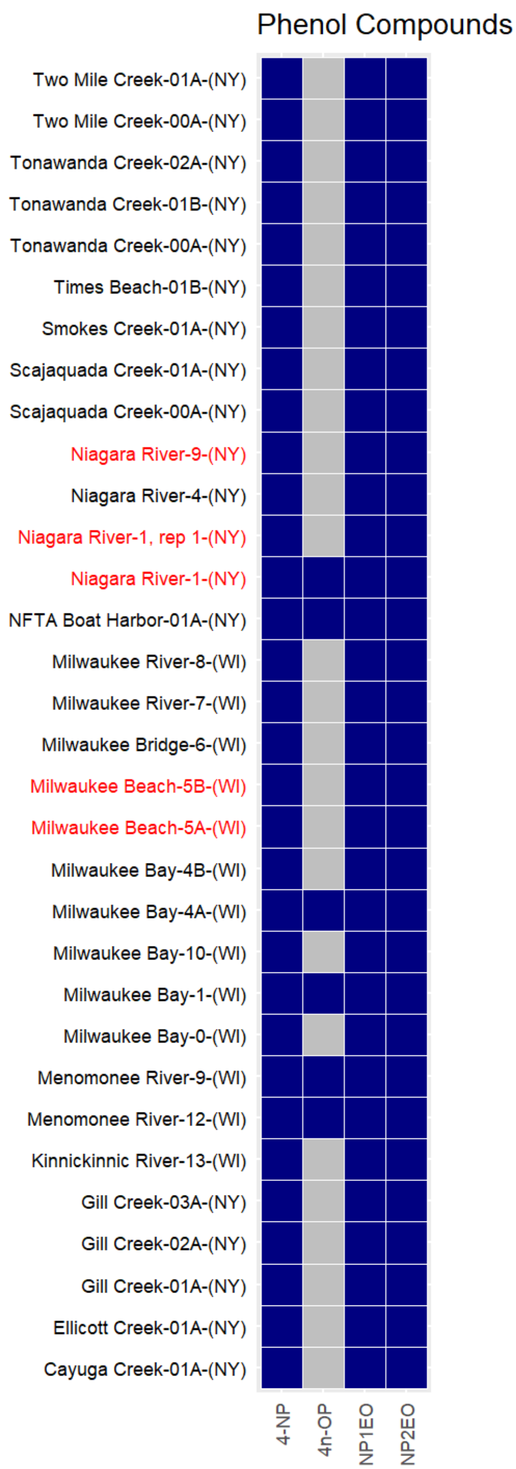

Figure B.10: Presence (■) and absence (■) of pharmaceutical and personal care products found in mussel tissue at various locations. The locations in red are nearshore sites and the rest are harbor-river-tributaries sites

Of the three most commonly detected compounds, 4-NP registered the highest concentration in dreissenid mussels (Figure B.11).

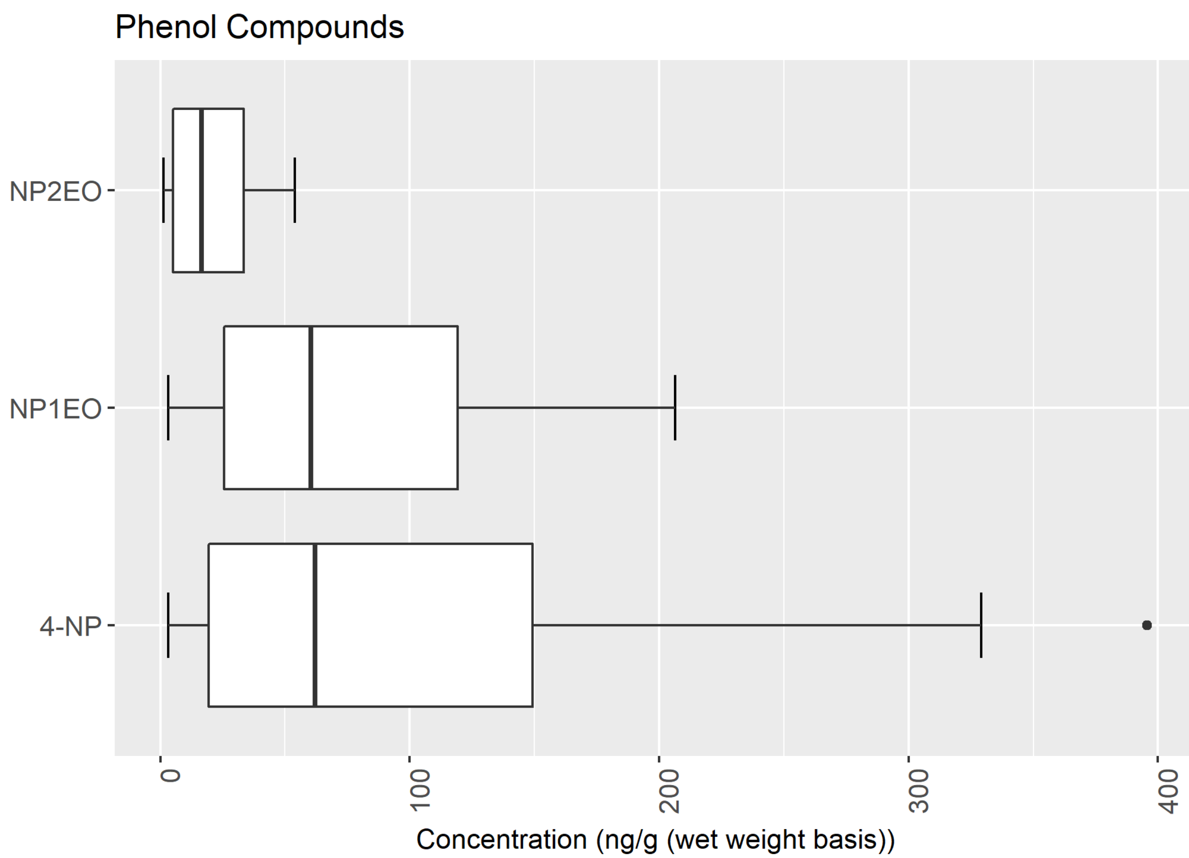

Figure B.11: Concentrations of alkylphenols (4-NP) and alkyl phenol ethoxylates (NP1EO and NP2EO) in dreissenid mussel tissue. Only chemical compounds found at more than five sites that had concentrations above 3 x detection limit are included. This plot provides perspective to the relative concentrations of most commonly found alkylphenols and alkyl phenolethoxylates in dreissenid mussel tissue.

B.3.5. Bivalve Health

The continued presence of legacy organic contaminants coupled with the threat of contaminants of emerging concern in the Great Lakes necessitates the incorporation of newer monitoring approaches, particularly effects-based tools to the traditional chemical-based contaminant monitoring. During Phase I monitoring, MWP conducted pilot studies to determine the feasibility of using DNA damage assays, metabolomics and cellular biomarkers in dreissenid mussels.

B.3.5.1. DNA Damage

Tissues of bivalves exposed to xenobiotic substances are threatened by the production of elevated levels of reactive oxygen and nitrogen species as a result of the metabolism or direct reactions of pollutants. Overproduction of free radicals can cause damage to biomolecules including DNA. Biomarkers of oxidatively induced damage, i.e., modified

DNA bases and nucleosides in DNA of dreissenid mussels can be used as bioindicators for environmental genotoxicity.

MWP in collaboration with National Institute of Standards and Technology (NIST) conducted a pilot project to study the applicability of quantitative mass spectrometric assessment of oxidatively induced DNA base damage in dreissenid mussels. The aim of this pilot was to see whether oxidatively induced DNA lesions could serve as early warning biomarkers for pollution and specifically, to determine whether samples from polluted sites can be differentiated from those collected from reference sites based on DNA damage. Mussel samples from two sites in the outer Ashtabula Harbor, a historically polluted harbor and a reference site in Lake Erie, approximately 6.5 km east of the Ashtabula River mouth were analyzed for DNA damage. Results show that the mussels from one site in the outer harbor had significantly greater levels of seven of the eight measured oxidatively induced DNA bases and nucleosides than those from the reference site (Table B.1; Jaruga et al., 2017). Further evaluation of this monitoring tool is planned in Phase 2 with mussels collected from other Great Lakes harbors in agricultural and industrial watersheds for comparison with reference sites as part of a larger strategic plan to identify and assess adverse impacts of CECs in Great Lakes tributaries.

Table B.1: The mean and SE of DNA bases and nucleosides measured in mussels from the harbor site (LEAR-1). All but one were significantly different from those measured at the reference site (LEAB).

	LEAB		LEAR1		
DNA base	**mean**	**SE**	**mean**	**SE**	**significant**
FapyAde	**3.26**	**0.41**	**7.15**	**0.49**	*
FapyGua	**7.49**	**1.05**	**15.7**	**0.78**	*
8-OH-Gua	**1.35**	**0.06**	**2.14**	**0.08**	*
ThyGly	**5.86**	**0.89**	**9.94**	**0.57**	*
5-OH-5-MeHyd	**6.65**	**0.33**	**8.38**	**0.42**	*
5,6-diOH-Ura	**8.21**	**0.37**	**8.41**	**0.57**	
***S*-cdA**	**0.039**	**0.003**	**0.216**	**0.033**	*
***R*-cdG**	**0.954**	**0.173**	**2.19**	**0.105**	*
***S*-cdG**	**2.32**	**0.40**	**5.89**	**0.29**	*

B.3.5.2. Metabolomics

Metabolomics is the systematic study of concentration profiles of endogenous metabolites in biofluids and tissues of a given biological system and has found applications in many fields including medicine, pharmacology, and more recently in environmental toxicology. Metabolomics can be used to investigate metabolic changes within an organism in response to toxicant exposure in laboratory conditions as well as in natural habitats. However, metabolomics data with respect to dreissenid mussels was lacking. During Phase1, MWP conducted a feasibility study to determine whether mussel metabolomics could augment standard practices for evaluating ecosystem impairment (Watanabe et al., 2015).

MWP partnered with NIST to study the application of NMR-based metabolomics to the analysis of the whole-body metabolome of dreissenid mussels collected from the three inner harbor sites of Milwaukee Estuary AOC and a reference site in Lake Michigan in 2012. One of the objectives of this pilot study was to examine whether there were differences in metabolite profiles between impacted sites and the reference site. A total of 26 altered metabolites with significant differences were successfully identified in a comparison of dreissenid mussels from an inner harbor site and the reference site (Figure B.12; Watanabe et al., 2015). This study has demonstrated the feasibility of NMR-based metabolomics approach to assess whole-body metabolomics of dreissenid mussels and are being explored further in Phase 2 activities.

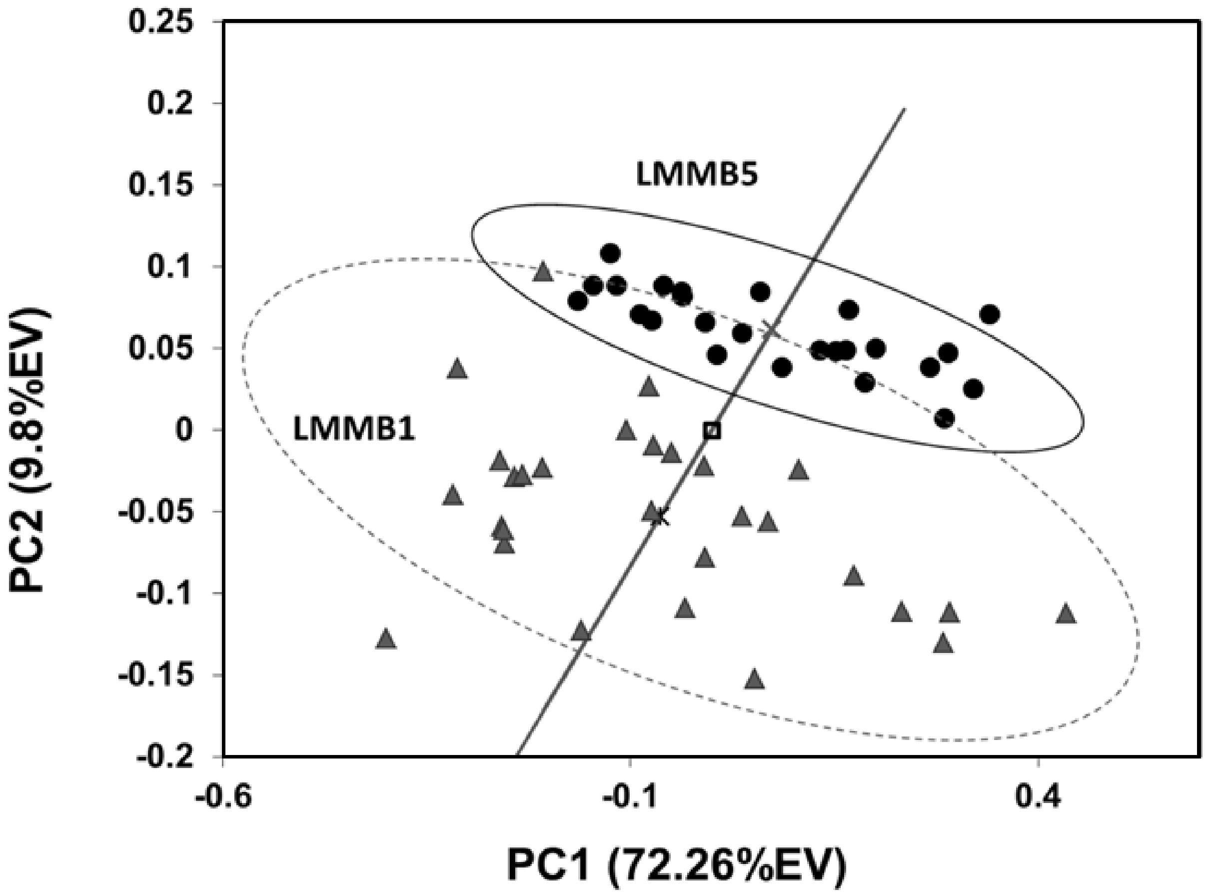

Figure B.12: PCA scores plot of the processed ^{1}H NMR spectra data obtained dreissenid mussels from impacted site LMMB1 (▲), and the reference site LMMB5 (●). The ovals indicate the 95% Hotelling's confidence interval. The solid line represents the projection of the scores onto the hybrid scores axis connecting the centers of each group. A Student's t-test for these projected points shows a significant difference between the groups (p= 5.65x10^{-6}).

B.3.5.3. Cellular Biomarkers

Biomarkers are quantitative measurements of biochemical and or physiological changes in organisms induced either by exposure to and or the adverse effect of xenobiotic substances. Bivalves respond to xenobiotics exposure by inducing enzymatic antioxidant defense mechanisms to detoxify excess reactive oxygen species. Therefore, antioxidant response and cellular damage can be useful biomarkers of oxidative stress in bivalves for environmental monitoring.

MWP ran a pilot project to examine the feasibility of using two cellular biomarkers, total glutathione (GSH) and lipid peroxidation (LPx), in wild populations of dreissenid mussels collected from around the Great Lakes to help identify highly impacted sites. The methods for analysis of these biomarkers in dreissenid mussels were optimized and tested for both whole body samples and hepatopancreas tissue. Our results indicated that the GSH and LPx biomarker responses were inversely correlated (ANCOVA, $r^2 = 0.40$; Figure B.13) and the pattern of response was identical for whole body samples and hepatopancreas tissue samples suggesting that the laborious work of isolating organ tissue can be avoided. Based on known biochemical mechanisms, high LPx and low GSH in animals are typically indicative of stress and in this study, we were able to rank the sites as 'normal', 'intermediate stressed' and 'highly stressed'. Additional results of analyses linking biomarker data and mussel tissue burden data will be summarized in Ringwood et al. (Manuscript in Preparation).

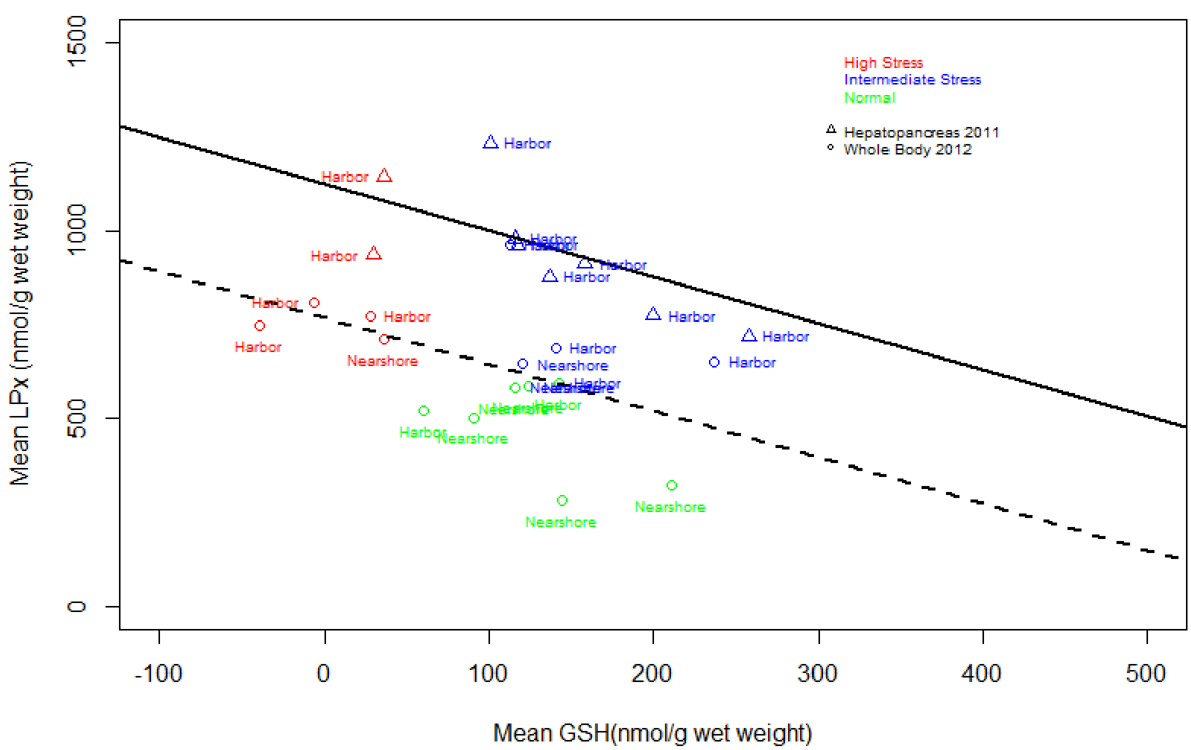

Figure B.13: Inverse correlation (ANCOVA, r2 = 0.40) of glutathione (GSH) and lipid peroxidation (LPx) in mussel samples (hepatopancreas and whole body) collected from several locations around the Great Lakes.

B.4. Key Findings

- Dreissenid mussels are a valuable bioindicator in the Great Lakes because of their basin-wide distribution and sessile nature, which allows us to characterize chemical contamination in three zones: offshore (open-lake), nearshore (shallow), and river-harbors. Mussels can be caged and strategically relocated for precise place-based assessments (monitoring along a pollution gradient, pre and post remediation/restoration assessments and/or contaminant source tracking) for both legacy and CEC monitoring.

- Our monitoring data indicates that dreissenid mussels accumulate both legacy contaminants and a broad suite of CECs, which were previously assumed to have low or no bioaccumulation potential. Furthermore, dreissenid mussels collected from hard substrates and hard natural rock bottom in nearshore and offshore locations reflect what chemical contaminants are bioavailable in the water column unlike other benthic organism that live in the sediment.

- Our preliminary work on mussel health metrics such as cellular biomarkers (GSH and lipid peroxidation), DNA damage, and metabolomics show that mussels can be utilized for effects-based monitoring and are able to discriminate impacted sites. The utility of these methods as practical and feasible tools for temporally and spatially robust CEC monitoring will be explored further during Phase 2 activities.

B.5. Management Implications

- Dreissenid mussels can be effectively monitored in all three zones of the Great Lakes for status and trends in chemical contamination, biological effects, and improve understanding of tropic transfer of contaminants. Compared to fish, mussels are known to tolerate high levels of pollution and have minimal ability to metabolize organic contaminants. Further, understanding the trophic relationships among mussels, fish and birds will likely lead to better predictive modelling of the distribution and effects of contaminants in the Great Lakes.

- The ubiquitous piers, jetties, revetments, and breakwaters that improve navigation into Great Lakes river-harbors support robust colonies of dreissenid mussels and provide for a standardized sampling approach for comparability of data across river-harbors and other stressed and polluted habitats in the Great Lakes.

- Mussel tissue and sediment data are available through the online database: Data Integration Visualization Exploration and Reporting (DIVER). For Great Lakes Mussel Watch chemical contaminant data 2009-2014. https://www.diver.orr.noaa.gov/

B.6. Knowledge Gaps

- Can the composition of CEC mixtures in mussels be predicted based on adjacent land use and point source discharges?
- How can the contribution of ubiquitous PAHs to bivalve health metrics be differentiated from other contaminants of emerging concern?
- How do multiple stressors interact to affect the biological effects in mussels?
- Is mussel monitoring data (contaminants and health metrics) predictive of conditions in other species (native mussels, fish and birds)?

B.7. Acknowledgements

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Appendix C

Exposure and Effects of Bioaccumulative Contaminants of Emerging Concern in Tree Swallows Nesting across the Laurentian Great Lakes

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C.1. Problem Statement and Study Overview

Contaminants of emerging concern (CECs) are a loosely defined group of chemicals whose wide-spread usage or presence in the environment has occurred more recently or for which there has been relatively little research done until recently. Many of these CECs are not currently regulated. The National Toxicology Program within the U.S. Department of Health and Human Services estimates that about 2000 CECs are introduced each year (https://ntp.niehs.nih.gov/about/). An unknown number may pose a risk to human or animal health. The Phase 1 (2010 – 2014) CEC work in birds, which is the subject of this report, assessed exposure across the Great Lakes to polybrominated diphenyl ethers (PBDEs), perfluorinated compounds (PFASs), and polycyclic aromatic hydrocarbons (PAHs), and put those exposures into context with data from biologically relevant endpoints such as reproductive success, as well as, physiological response indicators (bioindicators) to assess possible effects. The group of chemicals included in Phase 1 were mainly those chemicals that bioaccumulate in tissues. Phase 2 (2015 – 2019) CEC work with tree swallows was expanded to include CECs whose occurrence in the environment is more temporary or seasonal, and that do not necessarily bioaccumulate. These are often called pseudo-persistent, because, while they are not long-lived in the environment, there are often daily inputs via waste water treatment plants, and run-off from farm fields and storm drainages, thereby making them available to biota year-round. These include pharmaceuticals, personal care products, and newer pesticides including herbicides. Tree swallow work on these less persistent CECs will be reported in the future, however see other Appendices in this report for information on some of these types of CECs (Appendices A, B, D).

C.2. Introduction

Tree swallows (*Tachycineta bicolor*) are an avian species that is now being widely used to assess contaminant exposure and effects. Their natural history traits, such as willingness to use artificial nest boxes, their tolerance of human activity in the vicinity of their nest box, and their food habits (emergent phase of benthic aquatic insects), make them an ideal study species for examining contaminant exposure and effects. Additionally, there is now a wealth of information on exposure and concentrations at which adverse biological effects are likely (effect thresholds) for legacy contaminants, such as polychlorinated biphenyls (PCBs), trace elements such as mercury, lead, and cadmium, and organochlorine pesticides. More recently, effect thresholds are becoming available for some CECs as well.

For the study of CECs in birds, the lack of analytical chemistry methods to meaningfully quantify exposure in tissues often delays our ability to study them in the field. Biological matrices are complex and require considerable effort and time before new chemicals can be reliably quantified in biotic tissues. Another issue regarding the study of CECs in biota is that some CECs can cause adverse outcomes, but not be accumulated in tissues in a traditional dose-response manner, so different approaches to study exposure and effects are required. The choice of sampling matrices and methods to be used are dictated by the constraints mentioned above. The objectives of Phase 1 were to quantify exposure to selected bioaccumulative CECs and polycyclic aromatic hydrocarbons (PAHs), which are bioaccumulative in only some taxa, and to determine if there were any effects, at different levels of biological organization, associated with these CECs. Study sites were concentrated in Areas of Concern (AOCs), highly contaminated areas designated as such by the Great Lakes Water Quality Agreement (2012), but study locations also included 11 sites for comparative purposes that were not designated as AOCs (Figure C.1).

C.3. Methodology

Approximately twenty nest boxes were erected at each of ~70 sites across the Great Lakes (Figure C.1), protected from ground predators with metal cylinders, and then checked weekly to follow reproduction including the number of eggs laid, the number that hatched and the number of young that reached 12 days of age. Because tree swallows will readily nest in human-made boxes, this allowed data to be collected at all sites even in highly industrialized and urbanized landscapes where few other bird species readily nest. Tree swallows arrive in the Great Lakes region in April, lay their eggs in May, and rear their young in June. By early July, most have fledged from the nest boxes and have begun to disperse. Each site was studied for at least one year between 2010 and 2014; a few sites had multiple years of data when the level of chemical exposure was high, or remediation and restoration actions were in progress or planned. Egg, blood plasma, and other tissue samples were collected for chemical and physiological analyses at designated times each year. The types of CECs chemically analyzed in Phase 1 included polybrominated diphenyl ether flame retardants (PBDEs), perfluorinated substances (PFASs), and polycyclic aromatic hydrocarbons (PAHs), in addition to the standard suite of legacy and trace element chemicals. Contaminant analyses followed standard EPA methods with blanks, duplicates, and certified reference material analyzed with each batch. It is important when assessing effects that as many chemicals as possible be analyzed, especially those known to cause adverse effects in wildlife, to avoid drawing incorrect inferences. For this reason, a broad suite of legacy contaminants was also analyzed. The bioindicator analyses included enzyme analyses (EROD), oxidative stress measurements (GSH, GSSG, PBSH, TBARS, TSH), and somatic cell DNA damage. These bioindicators were analyzed by collaborators (Natalie Karouna-Renier, USGS and Cole Matson, Baylor Univ.) using published methods for each assay and incorporated standard assay-specific quality assurance methods. After the data had been assembled, univariate, multivariate, and multistate modelling statistical analyses were performed to compare among sites, between AOCs and nearby non-AOCs, and to assess whether there were adverse effects associated with the chemical contamination. The term non-AOC was used here, rather than 'reference' or 'control' sites, because the 11 sites were chosen to be nearby and indicative of sites in the Laurentian Great Lakes basin rather than choosing sites that were known to be pristine or lightly contaminated. Sites not designated as AOCs by the International Joint Commission under that program (Great Lakes Water Quality Agreement) could also be contaminated as evidenced by the area around Midland, MI which is highly contaminated with dioxins and furans, or Oscoda, MI which is highly contaminated by perfluorinated substances. The information presented here is published as indicated in the literature cited section; data are available in Science Base of USGS and easily visualized and accessed via a StoryMap (http://usgs.maps.arcgis.com/apps/MapSeries/index.html?appid=820ce23a0cb04dadb6525ace6ae4edc7).

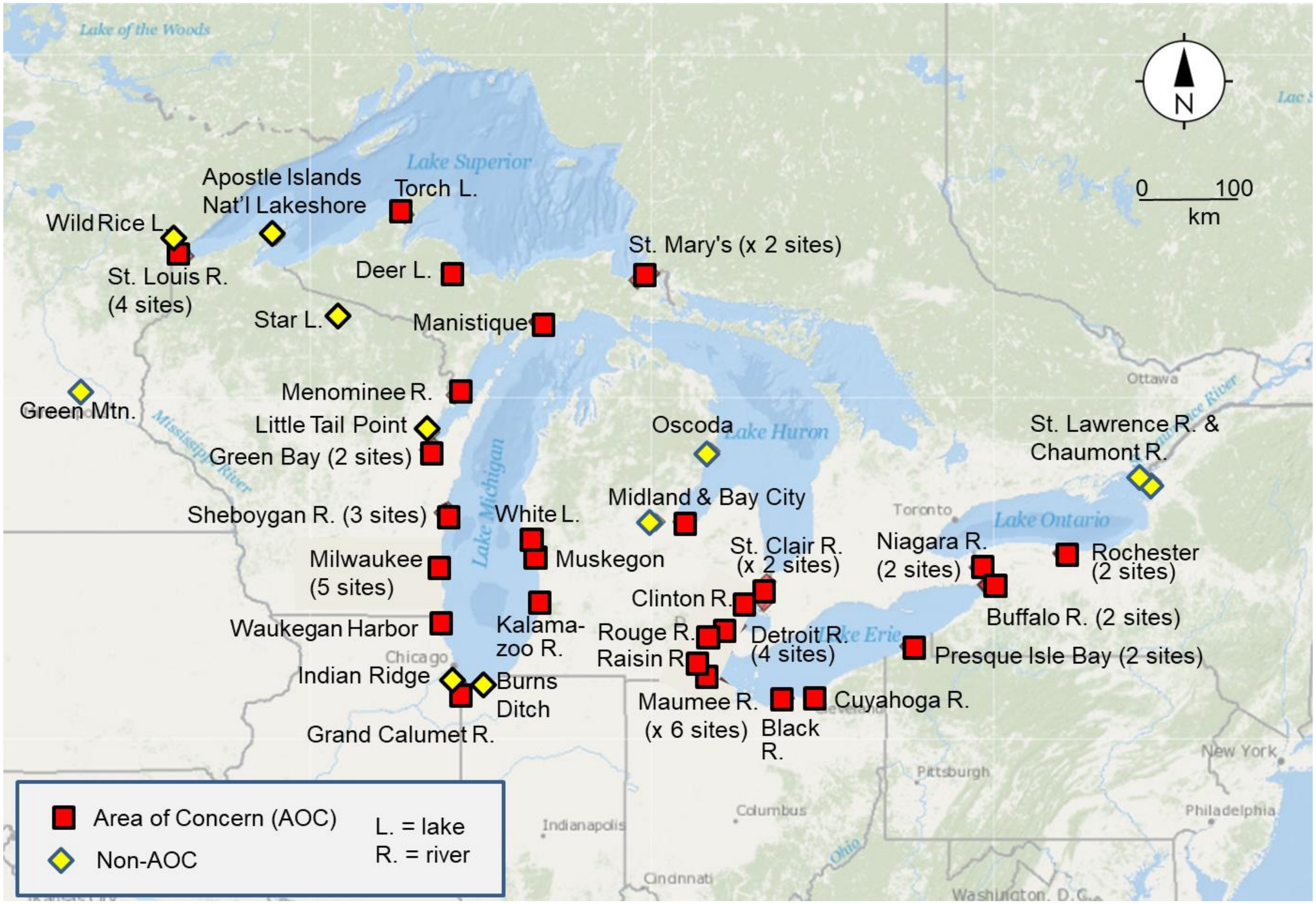

Figure C.1 Study locations for contaminants of emerging concern (CECs) in tree swallows across the Laurentian Great Lakes basin, 2010-2014.

C.4. Key Findings

This compendium of work is one of the most extensive and exhaustive studies of both legacy and CECs in birds from such a large geographic area (100,000 sq. miles), collected within a comparable timeframe, and using the same species. Contaminants of emerging concern were present in tree swallows nesting at these 70 sites across the Great Lakes, and at concentrations that varied by location and land use. These differential exposure patterns allowed for a rigorous assessment of both reproductive effects and physiological responses. Two of the CECs (PBDEs and PFASs) do not seem to be at exposure levels that affect either reproduction or physiological responses in nesting birds, however, one class of CECs, the PAHs, were associated with adverse reproductive, as well as, physiological responses.

C.4.1 Polycyclic Aromatic Hydrocarbons (PAHs)

In the context of contaminants in birds, PAHs are a CEC, not because they are a recently discovered contaminant, but because they have been little studied in vertebrate biota. This is because PAHs do not accumulate in a clear dose-response manner in birds, like some other CECs and most legacy organic contaminants do, so different approaches are needed to conduct field assessments in vertebrate biota. Because there is only limited metabolism of PAHs in aquatic insects and mussels (see Appendix B), both of these animal groups can serve as a measure of exposure to vertebrate biota that consume them. Therefore, PAHs were assessed in pooled samples of diet, primarily the aerial stage of benthic aquatic insects, from stomachs of nestling tree swallows, and then compared, on a site basis to background exposure levels and reproductive and physiological effects in swallows.

There are several hundred individual PAHs which differ in the number of benzene rings and attached methyl groups (see review in Abdel-Shafy and Monsour 2016). In this study we quantified 48 individual PAHs (detection limit = 0.25 ng/g wet wt., 20 parent and 28 alkylated PAHs, Custer et al. 2017a). Polycyclic aromatic hydrocarbons can be produced naturally, through burning of biomass in forest fires or internally in biota, but also through the actions of humans. Industrial production of PAHs has occurred since the Industrial Revolution, including in manufactured gas plants and through intensive fossil fuel burning. The PAHs which were the most prevalent and at the highest concentration were in the more highly industrial watersheds such as the Detroit River corridor and the Milwaukee Estuary, WI, and to a lesser extent in the Chicago, IL, Duluth, MN, and Toledo, OH areas (Figure C.2, Custer et al. 2017a). At many of the sites, the source of PAHs was a mixture of pyrogenic (resulting from combustion) and petrogenic (petroleum sources) based on the ratio of phenanthrene to anthracene. Pyrogenic PAHs tend to have more benzene rings (4 – 6) and are usually not alkylated (i.e. with few methyl groups attached). Petrogenic PAHs tend to be smaller molecules, 2 – 4 benzene rings, and have attached methyl groups. The sites with higher total PAH concentrations, such as the Rouge and Detroit Rivers, MI, and the Milwaukee, WI and Duluth, MN areas, had predominately pyrogenic sources (Custer et al. 2017a). These sites had an abundance of historic heavy industry including steel mills and other industries with a heavy reliance on fossil fuels. The Raisin and Maumee Rivers, both sites with moderately high PAHs exposure, had petrogenic-based sources predominantly. Most sites (>70%) had a mixture of PAH sources. Adding to the uncertainty of apportioning sources of PAHs is that there can be transformation of PAHs in sediments (Lei et al. 2005) and in biota (Näf et al. 1992).

There was sufficient exposure to PAHs, as measured in the diet, at some sites to illicit both a physiological response and a reproductive effect in the swallows. Whereas the physiological responses were anticipated based on previous avian work, the association with adverse reproduction was a surprise, in part because there have been very few studies that have tried to test for an association in a field situation. On the physiological level, both the alkylated and parent PAHs were the primary drivers separating high total sulfhydryl (TSH) from normal TSH levels (Custer et al. 2017b). Total sulfhydryl is a measure of cellular oxidative stress which is an imbalance created when there is an increased amount of reactive oxygen (free radicals) that exceeds the body's ability to detoxify them. This imbalance can lead to oxidative damage to proteins, molecules, and genes. Total PAHs in the elevated TSH group were 8 times higher compared to the normal TSH group. Protein bound sulfhydryl (PBSH), another measure of oxidative stress, followed this same pattern, likely due to the high degree of correlation between these 2 measures of oxidative stress. Ethoxyresorufin-*O*-dealkylase (EROD) activity, a liver enzyme used as an indicator of exposure to PAHs and other organic contaminants, was also higher in birds exposed to high concentrations of PAHs in their diets. This enzyme is activated when a toxin reaches sufficient levels to necessitate a physiological response by the organism to detoxify the contaminant. Geometric mean concentrations of total PAHs were 16 times higher in the highly-active EROD group compared to the normal group. Toxic equivalency (TEQ) is a method to quantify the toxicity of a group of related chemicals each of which may have a different level of toxicity. The TEQ value for the sum of the PAHs was 39 times higher in the EROD induced group compared to the normal EROD group. The coefficient of variation for DNA (DNA-CV), a measure of disruption of DNA division processes in somatic cells, in this case red blood cells, did not clearly associate with PAH exposure even though PAHs have been found to be associated with DNA-CV in other studies. Statistical analyses in these other studies were primarily single-variable analyses not multivariate analyses as was done in the current work (Custer et al. 2017b), which may possibly explain these differing results.

The association of PAHs and reproductive success in this study is one of the first such association found. While the detrimental effects of oil spills either on birds or their eggs is well known and documented, and the effects of injected petroleum in the laboratory on the survival of avian embryos is also well documented, the effects of ingested PAHs are much more difficult to study. This difficulty results because PAHs cannot be meaningfully measured in vertebrate tissue, because they are quickly metabolized and removed from the body. Because of this, a different approach was needed, namely to measure PAHs in the invertebrate food items that are being consumed. Getting adequate sample mass for chemical analyses of the food consumed is difficult and time consuming.

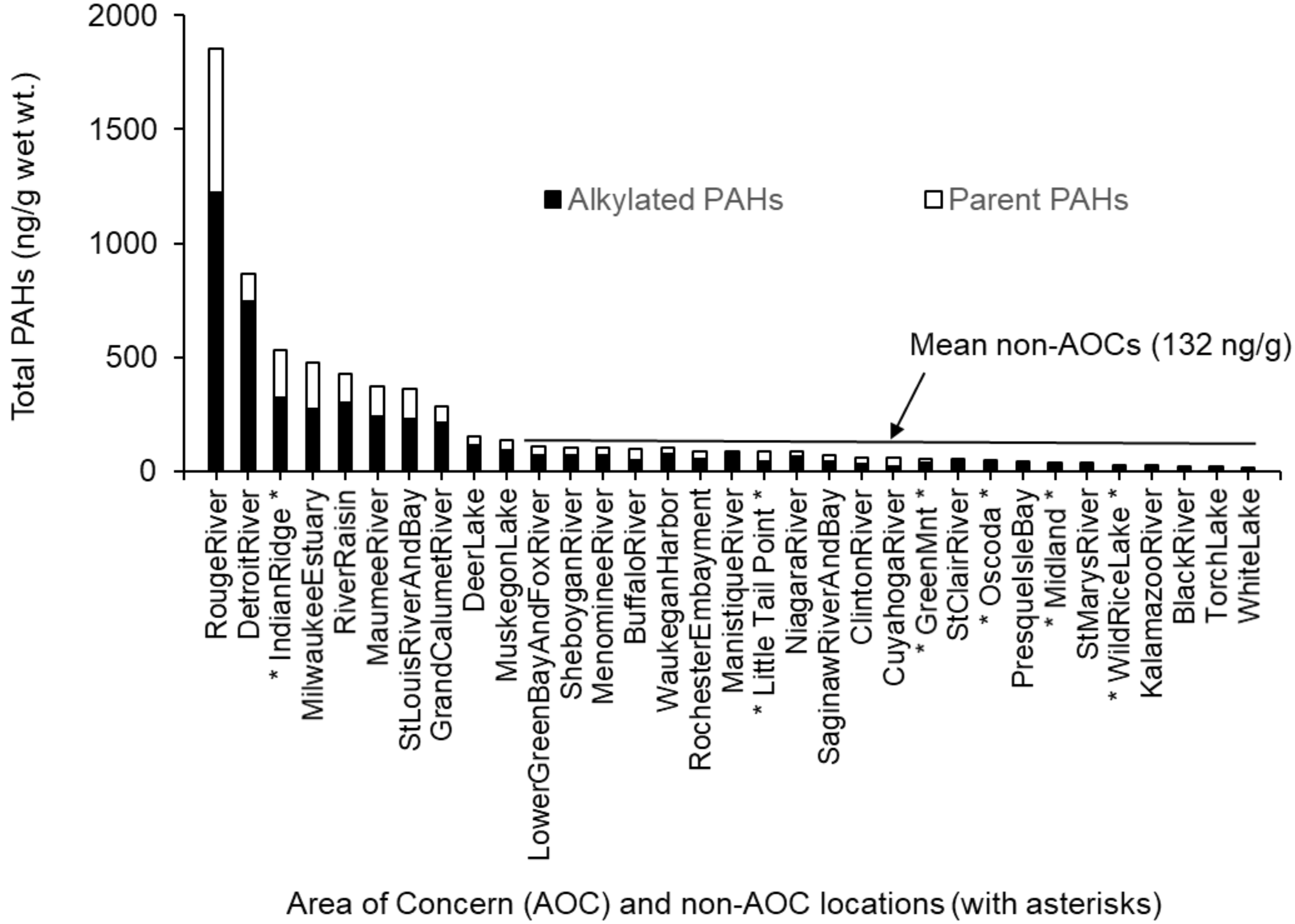

Figure C.2 Concentrations of total polycyclic aromatic hydrocarbons (PAHs) in the diet of tree swallows at study sites across the Laurentian Great Lakes basin, 2010-2014. Graph adapted from Custer et al. 2017a. Environ. Toxicol. Chem. 36: 735-748.

There was a decrease in reproductive success as PAH exposure, as measured in their food, increased. Reproductive success was quantified as the daily probability of egg failure (Custer et al. 2018) which accounted for adverse hatching outcomes, such as embryo death or infertility, and the timing of those adverse effects. As the probability of egg failure increased, the exposure to PAHs via their diet also increased (Figure C.3; Custer et al. 2018). Because of other factors known to affect reproductive success, including other contaminants such as the dioxin and furan TEQs, as well as ecological variables such as female age and date within season, the association with total PAHs was not strong, but warrants additional work.

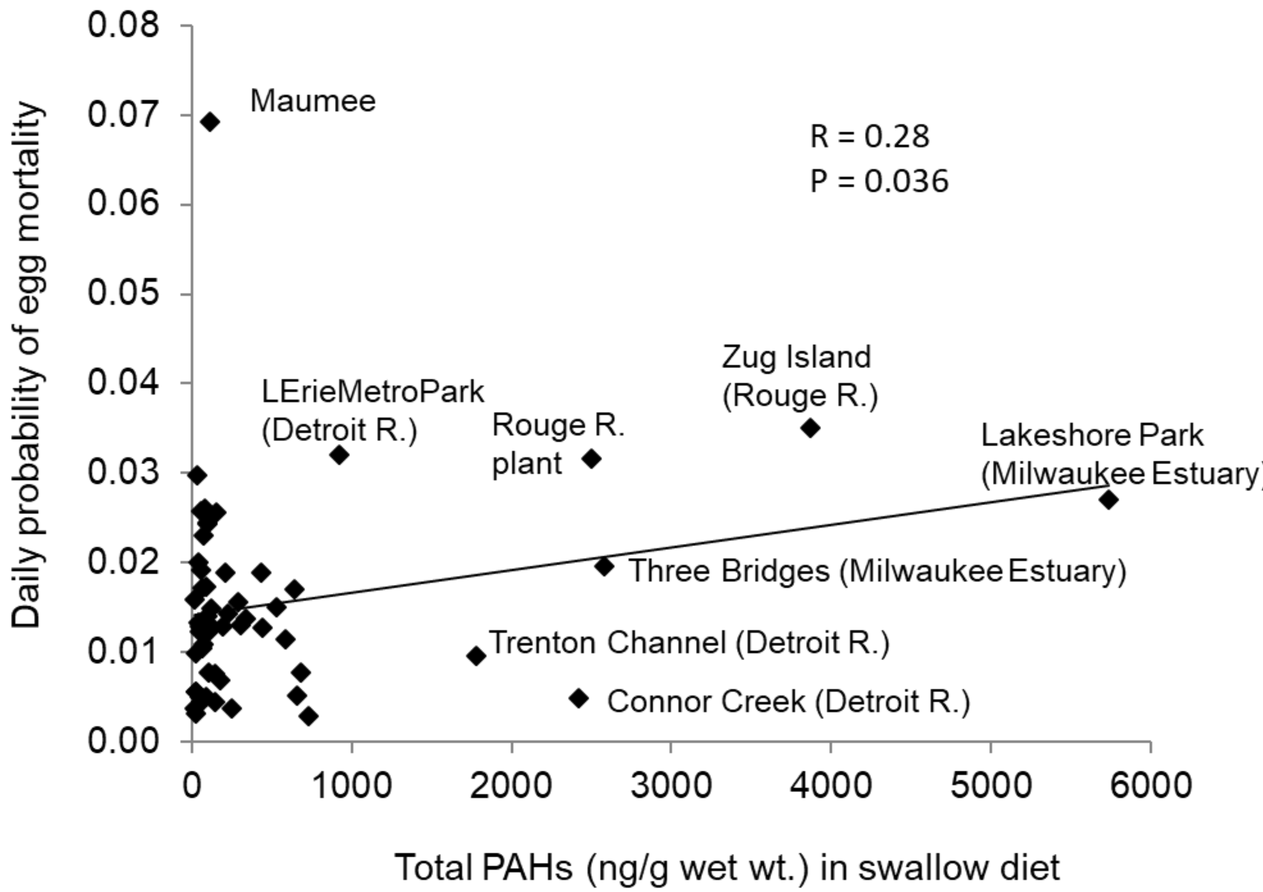

Figure C.3 Association of egg mortality and exposure of tree swallows to polycyclic aromatic hydrocarbons (PAHs) at sites across the Laurentian Great Lakes basin, 2010-2014. Area of Concern name in parentheses where appropriate. Graph adapted from Custer et al. 2018. Ecotoxicol. 27: 457-476.

C.4.2 Polybrominated Diphenyl Ethers (PBDEs)

The polybrominated flame retardants (PBDEs) were detected in all egg samples (detection limit = 0.004 ng/g wet wt.; 40 congeners analyzed, Figure C.4; Custer et al. 2016, 2017a) with the highest concentrations found in the highly industrialized, urbanized corridor along the Detroit River, MI and in Cleveland, OH. Another site with elevated exposure to PBDEs, and the only site that was statistically above background exposure in eggs, was near Midland, MI. The geometric mean concentrations at the rest of the sites were at or below the mean background concentration (96 ng/g wet wt.) which was recently established in a tree swallow study in Canada (Gilchrist et al. 2014). Sites not associated with AOCs had a mean concentration of 48.6 ng/g which can now also be considered a background value as well (Custer et al. 2016). There are few other studies that have established background concentrations in eggs of a passerine bird species. In both nestlings and diet, the site with the highest exposure was at Torch Lake, MI (Custer et al. 2017a) followed by other highly industrialized rivers such as the St. Clair and Niagara Rivers, and the Detroit River corridor. It is unclear what the source might be for the PBDEs in Torch Lake. A similarly small amount of data exists for effect thresholds for PBDEs, either reproductive effects or physiological responses. The lowest observed effect level (LOEL) for hatching success was estimated to be ~1000 ng/g in osprey eggs (*Pandion haliaetus,* Henny et al. 2009). Consistent with that LOEL value, and because the highest mean egg concentrations were >7 times lower than that threshold, we found no association of PBDE exposure with the daily probability of egg failure (Custer et al. 2018). We also did not find any biomarker responses associated with PBDEs (Custer et al. 2017b) indicating that exposure did not rise to the level that prompted a physiological response in the swallows.

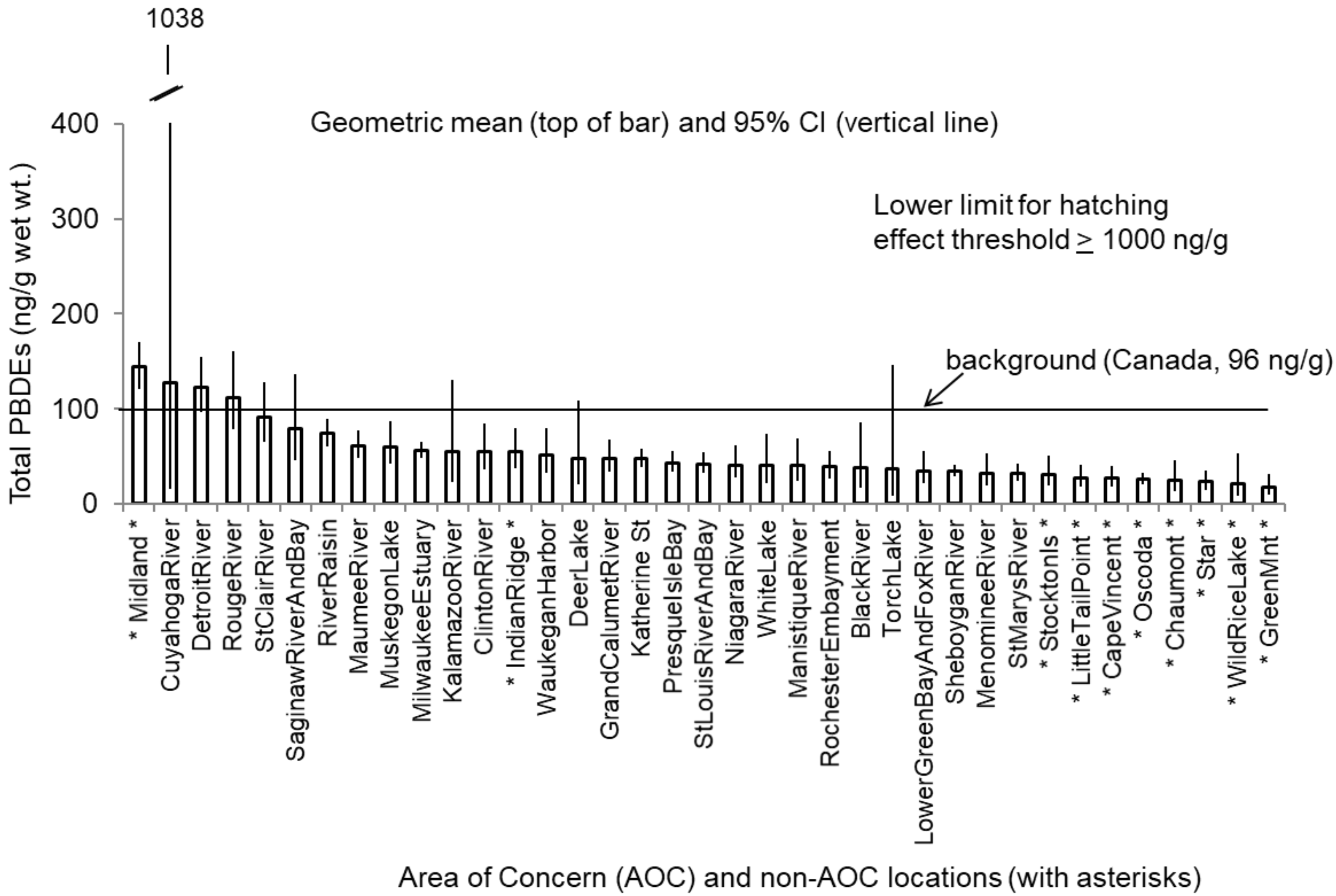

Figure C.4. Concentrations of polychlorinated diphenyl ethers (PBDEs) in tree swallow eggs (n=381) at study sites across the Laurentian Great Lakes basin, 2010-2014. Graph adapted from Custer et al. 2016. Environ. Toxicol. Chemi. 35: 3071-3092.

C.4.3 Perfluorinated Substances (PFASs*)*

Perfluorinated substances were detected in all tree swallow plasma samples (detection limit = 0.56 ng/mL;13 PFAS congeners separately analyzed), and at concentrations that varied among sites (Figure C.5). As with other CECs, there are limited data on background exposure levels, but in tree swallows background levels in plasma are $\leq$75 ng/mL (Custer et al. 2014). Only slightly higher was the mean exposure of 111 ng/mL at 7 non-AOCs across the Great Lakes (Figure C.5, Custer et al. 2017a). Sixty three percent of the AOCs studied had mean PFAS concentrations less than the mean concentrations at non-AOCs. There were 2 hot-spots of PFAS exposure, one near Duluth, MN (581.9 ng/mL) and the other near Oscoda, MI (1649.3 ng/mL); both locations were associated with airfields where film-forming fire-fighting foams were extensively used in fire-suppression training exercises. Like PBDEs, highly urban and industrial sites, including locations along the Detroit, Rouge, and Raisin Rivers in Michigan, the Chicago, IL area, and along the Niagara River, NY (Custer et al. 2017a), tended to have higher exposures to PFASs than less urban/industrial sites (Figure C.5). One PFAS in particular, perfluorooctane sulfonate (PFOS), dominated the suite of PFASs found in blood plasma. This result is similar to other studies world-wide that found that PFOS was the prevalent PFAS congener, especially in urban and industrial locations.

Similar to PBDEs, exposure to PFASs, except at one location, was below exposure levels that cause hatching effects in laboratory studies (Newsted et al. 2005). A predicted no-effect concentration in that study was set at 1000 ng/mL in serum, which was 5 – 10 times higher than mean exposure at many of the 70 sites across the Laurentian Great Lakes. Our field data were therefore consistent with the laboratory data. Even at the site near Oscoda, MI which had extremely high exposures, reproductive success of swallows was above average (Figure C.6; Custer et al. 2018); Oscoda had the 5th lowest egg failure rate among the 37 locations. There were also no biomarker responses associated with PFAS exposures (Custer et al. 2017b) indicating the lack of a physiological response induced by this class of contaminants.

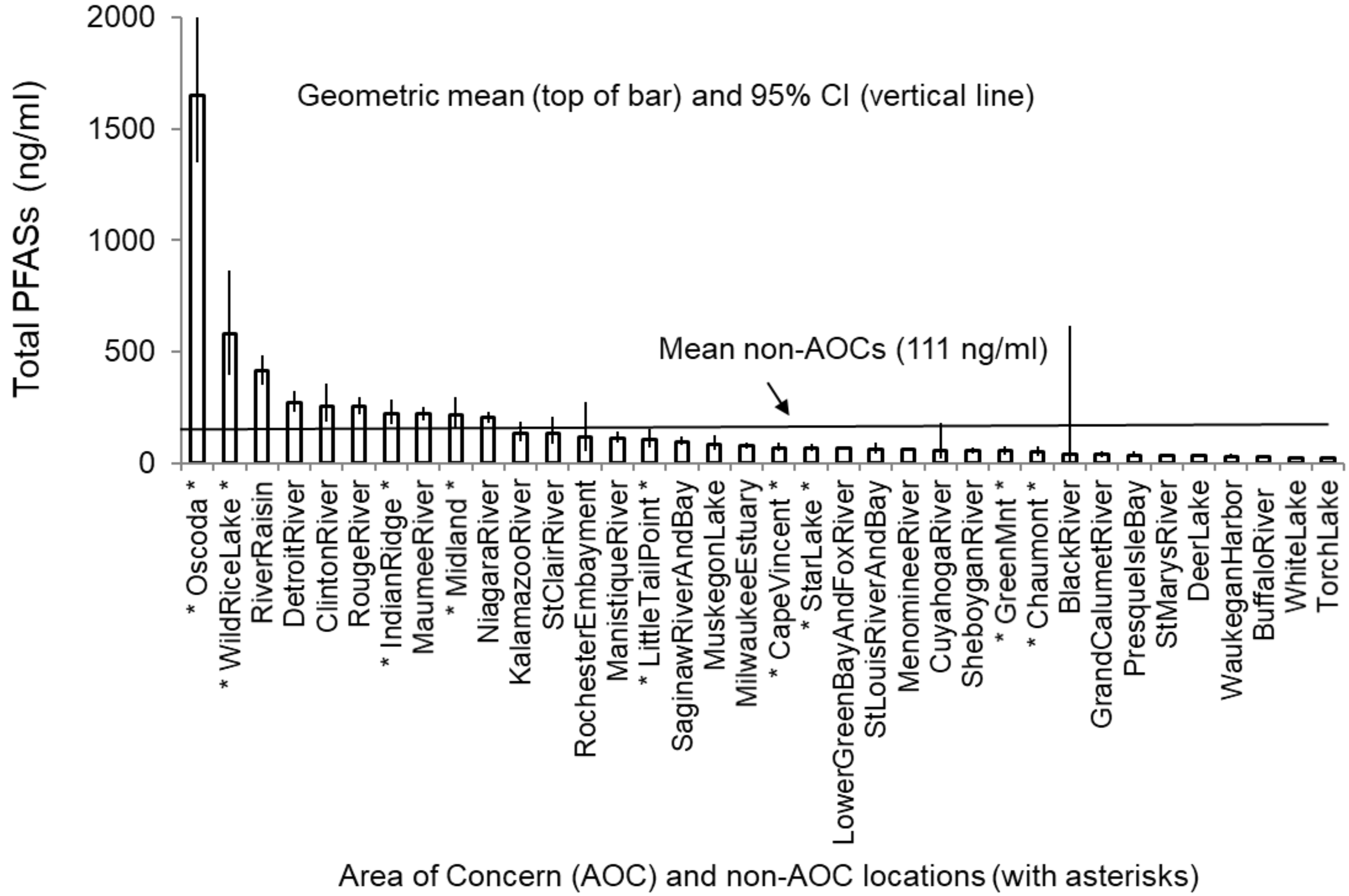

Figure C.5 Concentrations of total perfluorinated substances (PFASs) in tree swallow nestling plasma (n=566) at study sites across the Laurentian Great Lakes basin, 2010-2014. Graph adapted from Custer et al. 2017a. Environ. Toxicol. Chem. 36: 735-748.

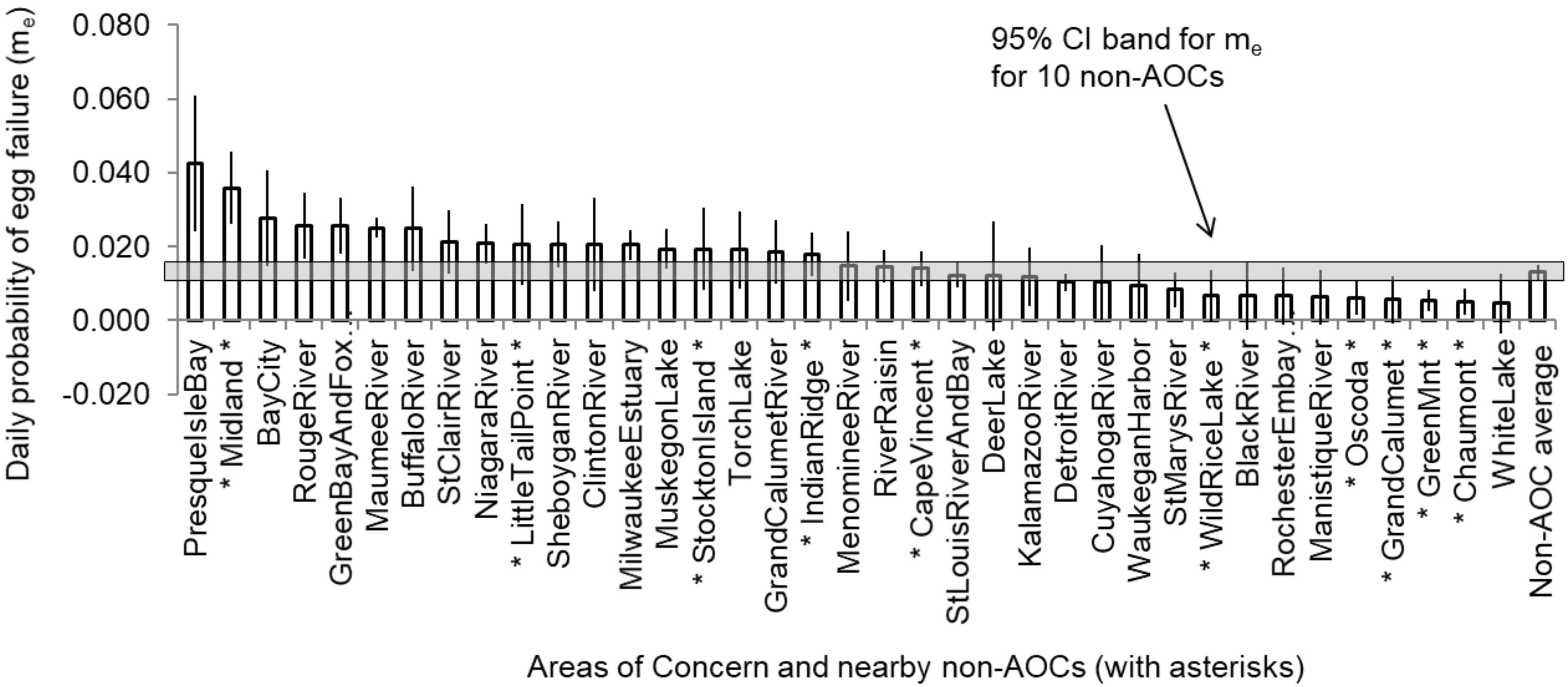

Figure C.6. Daily probability of egg failure (m_e) at study sites across the Laurentian Great Lakes basin, 2010-2014. Vertical lines are ± 1 standard error. Graph adapted from: Custer et al. 2018. Ecotoxicol. 27: 457-476.

C.5. Management Implications

The quantity of 2 of the 3 CECs documented in this Phase 1 work in swallows have either declined or stabilized in biotic tissues in the Upper Midwest including the Laurentian Great Lakes, generally because of either voluntary change by the manufacturer (PFASs), regulatory actions (PBDEs), or a combination of both. For example, PFOS concentrations in great blue heron (*Ardea herodias*) eggs declined by >50% between 1993 and 2010 and 2011 (Custer et al. 2013) along the Mississippi River. There has been a decline in PFOS in herring gull (*Larus argentatus*) eggs between 1990 and 2010 (Gebbink et al. 2011) in the Great Lakes. While PFOS declined in both studies cited above, some of the perfluoroalkyl carboxylates have tended to increase over those same timeframes. Continued monitoring of PFAS congeners, especially new ones entering the environment, as well as development of analytical methods for PFAS congeners that are currently not widely available seems warranted, as do focused studies on effects of those congeners that are increasing. The picture for the PBDEs is less clear (Gauthier et al. 2008), but should be resolved with analysis of the ongoing data collected as part of the Canadian Wildlife Services herring gull monitoring program (http://ijc.org/greatlakesconnection/en/2018/04/herring-gulls-are-sentinels-of-the-skies/; Hebert et al. 1999). There seems to have been a decrease of PBDEs in avian tissues once usage was restricted, but more current information is needed to confirm trends. The tree swallow data set presented here can become a baseline to access future trends in PBDE and other CEC exposure. Polycyclic aromatic hydrocarbons remain an underappreciated issue because PAHs do not bioaccumulate in vertebrates in the typical dose-response manner which makes that class of CECs difficult to study. Development of alternative methods should continue to more fully understand the possible effects of current exposure to PAHs, along with the inclusion of additional species in the effects studies (mussels and fish), and the addition of study sites where PAH exposure is high should be completed.

C.6. Knowledge gaps

While many of these bioaccumulative CECs are now being more widely studied in birds, CECs such as pharmaceuticals and personal care products, and next generation pesticides including herbicides and new types of insecticides, such as the neonicotinoids, need more research including new methods, in some cases, to quantify and study exposure and effects. These pseudo-persistent CECs are often more short-lived in the environment, but because they are continually entering the environment via non-point source run-off and passing through waste water treatment facilities, there is continual, but low-level exposure. Phase 2 CEC work on tree swallows is providing new data to fill these gaps for birds, including not only on exposure, but also to add information on possible effects of CECs on the activity of the thyroid hormone system (T3 and T4 concentrations) as well as the other commonly-used biomarkers. The use of metabolomics and transcriptomics to quantify perturbations in various physiological pathways and genes that may be impacted by these non-accumulative CECs is also ongoing.

C.7 Disclaimer

The USGS information has been peer reviewed and approved for publication consistent with USGS Fundamental Science Practices processes. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

C.8 Acknowledgements

We thank the over 75 landowners who allowed us access to their properties during the course of our field work and also thank the 30 field technicians and data entry support personnel for their help. The authors thank Heiko L. Schoenfuss and Ed Johnson for helpful comments on earlier drafts of this report. Funding was provided by the Great Lakes Restoration Initiative, as well as, the Environmental Health mission Area of U.S. Geological Survey.

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Appendix D

Survey of Contaminants of Emerging Concern and Their Effects to Fish and Wildlife in Great Lakes Tributaries

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D.1. Problem Statement and Scope

The Laurentian Great Lakes are a critical economic and environmental resource in North America. These lakes support an abundance of wildlife refuges; fish hatcheries, and a thriving commercial and recreational fishery (USFWS 2017, USFWS 2018b). The Great Lakes Basin is also home to 21 federally endangered species, 14 federally threatened species and many more species are threatened or endangered at the state level making restoration and conservation of the Great Lakes Basin’s natural resources important for the continuing benefit of current and future generations (USFWS 2018a).

The Great Lakes and their tributaries have been subject to historical and recent degradation associated with human development. Legacy pollutants from early industrial development of the Great Lakes Basin have been researched, regulated and, in some instances, mitigated. More recently, however, new classes of chemicals have been identified as being of emerging concern to fish and wildlife and human health. These contaminants of emerging concern (CECs) are a loosely defined group of chemicals whose wide-spread usage or presence in the environment has occurred more recently. Additionally, due to analytical chemistry limitations, cost, or other impediments relative little research has been completed on CECs, until recently. Little is known about the occurrence of CECs and their effects on wildlife.

Therefore the overall objective of this study was to assess the presence and biological consequences of CECs in US tributaries of the Great Lakes.

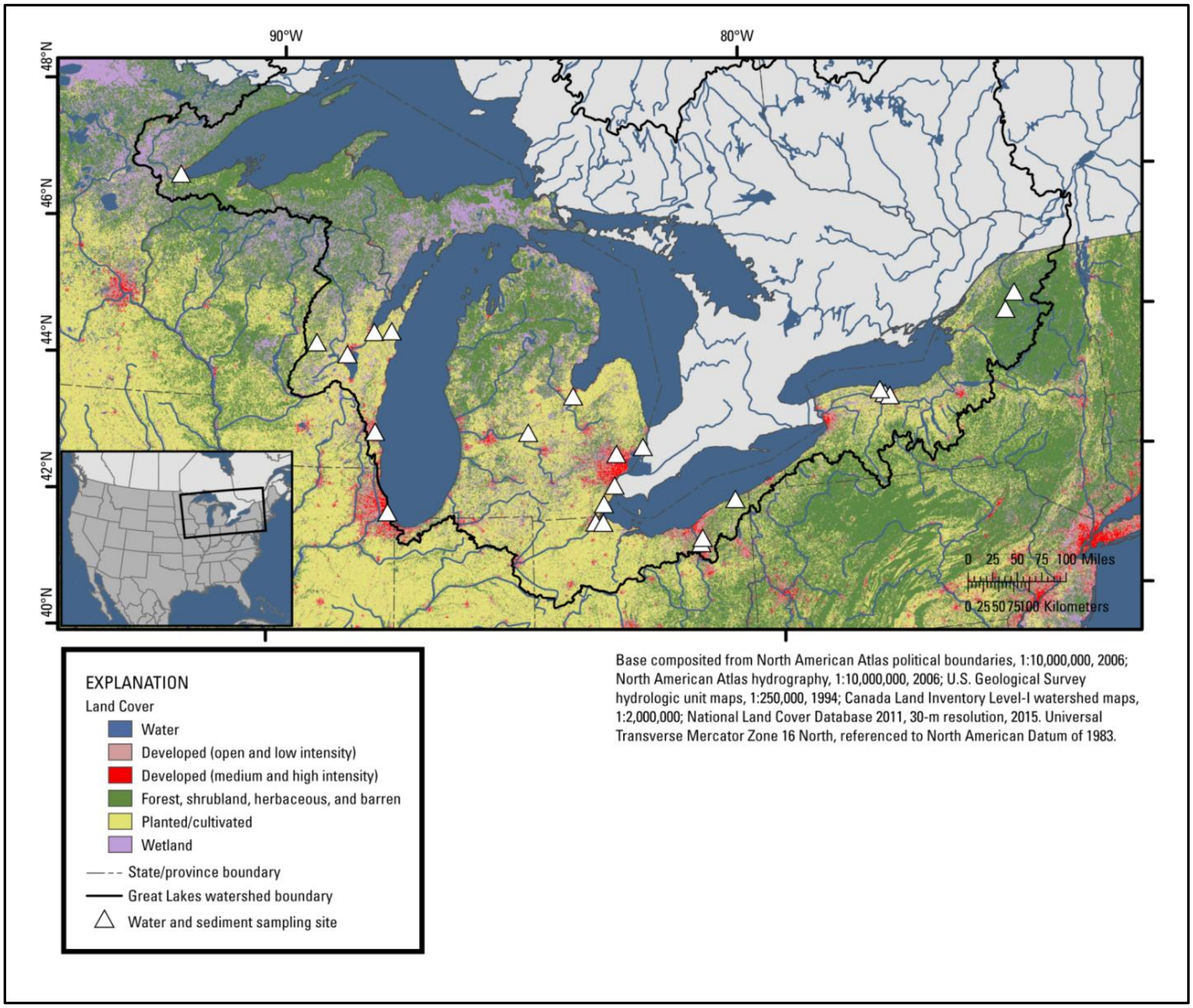

Figure D.1: Map of the 24 waterbodies sampled within the Great Lakes Basin. Water and sediment were sampled at all locations. Sampling occurred from 2010-2014 during spring, summer, and fall months. Each white triangle indicates a tributary sampled, but within each tributary 5-36 individual samples were collected. Sampling location names from west to east are as follows: St. Louis River, Waupauca Chain O' Lakes, Little Lakes Butte Des Mortes, Fox River, Kewaunee River, Milwaukee River, Chicago (North Shore Channel and Little Calumet), Grand/Maple River, Saginaw River, Swan Creek, Maumee River, Raisin River, Detroit River, Clinton River, St. Clair River, Cuyahoga River, Tinkers Creek, Ashtabula River, Long Pond, Genesee River, Irondequoit Bay, Oswegatchie River, and Raquette River.

As the human population has grown, so has the market for new and innovative products. With human population growth and industry projected to increase in the Great Lakes Region for the next two decades (Pendall et al. 2017) it can be assumed the threat of CECs is not easily understood or quickly solved. This population growth in turn causes increasing use of new products and chemicals and therefore new and increasing inputs into water sources from household use, direct point source inputs from industry, and run off from agricultural and urban areas. The overall presence and occurrence of CECs in the Great Lakes Basin and their biological effects remain largely unknown. Given the geographic scale (>260,000 km^2) of the study area and the diversity of aquatic habitats and surrounding land use, this study proceeded sequentially. A discovery phase (2010-12) focused on determining presence and concentrations of CECs in water, sediment and fish tissues in US tributaries across all five Great Lakes. Biological investigations of native fish health accompanied this focus on chemical detection. In 2013, the study progressed to a two-year focused assessment of the linkage between CEC presence, land use and biological effects. When integrated, these efforts produced the most in-depth and comprehensive assessment of the CEC exposure on native fish and wildlife ever conducted in the North American Great Lakes.

D.2. Methodology

At the onset of this study, only scant and discontinuous knowledge of the presence and concentrations of CECs in Great Lakes tributaries and its native fauna existed. To meet the project objective, it was necessary to select representative US tributaries to the Great Lakes across all five lakes and encompassing a variety of land uses. This list included 24 rivers, impoundments and embayments across the Great Lakes Basin (Figure D.1).

To assure consistency and quality of sampling, all samples were collected using peer-reviewed standard sampling protocols for water, sediment and fish tissue (Lee et al. 2012; Elliott et al. 2015, 2016). These protocols required the collection of duplicate samples, split samples, and blank samples to assess variability and detect contamination of samples from other sources. All chemical concentration data reported here are the result of these rigorous quality assurance practices and exclude any data not meeting minimum quality standards.

As the investigations moved beyond the survey of CEC presence and concentration to the assessment of biological effects in native fishes, similar quality assurance protocols were implemented (Blazer et al. 2014; Thomas et al. 2017; Jorgenson et al. 2018). The biological analysis between 2010 and 2012 focused on small numbers of fish in a few selected sites (Blazer et al. 2014) and expanded to a broader assessment of native and caged fish from six rivers over two years in 2013 and 2014 (Thomas et al. 2017; Jorgenson et al. 2018). Similar to the selection of rivers for chemical analysis, rivers for biological assessment of fish were chosen to represent a continuum of land use from forested reference streams (Raquette River, NY), to intensely agricultural watersheds (Fox River, WI), and densely urban watersheds (Chicago River, IL; Clinton River, MI). In each river multiple sites along the rivers' length were chosen to bracket known point sources (specifically treated wastewater effluent discharge) and transitions in land use. Using existing protocols established by Environment Canada (Munkittrick, 1992) to assess fish health in effluent affected streams, an effort was made to collect bass, suckers and sunfish at each stream site. These collections were augmented with two-week caging of hatchery-reared sunfish at five to six sites in each river to provide a more controlled biological integrator of exposure and biological effect.

Once fish were processed, rigorous quality assurance and quality control protocols were implemented (Lee et al. 2012; Blazer et al. 2014c; Elliott et al. 2015, 2016) to avoid sampling or analysis bias. These measures included duplicate and triplicate sample analysis, randomizing of sample analysis and “blinded” analysis of samples to assure that the researcher is unaware of the source of each sample. Lastly rigorous statistical methodology was applied to reduce the likelihood of “false positive” results.

D.3. Key Findings

D.3.1 CECs Are Ubiquitous in Great Lakes Waterbodies

The objective of Phase 1 of the study was to characterize CECs across the Great Lakes basin to better understand their presence and concentration. CECs were found in surface water and sediment of all 24 waterbodies sampled from 2010 to 2014 (Figure D.1). At each of the 24 waterbodies between two and 36 different sampling sites were established. Samples were analyzed for over 200 chemicals which were then grouped into nine chemical classes:

1. *Alkylphenols*, building block materials and additive chemicals used to make other chemicals and products such as resins, fuels, fragrances, lubricants and adhesives to name a few
2. *Flavors and fragrances*, found in perfumes, soaps, and detergents, and food products with artificial flavors
3. *Hormones*, naturally occurring and man-made pharmaceutical products for human and animal use
4. *PAHs*, stands for polycyclic aromatic hydrocarbon, and are found in coal tar residues and are also from incomplete organic matter combustion such as vehicle exhaust, wood burning, and coal-tar pavement sealcoat. Although not an emerging contaminant this chemical class was included in analyses because PAHs remain an environmental concern. New levels or their interactions with other chemicals could be a cause for concern and warranted investigation
5. *Pesticides*, found in commercial, agricultural, and household products
6. *Pharmaceuticals*, prescription and over the counter medications for humans and animals such as anti-depressants, anti-seizure, blood thinners, and pain relievers
7. *Plasticizers and flame retardants*, additive chemicals in products which produce flexibility in plastics and reduce flammability in commercial and household products such as carpets and upholstery
8. *Sterols*, naturally occurring in plants and animals, and can be an indicator of waste water
9. *Other*, miscellaneous category for chemicals which did not fit the above descriptions

Although CECs were detected in all waterbodies sampled, the concentrations and individual chemicals detected differed substantially. Despite these differences, sampling sites within each tributary tended to have similar chemical profiles (Choy et al. 2017, Elliott et al. 2017). This indicates other drivers such as land use, human population, number or types of point sources, size of watershed, or other factors, are influencing the presence and concentration of CECs within each waterbody.

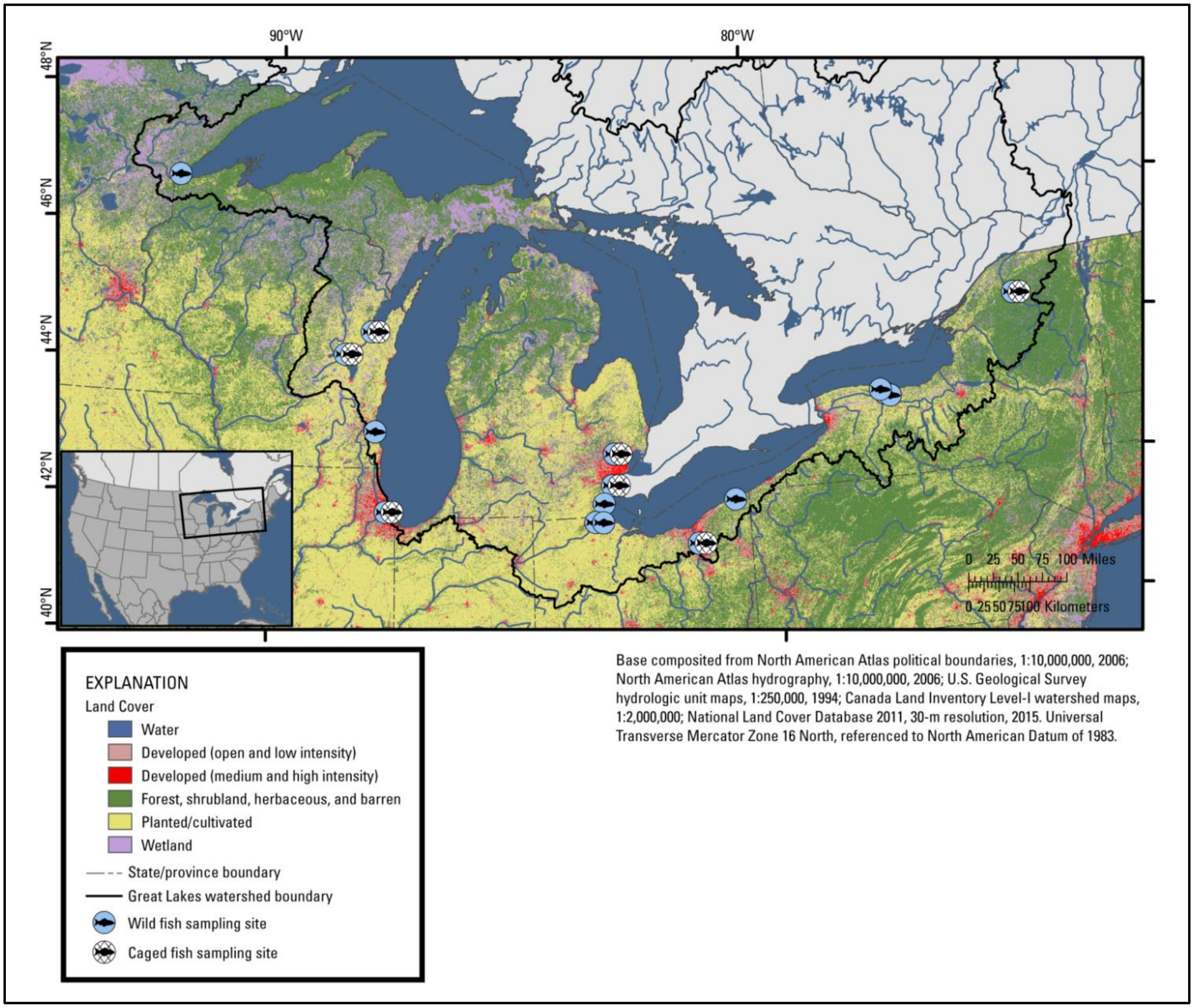

Figure D.2: Sampling locations for wild and caged fish. Wild and caged fish were both sampled for at the following locations from west to east: Fox River, Little Lake Butte Des Mortes, Chicago (North Shore Channel and Little Calumet), Detroit River, Clinton River, Cuyahoga River, and Raquette River. Only wild fish were sampled at (from west to east): St. Louis River, Milwaukee River, River Raisin, Swan Creek, Ashtabula River, Maumee River, Genesee River, Irondequoit Bay, Long Pond, Raquette River.

Types of chemicals frequently detected in sediment varied from those frequently detected in water. Pesticides, pharmaceuticals, and plasticizers/flame retardants were detected more frequently in water than sediment. Commonly detected CECs in sediment included diphenhydramine (pharmaceutical sleep aid found in common over-the-counter medications), indole (a fragrance and fecal indicator), estrone (hormone), and carbazole (pesticide). The St. Louis River (MN) had the greatest frequency of pharmaceuticals detected in water and sediment, while Irondequoit Bay (NY) had the lowest frequency of pharmaceuticals detected. The Maumee River (OH), Fox River (WI), River Raisin (MI), and Long Pond (NY) had the highest frequency of pesticides detected in the water samples while the Detroit River (MI) had the least. Detailed explanations of findings and site descriptions can be found in Choy et al. (2017) and Elliott et al. (2017).

Even though concentrations of detected CECs were low (detections were in ng/L or parts per trillion), some exceeded concentrations previously shown to impact fish and wildlife as did measured concentrations of some PAHs that exceeded water quality benchmarks (Elliott et al. 2017). The highest detected concentrations of Metformin (a diabetes medication) has been shown by previous studies to alter fish physiology which can lead to population declines (Crago et al. 2016). Much of what is known about the effects of CECs on fish and other wildlife are the result of laboratory studies. However, it is still unclear how these chemicals impact fish and wildlife when they are present in complex mixtures and under fluctuating environmental conditions. Therefore, the impacts to aquatic life could be more (or less) severe in natural habitats than in laboratory investigations. This knowledge gap requires further study.

The observations and findings described above are not unique to this study or the Great Lakes Basin. CECs have been found in water and sediment across the globe, in freshwater and marine systems (Herberer et al. 2002; Maruya et al. 2012; Pritz et al. 2014; Yager et al. 2014; Odendall et al. 2015; Morales-Caselles et al. 2017; Edwards et al. 2018). Since CECs are widely found at detectable concentrations and many studies show even low concentrations of CECs impact aquatic life, it is possible that the CECs and the associated observed concentrations are negatively impacting fish and wildlife residing in the Great Lakes' tributaries. CECs have also been detected in plant and animal tissues (Maruya et al. 2016, Sengupta et al. 2016) raising the specter of food-web interactions (reviewed in Nilsen et al. in press). The observed ubiquitous presence of CECs justified the next step in this investigation: an assessment of the biological effects of CECs in fish residing in Great Lakes Tributaries in order to provide the basis for informed management decisions for the continuing benefit of Great Lakes natural resources.

D.3.2 Biological Effects Are Subtle and Widespread

<u>Fish Tumors in Native Fish</u>

Assessment of fish health began in 2010 with the collection of brown bullheads (*Ameiurus nebulosus*), white sucker (*Catostomus commersonii*), and large or small mouth bass (*Micropterus salmoides* or *dolomieu*), in federally designated Great Lakes Areas of Concern (AOCs). Histopathological investigations revealed that fish in AOCs had a higher prevalence of skin tumors than fish at reference sites. The prevalence of liver tumors matched or was incrementally higher at AOC sites (Blazer et al. 2014b), especially those in highly urbanized tributaries. Similar, subtle changes in tumor prevalence were found in white suckers (*Catostomus commersonii*) collected from an AOC in a Lake Superior tributary (St. Louis River) when compared to fish outside the

AOC (Mazik et al. 2015; Blazer et al. 2014a). These histopathological findings provided some evidence that pollutant exposure in Great Lakes tributaries has adverse effects on exposed fish populations. Further evaluation of specific chemicals and comparison to reference sites is still needed to draw concrete conclusions.

Biological Effects

To provide greater linkage between biological effects and CEC presence in Great Lakes tributaries, a concerted effort was made in 2013 and 2014 to collect up to 40 sunfish (*Lepomis spp.*) at multiple sites in each of six Great Lake tributaries. These resident fish collections were paired with two-week long caging of hatchery-reared bluegill sunfish (*Lepomis macrochirus*) to provide a more controlled exposure scenario of known length to a group of fish of similar age and exposure history. Resident as well as caged sunfish were found to have increased blood glucose concentrations at sites with higher CEC presence and concentrations (Figure. 6.3). The increased glucose concentrations are likely the result of pollutant-induced metabolic stress, and were associated with increased liver size and cellular changes in liver tissue (Thomas et al. 2017). These indicators of pollutant exposure were inversely correlated (had opposite trends) with indicators of reproductive potential. In fish with higher blood glucose concentrations and altered liver anatomy, reproductive organs (testis, ovary) were smaller and less mature (Thomas et al. 2017). These altered fish health parameters were more commonly associated with sites containing greater CEC presence and concentrations (Thomas et al. 2017).

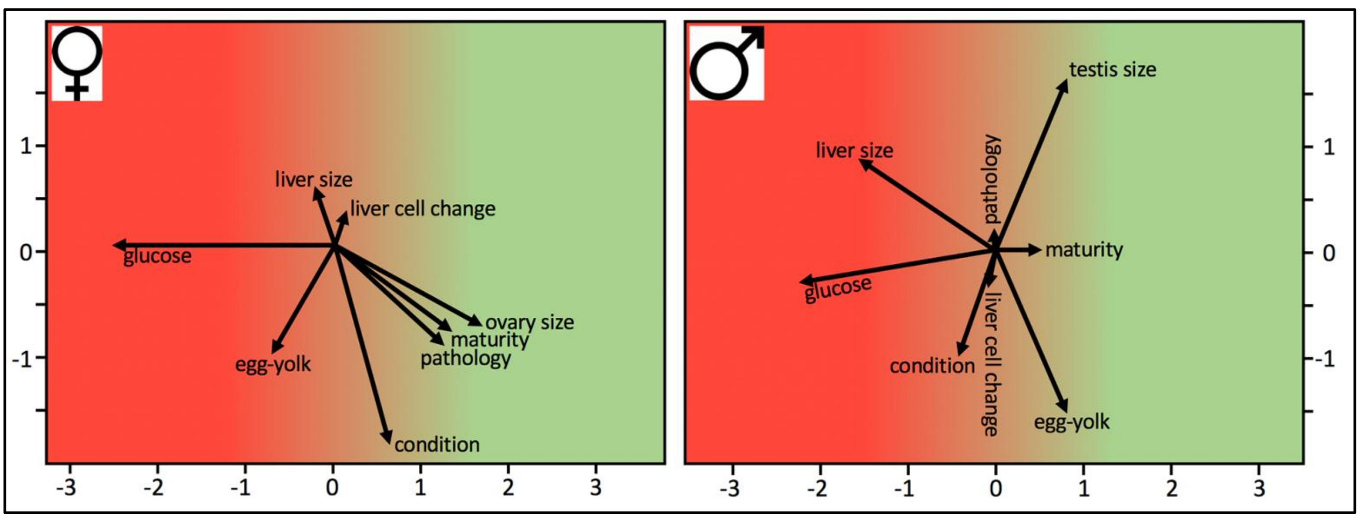

Figure D.3. Relative changes in vital functions in caged female (left) and male (right) sunfish are associated with locations in Great Lakes tributaries that experience greater (red) or lesser presence of Contaminants of Emerging Concern. Greater arrow length indicates more pronounced effects. Arrows pointing similar directions indicate positive associations while arrows pointing in opposite directions indicate negative associations. For example, larger glucose values are associated with less maturity. Associations along the horizonal axis are stronger than along the vertical axis. Comparisons are between fish caged at sites in the study area.

The analysis of CECs at sites where fish were collected and caged also suggested that the presence of PAHs and pharmaceuticals can explain most of the variation in blood glucose concentrations, altered liver anatomy, reduced reproductive organ size and lesser maturity. These findings were consistent for resident and caged fish of both sexes. Unfortunately, the data set was not sufficient to determine which classes of pharmaceuticals (for example, antidepressants, antibiotics, NSAIDs) were most responsible for the observed changes. However, the strength of a negative association between blood glucose concentrations and reproductive endpoints (maturity and ovary/testis size) suggested that there is an energetic cost associated with exposure to CECs (likely as a result of the recruitment of detoxification pathways by the exposed organisms) that may divert energetic resources from reproduction.

D.3.3 Effects of CECs Differ between Species

In addition to the resident and caged sunfish described above (3.2), largemouth bass and white suckers were collected across 16 sites (Jorgenson et al. 2018). These fish were analyzed for many of the same biological indicators of exposure as the sunfish described above. Similar to findings in sunfish, liver size and liver structure were often altered in fish living at sites downstream from point source pollution were more likely contaminated by CECs. In addition, this analysis identified different response patterns in different fish species. Patterns of biological response to CEC exposure clustered around three biological processes (Figure D.4): (i) organismal health as assessed by the overall nutritional status of the fish (condition factor) and the presence of pathologies such as parasite infestation; (ii) liver health as indicated by plasma glucose concentrations, changes to liver cellular structure and liver size; and (iii) reproductive health as indicated by differences between sites in the size and maturity of the reproductive organs as well as the presence of the egg yolk pre-cursor protein vitellogenin in male fish. Changes in organismal health indices were more closely associated with female sunfish. Changes in liver health indices were more closely associated with female largemouth bass. Male white suckers were also closely associated with changes in liver health, while both sexes of white suckers were found to have close associations between CEC presence and changes in reproductive health indices (Jorgenson et al. 2018). These results suggest that subtle changes in fish health could be a response to CEC exposure may differ between species. Other factors such as chemicals not measured, altered stream characteristics, and habitat could also be factors contributing towards the effects seen.

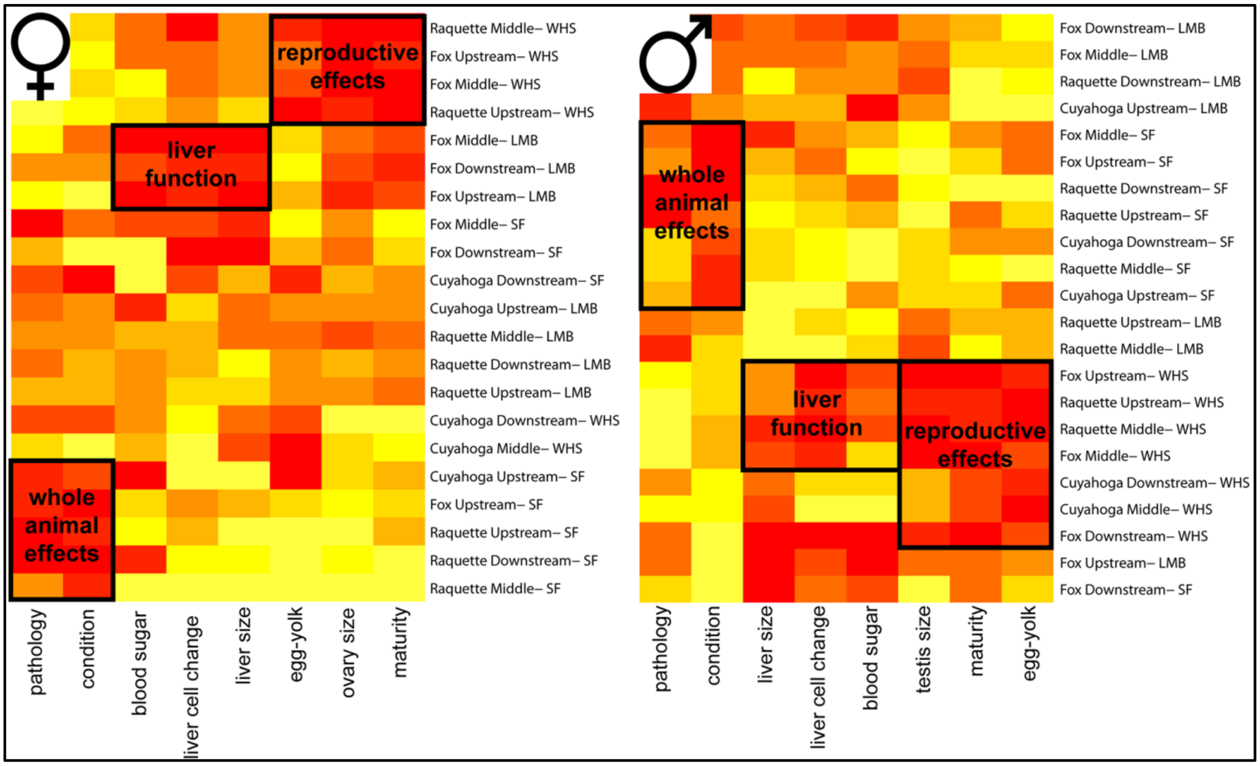

Figure D.4. Clustering of adverse effects in resident white suckers (WHS), largemouth bass (LMB) and sunfish (SF). Darker red colors indicate stronger effects. Results from female fish (left) and male fish (right).

D.3.4 Some CECs Occur at Concentrations Predicted to Cause Biological Effects

As a result of detecting CECs in in water and sediment, and finding that CECs may be a contributing factor impact the biology and physiology of fish living in areas polluted by CECs, screening values were developed as a tool for natural resource managers. These screening values will help managers evaluate the water at their locations to better understand if fish in those locations are at risk for impacts by CECs. Effect-specific pairs of mean Screening Values (SV) were developed for 14 CECs in water for which there was adequate peer reviewed literature available (Figure D.5). Screening values are estimated threshold concentrations of chemicals that define our expectations about adverse effects in target biota. A set of Comprehensive SV_{HIGH} and SV_{LOW} values was developed for multiple effect categories using all adverse effects reported in peer reviewed literature for a given CEC. A subset of adverse effects was used to derive a set of Population-relevant SV_{HIGH} and SV_{LOW} values, to focus Ecological Hazard Assessments (EHA) on the potential for population-level impacts. The SV_{LOW} is the threshold concentration of a CEC at or below which it is unlikely to cause an effect to freshwater fish, while an SV_{HIGH} is the threshold concentration above which it is likely that an adverse effect has occurred. For values that fall between these two thresholds, it is uncertain if an effect will occur.

SVs for each of the 14 chemicals were developed for five population-relevant biological effect categories (Gefell et al. 2019a):

1. Mortality
2. Reproductive
3. Developmental
4. Behavioral
5. Growth.

Seven additional ‘comprehensive’ effect categories were evaluated for potential impacts to fish health.

An EHA was then conducted for the 24 waterbodies sampled for CECs in 2010-2014 (Figure D.1), using the CEC concentrations detected in the water samples. The primary purposes of the EHA were to determine a location’s relative hazard from CECs, and then rank CECs and sampled sites in terms of potential for direct impacts to fisheries from water-borne CECs. It was evaluated whether ecological hazard to fish due to CEC exposure could be discerned in the dataset, and if so, what is the nature and extent of that hazard. For each exposure data point, a set of hazard scores was developed from simple comparisons of CEC concentration to the corresponding pair of CEC-specific SVs. A hazard score of 1 was assigned where the individual CEC concentration was less than the SV_{LOW}, a score of 3 was assigned where the CEC concentration exceeded the SV_{HIGH}, and CEC concentrations that fell between the SV_{LOW} and SV_{HIGH} received a score of 2. At each project location an exceedance of SV_{LOW} was observed for at least one of the CECs, in one of the effect categories (Gefell et al. 2019b), while an SV_{HIGH} exceedance in at least one effect category was observed in 17/24 locations. These results indicate environmental concentrations likely impact native fish health, but much is unknown and more information is needed to fully evaluate the impacts of CECs on native fish communities. Detailed information for each of the 14 chemicals and associated screening values and EHA can be found in Gefell et al. (2019a and 2019b).

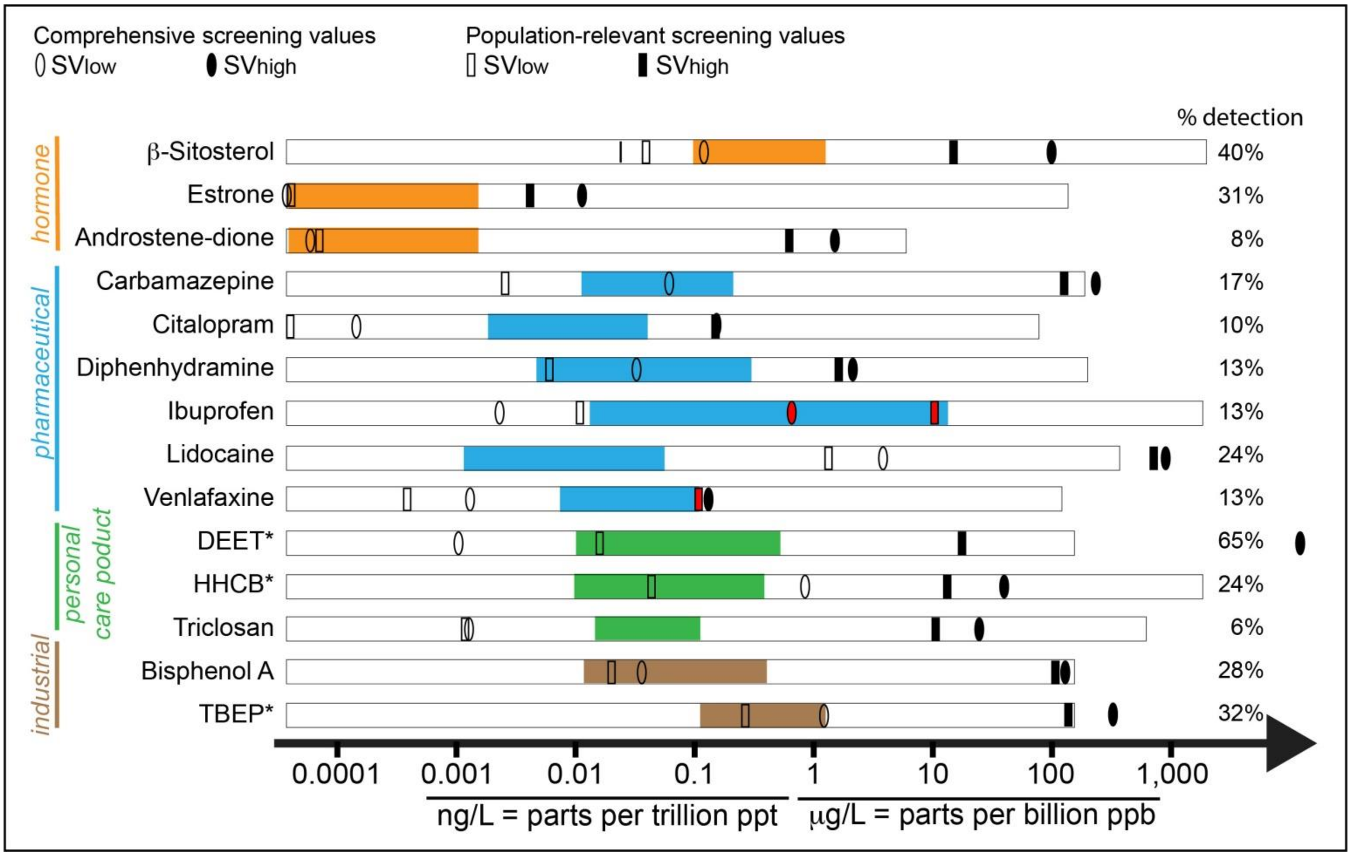

Figure D.5. Comprehensive (ellipse) and Population-relevant (rectangle) Mean screening values (high=solid) for fourteen Contaminants of Emerging Concern (CEC) commonly detected in water samples in tributaries to the Laurentian Great Lakes. Measured detected concentrations for each CEC in water samples (Choy et al. 2017) are indicated by the horizontal colored columns. Red rectangles/ ellipses indicate that environmental concentrations exceeded screening values. Percent detection for each CEC (out of 150 water samples) is indicated in the right hand side column. Black outlined hollow bars for each chemical indicate range of concentrations measured by Bradley et al. 2017 in 38 US streams.

D.4. Management Implications

Since CECs are ubiquitous throughout Great Lakes tributaries, and fish are exhibiting effects associated with exposure to CECs, it is important for fisheries and natural resource managers to be aware of CECs. Natural resource managers need to have a good understanding of where and what CECs are found in their management areas, and how those CECs could impact fish species and populations in order to make sound management decisions. Understanding and accounting for CECs in the environment is one more piece of the puzzle in natural resource management. Possible considerations for natural resource management could include:

- Surveying for CECs before beginning restoration or mitigation projects to understand how the CECs in the project area may impede the success of the project and applying screening values to the samples taken to identify potential risk.
- Prioritizing restoration, mitigation, or release sites with lower occurrence and presence of CECs to reduce the risk of impacts and ensure success to those projects.
- Surveying/monitoring for CECs if incidences of concern arise, such as altered age structure or decreases in populations, especially when all other environmental factors are favorable.
- Working together with state, federal, local, or tribal agencies, private land owners or industries to spread awareness of the impacts CECs have on the environment.
- Education campaigns on improving disposal and use of CECs to reduce presence in aquatic systems.
- Providing information to WWTPs or other industries to help them formulate wastewater treatment processes to reduce loading of CECs into the environment.
- Working with USFWS National Wildlife Refuges, Private Lands Division, Fish Hatcheries, Fish and Wildlife Conservation Offices, Fish Health Centers, to name a few, to help prioritize areas for projects and best use of restoration efforts.

Best management practices and strategies need to be adaptive and change as new information arises. These suggestions are based on current information and may be revised as new results and information become available. However, it is important for natural resource managers to understand that CECs are present in most water and sediments in Great Lakes tributaries, and have the potential to negatively impact natural resources. As this team continues to investigate and assess those impacts and risks to aquatic life, more information on how to adjust best management strategies will become available.

D.5. Knowledge Gaps

- Is the potential for severe CEC-related impacts at measured CEC exposure levels masked in current fish populations and communities? That is, have the most dramatic CEC-related impacts to resident fish communities already occurred in waterbodies that have received long-term CEC inputs, leaving only the most CEC-tolerant species and strains within species to investigate in our current field assessments?
- Can the composition of CEC mixtures be predicted based on land-use characteristics?

- If fish are exposed to complex mixtures of CECs, what are the aggregate biological effects of CEC mixtures and are mixture effects predictable from individual compound exposures?

- As CECs occur in Great Lakes tributaries year-round, what are the aggregate life-time exposure effects?

- As CECs co-occur with other environmental stressors (such as nutrient pollution and changing water temperature), how do these multiple stressors interact to affect aquatic biota?

- Can aggregate effects of life-time and complex mixture exposures be extrapolated to population level consequences for exposed populations of fishes?

- Can the aggregate life-time complex CEC mixture exposure effects be extrapolated to species that are difficult to study directly (i.e. listed species)?

D.6. Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the U.S. Fish and Wildlife Service or the U.S. Environmental Protection Agency

D.7. Acknowledgements

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Appendix E

Detecting and Evaluating Biological Effects of Contaminants of Emerging Concern in Great Lakes Tributaries

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E.1. Problem Statement and Scope

As analytical methods for detecting chemicals in the environment improve, an increasing number and diversity of contaminants can be detected in surface waters, sediment, and biota across the Great Lakes watershed. Unfortunately, for many of these contaminants traditional toxicity benchmarks (e.g., water quality criteria; guidelines; standards) are unavailable. This makes it challenging for federal, state and/or tribal resource managers, and local communities to understand which, if any, of these contaminants may potentially cause adverse effects on Great Lakes ecosystems and the valuable services they provide to both local communities and the Nation. This challenge is further complicated by the fact that contaminants in the environment occur in complex mixtures, and toxicity benchmarks for individual chemicals, even when available, may not adequately reflect toxicity of the mixture due to interactive effects.

The goal of this research, undertaken as part of the Great Lakes Restoration Initiative (GLRI) Action Plan I, was to develop a range of approaches and tools that could (1) predict or detect biological activities associated with mixtures of contaminants of emerging concern (CECs) in Great Lakes tributaries, (2) understand what contaminants and/or sources are likely, or unlikely, to be driving those activities, (3) define the types of adverse effects to Great Lakes wildlife that may be associated with those exposures, and (4) understand which classes of organisms may be vulnerable. While this may not necessarily provide resource managers with a definitive toxicity benchmark to incorporate into an ecological risk assessment, the approaches can help them triage the sites, contaminants, and biological effects under their purview and focus limited resources for monitoring on the issues most likely to be of concern.

To achieve this, we leveraged the rapidly growing sources of biological pathway-based data, information, and knowledge that are being developed as part of an on-going paradigm shift in chemical safety assessment (NRC 2007). Traditional toxicity testing approaches, which involve dosing animals and observing the concentrations at which statistically significant impacts on survival, growth, and/or reproduction occur, cannot tractably be performed for 10,000s of chemicals that occur in the environment, let alone the array of complex mixtures in which they occur. Therefore, concerted efforts are being made to use both our steadily improving understanding of biological systems and new technologies for evaluating chemical safety. Understanding of fundamental mechanisms of toxicity is being used to infer likely biological hazards associated with chemicals based on molecular, biochemical, and/or cellular effects (collectively termed pathway-based effects) the chemicals may elicit in assays that can be more rapid, cost effective, and/or information-rich than traditional toxicity tests. For example, advances in automation are being used to conduct assays with proteins, cells, or even small organism like fish embryos or nematode worms in high throughput multi-well (e.g., 96-, 384-, 1536-well plates) formats (Kavlock et al. 2012). Likewise, advanced analytical instrumentation and biotechnologies enable measurement of hundreds to thousands of biologically relevant features like mRNA transcripts, proteins, or other small endogenous metabolites like lipids, carbohydrates, amino acids, etc. in a single biological sample, providing very rich data content for each sample. Both high content and high throughput approaches are facilitated by advances in computing power and increased capacity to store, aggregate, and disseminate data and knowledge via cloud computing and the internet. Additionally, translational tools, like the adverse outcome pathway (AOP) framework, which organizes information concerning the plausible and evidence-based linkages between molecular, biochemical, and/or cellular level perturbations and the adverse outcomes they may cause (Ankley et al. 2010; Villeneuve et al. 2014), are being used to aid interpretation and application of these new data sources. While most research and development has focused on the application of these tools for single chemical assessments, there are many opportunities to utilize new approach methods, and the data they provide, to address on-going challenges associated with detecting and evaluating biological effects of mixtures of contaminants of emerging concern (CECs) in the Great Lakes. Consequently, as part of GLRI Action Plan I, our major aim was to develop approaches and tools to help harness and apply data from these new approach methods. Pilot experiments and case studies were conducted to demonstrate how these approaches can be applied in a real-world setting, evaluate their strengths and limitations, and determine how they can be refined, optimized for implementation, and used by Great Lakes resource managers.

E.2. Detecting Biological Activities Associated with Mixtures of CECs in Great Lakes Tributaries

When toxicity benchmarks are lacking (as is the case for many individual CECs, as well as for complex mixtures whose composition is often only partially known), the most direct method for associating exposure with effects is to conduct a bioassay with the chemical or mixture in question. This is the basic rationale that led to programs like US EPA’s Whole Effluent Toxicity (WET) Testing program, which has long been used to evaluate the toxicity of complex mixtures of unknown and variable composition for which development of chemical concentration-based standards is impractical (Grothe et al. 1996). However, laboratory-based toxicity testing of complex mixtures in ambient waters can be prohibitively expensive, and logistically difficult (e.g., requiring transport of large volumes of water). Additionally, testing that focuses only on direct effects on survival, growth, or reproduction generally provides little insight into biological mechanism(s) through which those effects are occurring, making it difficult to narrow the field of potential agents contributing to the toxicity. Likewise, such tests may miss potential chronic, sublethal effects that are nonetheless significant to ecological fitness. Consequently, our aim was to develop effects-based monitoring approaches that were logistically tractable, but also information-rich with regard to identifying both obvious and subtle signs of exposure and/or toxicity and associating those effects with specific stressors so that sources and possible management actions could be identified.

In developing effects-based monitoring tools, there were two scenarios we wanted to account for (Figure E.1; Ekman et al. 2013). The first was a surveillance scenario where there is little previous knowledge regarding what concern(s) may be of interest for a given site. The key need for surveillance is to “cast a broad net” that maximizes the ability to detect any biological effect(s) of potential concern. Such an approach is also referred to as *untargeted* monitoring, since a specific effect is not monitored for. In contrast, the second scenario was *targeted* monitoring. In the targeted scenario, there is pre-existing information that can inform problem formulation and has already helped narrow the field of potential biological activities or effects of concern. An overall goal of this research was to demonstrate the complementary relationship between these scenarios, where untargeted, surveillance-oriented, approaches could be used to inform subsequent targeted monitoring to ensure greater protection while reducing overall risk assessment costs.

For each of these scenarios, two primary monitoring strategies were explored (Figure E.1; Ekman et al. 2013). The first strategy relied on the use of in vitro assays to screen samples for specific biological activity that may be plausibly linked to adverse toxicological outcomes in vivo. A primary advantage of this strategy is that it is logistically easy to implement, generally requiring collection and extraction of only a small volume or mass of sample matrix (e.g., a liter of water, a few grams of sediment). Second, because samples and resulting extracts are tested in a laboratory environment, the assays can be conducted under highly standardized conditions, facilitating less confounded comparisons among multiple samples. A disadvantage of the in vitro assay-based approach is that it employs a simplified biological system that may not capture all aspects of the biology that may influence toxicological outcomes (e.g., chemical absorption, distribution, metabolism, elimination). Likewise, standardization under laboratory conditions comes with a loss of realism in terms of variable and fluctuating conditions that may influence responses in a real-world context. Additionally, it is recognized that any extraction of a sample matrix involves loss of some sample components.

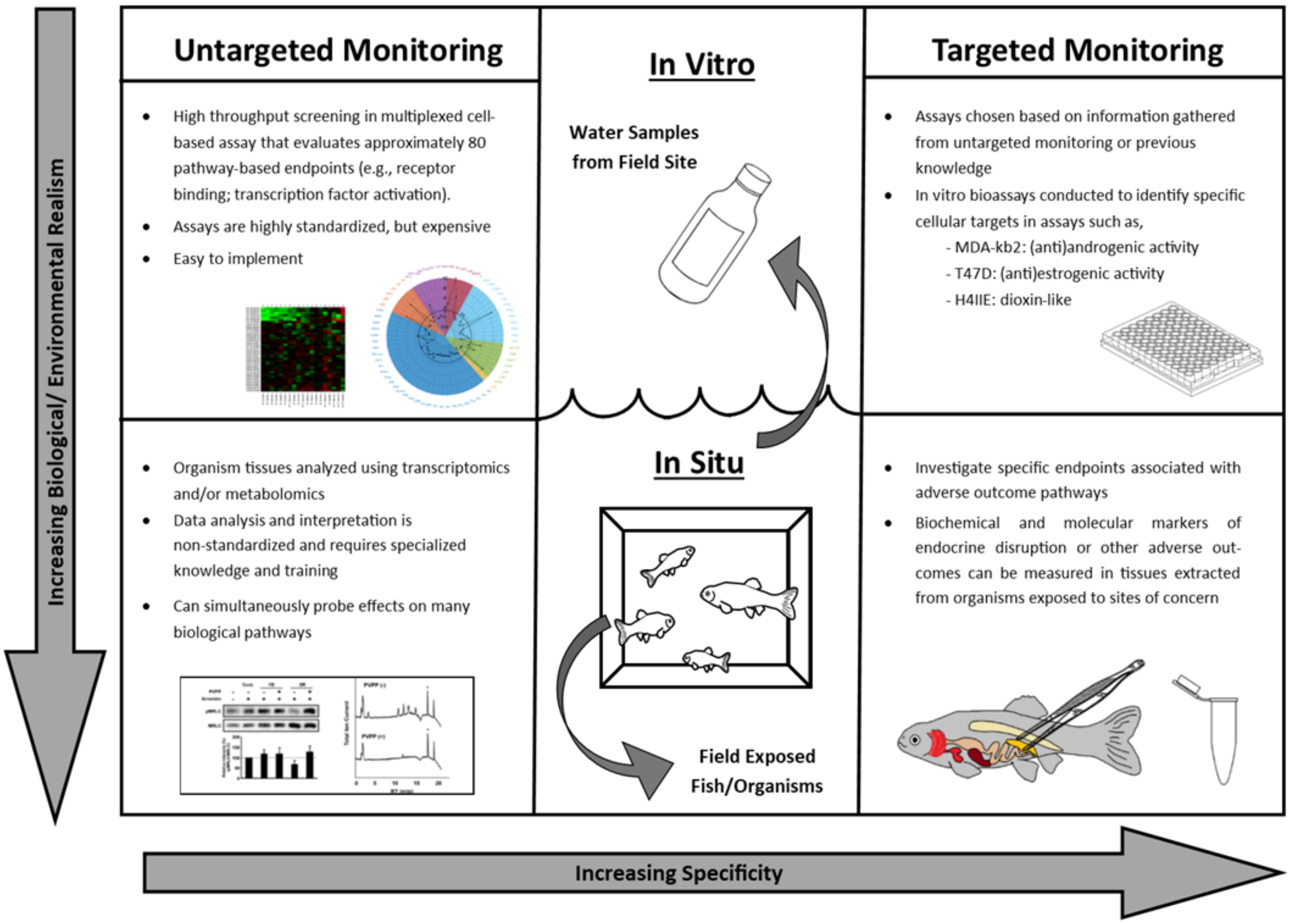

Figure E.1. Targeted vs untargeted effects-based monitoring scenarios. Each scenario can be addressed with in vitro and/or in situ approaches, represented here with increasing biological realism from top to bottom and specificity left to right.

A second complementary strategy that was developed employs caged organisms exposed at the site (in situ). This in situ approach is logistically more complex to implement. Organisms with known history must be transported from a laboratory or commercial supplier, held at a site of interest for some period and then retrieved for sampling. The comparative advantage to this approach is greater biological realism in terms of utilizing a complex organism with all its attendant integrated biological functions. Additionally, an in situ approach provides a more realistic representation of the exposure profile including multiple stressors, differences in the potential viability and route(s) of uptake for different chemicals, and variation in the exposure characteristics over time.

E.2.1. Strategy – In Vitro Assays

Under GLRI-Action Plan I, targeted monitoring with in vitro assays focused on two endocrine pathways with direct relevance to EPA's Endocrine Disruptor Screening Program. The T47D-kbluc assay (Wilson et al. 2004) was used to screen water samples or extracts for chemicals that could either activate or antagonize the estrogen receptor (ER). The MDA-kb2 assay (Wilson et al. 2002) was employed to screen for compounds that could activate the androgen receptor (AR). Activation or antagonism of either of these receptors by contaminants are of concern due to the important roles that androgens and estrogens play in regulating reproduction, sexual development, sex-specific behaviors, and a variety of other critical biological functions.

However, interactions with estrogen receptor and androgen-receptor are not the only pathway-based effects of chemicals that are of potential concern to Great Lakes wildlife. Consequently, for the purposes of broader, non-targeted, biological surveillance, we piloted the use of commercial, multi-factorial, assays developed by Attagene (Romanov et al 2008; http://www.attagene.com/technology.php) to evaluate complex mixtures. The Attagene cis-Factorial assay probes the ability of a sample to activate any of 46 different transcription factors known to regulate pathways relevant to toxicity. The Attagene trans-Factorial assay evaluates the ability of a sample to activate any of 25 human nuclear receptors (including AR and ER). Employed together, the Attagene assays allow for evaluation of 71 different endpoints using two in vitro bioassays, thus, providing much broader coverage of biological targets/pathways than the targeted assays. The primary trade-off with the Attagene assay, compared to the less information-rich targeted assays, is greater cost per sample. The Attagene approach is more cost effective if the goal is to cover a broad diversity of biological targets/pathways for a small number of samples. In contrast, the more targeted assays are better suited for evaluating a single activity for many samples.

A second in vitro assay-based surveillance approach involved metabolomics analyses on cells exposed to surface water samples in the laboratory (Zhen et al. 2018). The term “omics” broadly refers to methods that can measure a “totality” of a certain type of biological feature in an organism. In the case of *metabolomics*, the biological feature under investigation is the collection of small endogenous molecules, or metabolites, that make up and control an organism’s metabolism. To conduct in vitro metabolomics studies, cells were exposed to individual water samples for 48 hours, after which they were rapidly quenched to stop all metabolic activity. The cellular contents were then analyzed by advanced analytical instrumentation (e.g., nuclear magnetic resonance spectroscopy and/or high-resolution mass spectroscopy) to quantify the different endogenous metabolites that the cells were producing. Where specific metabolites can be identified, pathways impacted by exposure to the sample can be inferred based on the biochemical pathways that generate or regulate the impacted metabolites. However, even where specific metabolites cannot be identified, useful information can be extracted by comparing metabolite profiles among samples. The relative numbers and magnitude of metabolite changes associated with exposure to a sample can suggest the relative severity of biological impact the sample has on the cells. Likewise, the similarity in metabolite profiles among samples, or between a given sample and that for a reference chemical, can be used to infer whether the bioactive chemical composition among samples may be similar.

E.2.2. Strategy – In Situ Exposure of Model Organisms

In addition to examining biological responses in cells following exposure to samples from the Great Lakes, we also examined responses in fish exposed in situ. Fathead minnows

(*Pimephales promelas*) are a widely used aquatic toxicity test model that is amenable to laboratory culture and resident in freshwater lakes and streams across much of North America (Ankley and Villeneuve 2006). They are available from numerous commercial suppliers and contract labs. Consequently, the fathead minnow was selected as our model organism for in situ exposure. Nonetheless, the basic approach developed could be used for nearly any available small bodied fish.

Under Action Plan I, caged fish exposures were conducted at 31 sites spread across five Great Lakes Areas of Concern (Figure E.2). To probe the utility of this in situ monitoring strategy for either targeted monitoring or surveillance, endpoints and approaches suited for each were included in each case study. Kahl et al. (2014) reported on the development of a simple cage and buoy system employing commercially-available minnow traps as a successful system for deployment and in situ exposure of fathead minnows in Great Lakes waters. As part of Action Plan I, dozens of in situ exposures were conducted using this system, or slight adaptations thereof (Figure E.3). These deployments were highly successful in terms of recovery of the fish and associated biological samples. The system has also proven to be adaptable to a range of environmental conditions (Kahl et al. 2014). Additionally, all equipment and supplies needed to construct and deploy the system are low-cost and commercially available from hardware and sporting good suppliers, making them a practical system for resource managers to deploy.

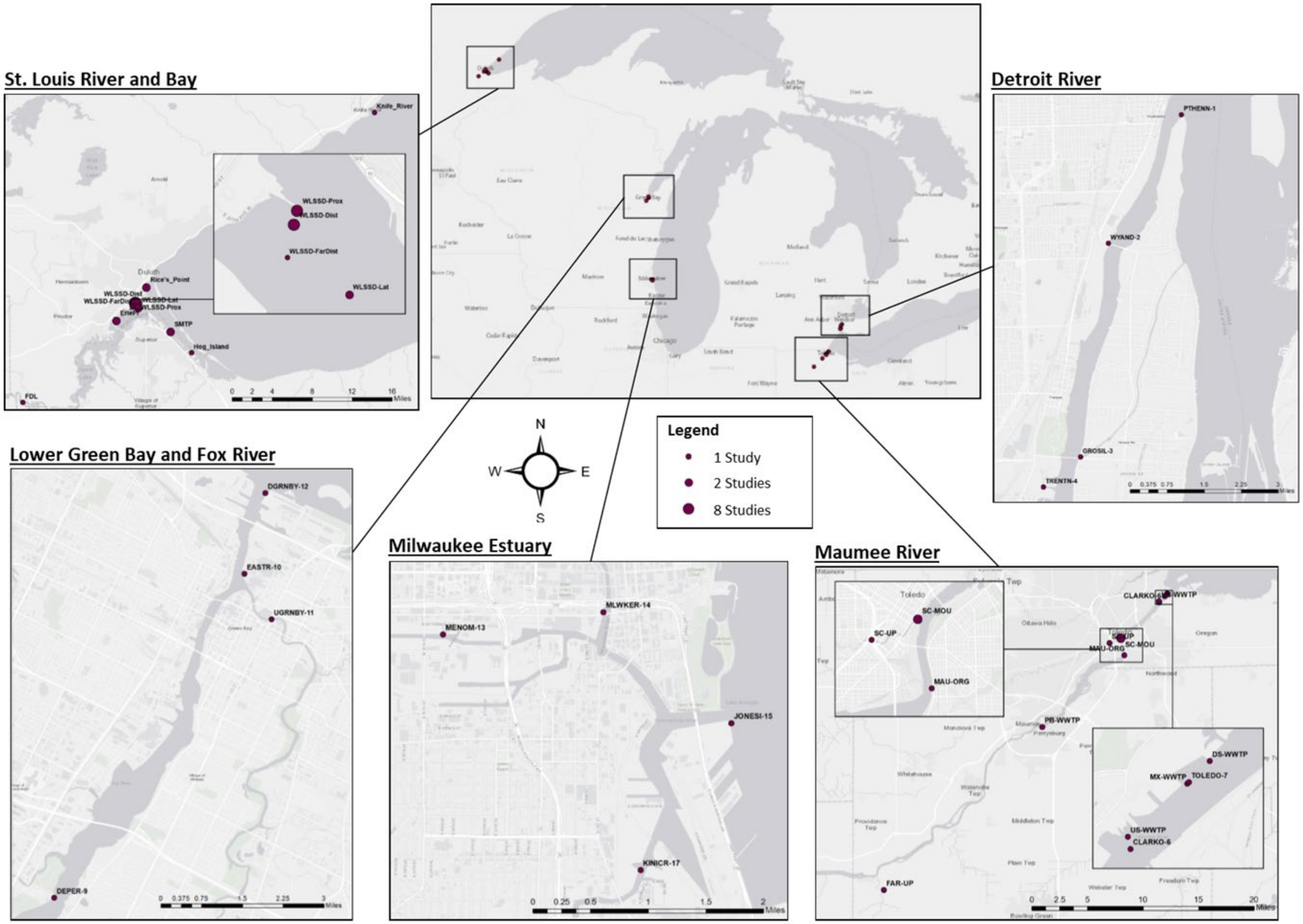

Figure E.2. Locations of case studies conducted as part of Action Plan I and corresponding study sites. Size of each marker reflects the number of caged fish exposures conducted at each site. Map surface layer credits: ESRI, HERE, Garmin, OpenStreetMap Contributors, and the GIS user community. Map created by Kendra Dean (10/24/18).

Figure E.3. The fathead minnow caging system consisting of (A) modified minnow traps held in a mesh bag, (B) suspended from a buoy system anchored to cinder blocks. (C) An autosampler was also tethered to each caging system to collect a composited water sample representative of the in situ exposure.

E.2.2.1. Endpoints and Analyses

The targeted monitoring scenario that we examined for in situ exposures conducted under Action Plan I focused on impacts related to reproductive endocrine functions. Specifically, we monitored for changes in the expression of genes important to reproductive steroid biosynthesis in gonads and to the production of egg yolk precursor proteins in liver (e.g., vitellogenin [VTG] and estrogen receptor alpha). Gonad tissue collected from the fish exposed in situ was used to examine effects on relative rates of steroid biosynthesis, while concentrations of two key reproductive steroids, 17β-estradiol (an important estrogen) and testosterone (an important androgen), were measured in blood to evaluate overall impacts on circulating reproductive steroid concentrations. Finally, circulating concentrations of the egg yok precursor protein, VTG, in blood were also evaluated. The focus is largely on occurrence of elevated concentrations of VTG in males. Under normal conditions male fish do not synthesize appreciable amounts of VTG protein, consequently elevated concentrations of this estrogen-inducible protein in males are viewed as a classic biomarker of exposure to environmental sources of estrogen.

Recall that in the surveillance/untargeted scenario, the goal is to cast as broad a net as possible for detecting potential pathway-based biological activities. The caging strategy used for surveillance was identical to that employed for the targeted scenario. However, rather than incorporating additional targeted analyses, metabolomics as well as transcriptomics were employed. Recall that “omics” broadly refers to methods that can measure a “totality” of a certain type of biological features in an organism. Metabolomics measures the totality of small endogenous metabolites (e.g., sugars, amino acids, certain lipids, vitamins, co-factors). Transcriptomics is used to measure the totality of messenger RNA molecules expressed in a tissue sample. In practice, neither approach captures the true totality of these features, but they do successfully probe the simultaneous expression of thousands of genes or hundreds of metabolites in a single sample. By measuring changes in gene expression and/or metabolite abundance in the fish following in situ exposures, one can conceptually infer impacts on a potentially broad diversity of different biological pathways and/or functions. The breadth of biological responses that can be simultaneously probed and considered is the strength of the approach. The challenge or limitation is the cost, specialized nature of the instrumentation or equipment required for the analyses, and the lack of efficient and standardized pipelines for analysis and interpretation of the results.

While most of the analyses employed by the project are destructive, requiring that the exposed fish be killed to collect the tissue or biofluid (e.g., plasma) for analysis, a novel, non-destructive surveillance approach was also developed. The method involves extracting and measuring metabolites found in fish skin mucus (Mosley et al. 2018). Because sample collection simply involves blotting the skin of a fish with a piece of filter paper of known surface area, it can easily be used without harm on a wide variety of fish species, either caged or resident, including endangered species.

E.2.3. Key Findings/Progress – Detecting Biological Activities

- **Levels of estrogenic activity detected were generally below those expected to raise significant concern regarding potential human or ecological effects***.* While definitive benchmarks for total estrogenic activity have not been established, 3.8 ng 17β-estradiol equivalents (E2-EQ) /L has been suggested as a trigger value for drinking water (Brand et al. 2013), while activities up to 0.4 ng E2-EQ/L were suggested as a safe level for most waste water treatment effluents for fish (Jarošová et al. 2014). Based on analysis of 256 samples from 31 locations in 2010-2013 (Figure E.2), only one source (located in the St. Louis River AOC) consistently exceeded 0.4 ng E2-EQ/L. However, estrogenic activity greater than 0.4 ng E2-EQ/L was commonly detected only in effluent samples, or within 100 m of effluent discharge where mixing and dilution was variable (Figure E.4).

  A subset of the samples were analyzed for both estrogenic activity and known estrogenic chemicals including 17β-estradiol, 17α-ethynyl estradiol, estrone, estriol, 17α-estradiol, 4-nonylphenol, 4-octylphenol, and bisphenol A. Based on the chemical analyses, total expected estrogenic activity was calculated by multiplying chemical concentrations by relative estrogenic potency in the T47D-kbluc assay. The estrogenic activity expected based on the known chemical composition of the sample was compared with estrogenic activity detected using the in vitro T47D assay. Results suggest that the set of compounds measured analytically accounted for most estrogenic activity detected in the bioassay. This suggests that the major drivers of estrogenic activity measured in the Great Lakes tributary samples collected 2010-2013 are known.

  It should be noted that over half of the samples analyzed for estrogenic activity were collected in the St. Louis River AOC. Likewise, sampling in four other AOCs was less extensive in terms of spatial and temporal coverage and collectively the locations directly surveyed for estrogenic activity using the T47D assay represent only of faction of those studied by the broader group of partners. Therefore, conclusions drawn based on this subset do not necessarily apply across the basin.

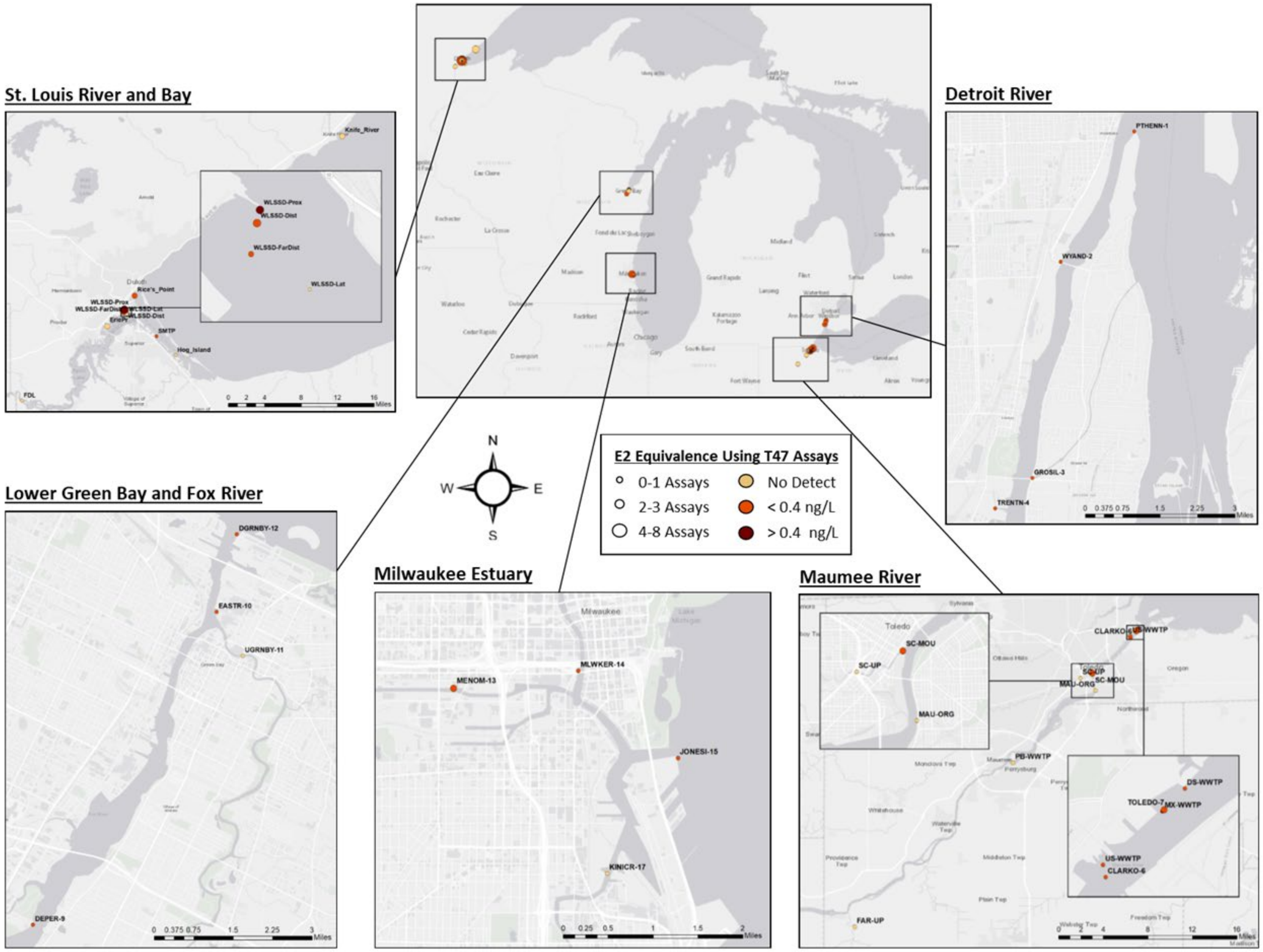

Figure E.4. Total estrogenic activity associated with water samples collected within five Great Lakes case study locations as measured using the T47D-Kbluc bioassay. Marker size indicates number of water samples collected and analyzed at a given site. Color indicates the median total estrogenic activity reported in 17β-estradiol equivalents (E2-

EQs) at a given site. Map surface layer credits: ESRI, HERE, Garmin, OpenStreetMap Contributors, and the GIS user community. Map created by Kendra Dean (10/24/18)

- **Androgenic activity was generally non-detectable in surface waters surveyed.** Among 118 samples from 31 locations sampled 2010-2013, androgenic activity in the MDA-kb2 assay was only detected in undiluted effluent samples or water samples spiked with an androgenic reference chemical. Our results provided no evidence of wide-spread androgenic contamination in the areas surveyed.

- **Among 71 endpoints evaluated using the multi-factorial Attagene assays, nine different biological activities were associated with samples from Great Lakes tributaries.** Under GLRI Action Plan I, we conducted initial pilot testing of the Attagene cis- and trans-Factorial assays as tools for effects-based environmental surveillance. Fifteen samples, including 10 from the St. Louis River AOC and 5 from the Maumee River AOC, plus one extraction blank were analyzed. The most commonly observed biological activities were associated with pregnane X response element (PXRE), aryl hydrocarbon receptor (AhR), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), estrogen response element (ERE), glucocorticoid receptor (GR; NR3C1), peroxisome proliferator activated receptor gamma (PPARg), estrogen receptor alpha (ERa), pregnane X receptor (PXR), and retionoid x receptor beta (RXRb) activation (Figure E.5). Aryl hydrocarbon-receptor and ERa-mediated activities detected in these multi-factorial assays agreed well with results from targeted in vitro assays probing the same targets, lending confidence that the multi-factorial assays were sensitive and suitable for use with complex extracts. Given the promising results, use of the Attagene Factorial assays was expanded under GLRI Action Plan II. Additionally, the novel bioactivities identified in the pilot study were prioritized for adverse outcome pathway development (see section E.4.1 below).

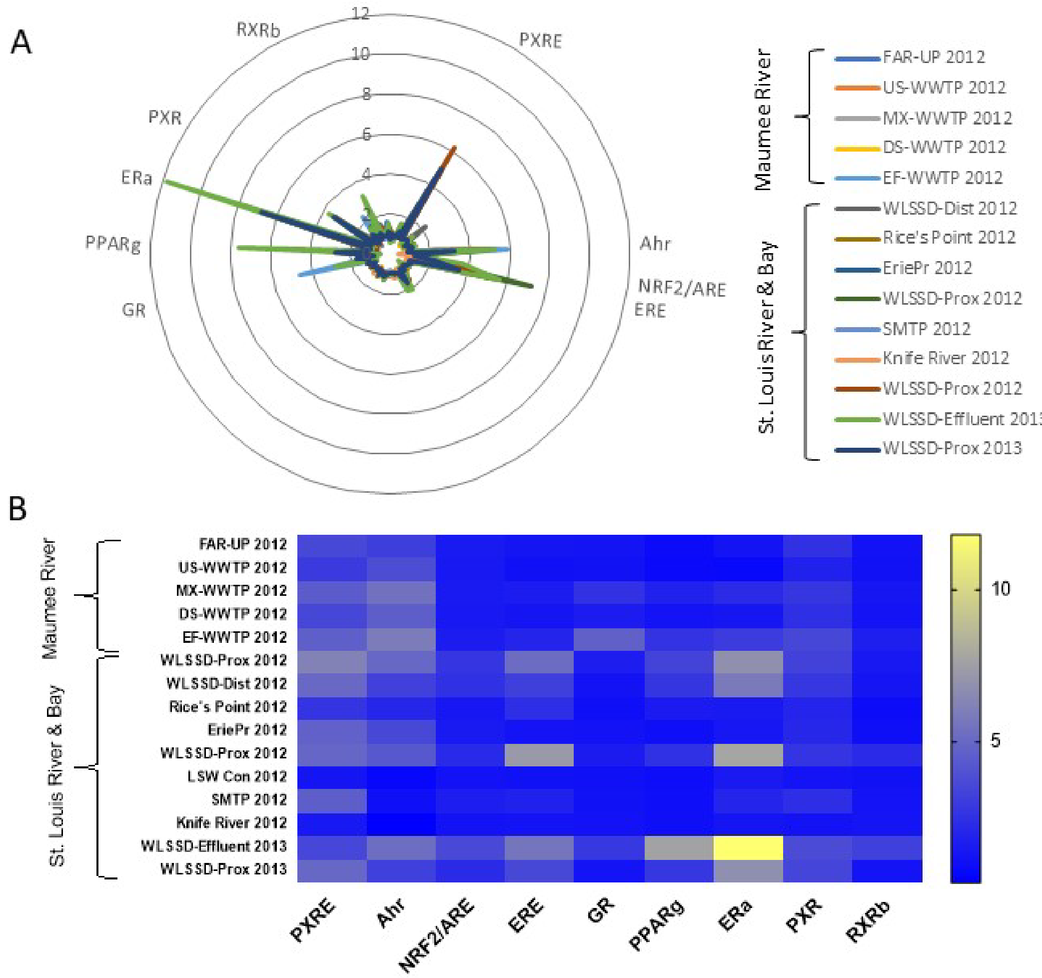

Figure E.5. Bioactivities measured in extracts of surface water samples from the Maumee River and St. Louis River Areas of Concern in 2012 and 2013 using Attagene Facotrial assays, and example of an in vitro bio-effect surveillance approach. A) Radar plot showing bioactivities of all 71 endpoints. For clarity, endpoints labels are only shown for activities that were 3-fold above the extraction blank for one or more samples. B) Heat map summarizing the nine bioactivities with at least one response 3-fold above the extraction blank. Color scaling indicates fold-change above the assay extraction blank.

- **In vitro metabolomics revealed site-specific biological responses and general agreement with contaminant data.** Under GLRI Action Plan I, zebrafish liver cells (ZFL) were exposed to composite water samples collected at sites within the St. Louis River AOC. Impacts on biochemical pathways were determined using a high-throughput metabolomics approach that incorporates both high-field nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry for determining metabolite changes in response to environmental exposures (Figure E.6). Sites with high levels of contamination displayed considerable disruption of glutathione synthesis, as well as biochemicals in related pathways (e.g., glutamate and glutamine), suggesting oxidative stress and increased use of detoxification pathways. Notably, this disruption was also observed at one site that displayed relatively low numbers and levels of targeted contaminants, highlighting the utility of biological monitoring and recommending further investigation of this site for the culprit untargeted contaminants. Metabolites previously identified as indicative of estrogenicity did not appear to be impacted. As a result of the success of this study, the in vitro metabolomics component was expanded under GLRI Action Plan II.

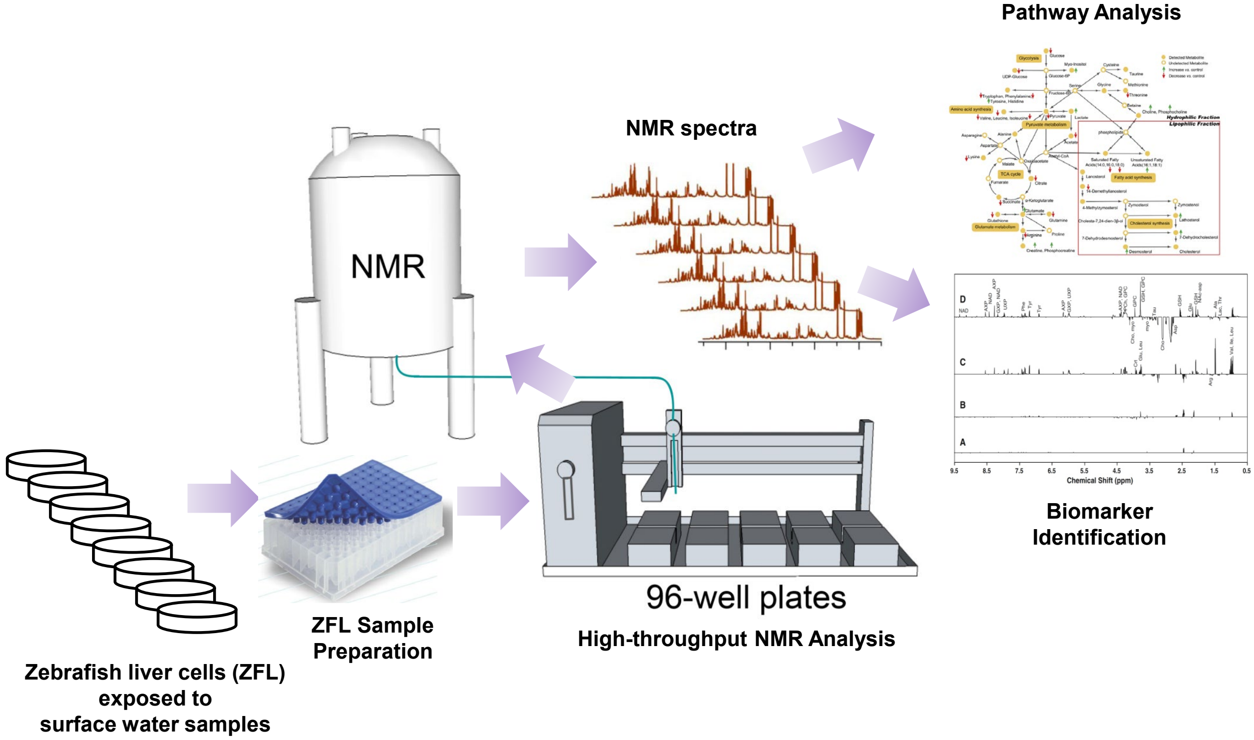

Figure E.6. High-throughput in vitro-based metabolomics approach for assessing biochemical impacts of exposures to Great Lakes surface waters. Only nuclear magnetic resonance (NMR)-based analysis is shown for simplicity.

- **There was little evidence of significant disruption of reproductive endocrine physiology in fish caged at 31 Great Lakes tributary locations, 2010-2013.** Fathead minnows were caged at 31 locations distributed among 5 Great Lakes areas of concern, 2010-2013 (Figure E.2). There were some instances where biological responses in the caged fish differed from those of fish housed in the laboratory over for the same duration. However, targeted endpoints indicative of endocrine disruption generally did not vary consistently or systematically as a function of time or exposure (i.e., proximity to known sources). This suggested either transient effects or random false detection rather than strong evidence for exposure to endocrine active chemicals at concentrations that would impact reproductive function. Induction of VTG gene expression was observed in some males, particularly those caged near wastewater discharges, but there was little evidence for widespread VTG induction throughout the system and rarely any evidence for induction of significant circulating VTG protein concentrations in plasma. The most notable finding from the targeted monitoring with caged fish was a significant increase in circulating 17β-estradiol concentrations in male fish exposed near certain waste water discharges. This observation led to the hypothesis that exogenous estrone associated with waste water discharges was being converted to endogenous 17β-estradiol by the fish (see section E.5.3 below; Ankley et al. 2017). Estrone is a steroidal estrogen, naturally produced and excreted by vertebrates, including humans, that is commonly detected in domestic waste water. Estrone is typically estimated to be between 10 and 100 times less potent than 17β-estradiol in terms of its ability to activate the estrogen receptor. However, estrone can be converted to 17β-estradiol through enzymatically catalyzed reactions that are part of normal steroid biosynthesis pathways.

- **Skin mucus-based metabolomics was an effective, non-destructive, method for profiling and comparing biological responses among sites.** Methods for extracting and analyzing metabolites found in fish skin mucus were successfully developed and employed for surveillance and monitoring. The skin mucus is metabolite rich, with over 300 metabolites detected in skin mucus collected from fathead minnows and clear sex-linked differences in mucus metabolite profiles (Mosley et al. 2018). Additionally, both metabolites associated with endogenous physiological processes as well as biotransformation products of exogenous CECs can be detected in mucus. Thus, the method is both suitable for inferring biological effects of exposures and collecting evidence related to bioavailability and biotransformation of certain contaminants. This non-destructive sampling and analysis technique, developed in support of Action Plan I, is now being used to investigate potential drivers of low reproduction and recruitment in lake sturgeon (*Acipenser fulvescens*) located at the St. Louis River AOC.

E.3 Associating Chemicals with Specific Biological Activities

While detecting biological activity can be a highly useful and informative component of environmental monitoring in the Great Lakes, ultimately to inform management actions, it is important to be able to associate biological activities of concern with the chemical contaminant(s) driving those responses and their sources. As part of Action Plan I, we developed a variety of approaches to help evaluate and establish potential links between chemicals detected in Great Lakes tributary samples and biological effects that were either observed, or may be expected. This included development of novel collection systems, employing statistical approaches, and leveraging the steadily increasing access to chemical-biological association data that are publicly accessible on-line.

E.3.1 Sample Collection Approaches that Minimize Variables that Can Confound the Association of Chemical Stressors and Biological Effects

To associate measured chemical concentrations with biological effects, it is important that the samples collected for chemical analyses are representative of the water to which either cells (in the case of in vitro assays) or fish were exposed. For in vitro assays, this was achieved by simply collecting a volume of water, then splitting the sample for chemical analysis or bioassay. Slight differences in extraction efficiencies for different compounds may confound the association to some degree, but these differences are generally minimized by using similar extraction methods for both chemical and biological analyses.

Obtaining water samples that align well with the in situ monitoring approach (i.e., caged fish deployments) is more challenging. In this case, fish are typically exposed continuously to site water over a period of days (e.g., 96 h in most of our studies). Grab samples just capture an instantaneous snapshot in time and may not be representative of a multi-day period of exposure that can involve diurnal fluctuations, weather events (e.g., heavy rainfalls, changes in wind direction and speed), or changes in system hydrodynamics. Passive sampling devices like Polar Organic Chemical Integrative Samplers (POCIS), semi-permeable membrane devices (SPMD), or polyethylene sheets (PEDs) can obtain a time-integrated sample from a site. However, these methods are selective for either hydrophilic or hydrophobic chemical fractions and operate on chemical partitioning principles (Booji et al. 2016). Consequently, back-calculation of an equivalent water volume sampled and associated water concentration can be technically difficult, particularly if one is interested in a broad range of chemicals with differing physico-chemical properties. An alternative, developed over the course of our research under Action Plan I, was to collect time-integrated composite water samples. To achieve this, an inexpensive (<$250 per unit) portable auto-sampling device was designed and constructed (Kahl et al. 2014). These submersible devices can be deployed in direct proximity to caged fish, can be programmed to pump a standardized volume of water at regular time intervals (e.g., every 15 min over 4 d) into a water tight cubitainer, teflon bag, or other type of collection vessel (Figure E.3C). While still subject to some confounding issues like potential degradation of certain chemicals in the collection vessel, the devices provide a sample of known volume that is closely representative of time-averaged concentrations over the duration of in situ fish exposure. The composite volume collected can then be split for both chemical and biological analyses, minimizing confounding variables associated with comparing in vivo responses in caged fish to either measured chemical concentrations or in vitro assay results.

E.3.2 Multi-Variate Statistical Approaches for Associating Chemicals with Biological Effects

With potentially confounding sample collection variables minimized, multivariate statistical methods can be employed to assess co-variation between the concentrations of CECs detected via analytical chemistry and the magnitude or intensity of a given biological response. For example, Davis et al. (2016) used partial least squares regression to analyze the association between chemical concentrations detected at 18 sites across the Great Lakes basin and alterations in endogenous metabolite profiles (i.e., metabolomics) from fish exposed in situ at those sites. Based on our analysis, we eliminated over 50% of the chemicals measured as likely contributors to the biological responses observed and ranked and prioritized those chemicals that showed the strongest co-variance with the biological response. Similar statistical approaches were employed with transcriptomics data from the same caged fish (see Appendix F).

One of the primary limitations to the multi-variate statistical approaches is that they generally require large numbers of samples. For many smaller scale surveillance or monitoring efforts, total sample numbers may not be adequate to support robust statistical associations. This is particularly true when many of the chemical concentrations and/or biological responses of interest are at or near detection limits or background levels. When the bulk of data points are skewed toward one small part of the overall distribution, it can be difficult to elucidate statistically robust associations without impractically-large sample numbers.

E.3.3 Evidence-Based Approaches for Associating Chemicals with Biological Effects

An alternative to multivariate statistical approaches is to look for existing evidence for an association between a given chemical and a specific biological effect. For a small list of chemicals and/or biological effects of interest this can reasonably be achieved through a systematic literature review. However, for lists of tens to hundreds of chemicals, and similar numbers of potential pathway-based biological effects of concern, systematic review of open literature sources can quickly become too onerous and time-consuming for practical use. Under the latter scenario, querying associations that are already compiled in curated, machine-readable, databases can provide a more tractable approach. Consequently, as part of GLRI Action Plan I, we piloted the use of two novel evidence-based approaches for associating specific chemicals, identified through analytical monitoring, with pathway-based biological responses (Schroeder et al. 2016).

E.3.3.1 Chemical-Pathway Interaction Networks

There are a growing number of publicly accessible on-line databases that compile and curate information on the association between chemicals and biological responses (Schroeder et al. 2016). These can serve as one line of evidence for linking specific chemicals measured in a sample with observed biological outcomes. Schroeder et al. (2017) demonstrated how a list of chemicals detected at a given site could be used to query a chemical-pathway association database like the Comparative Toxicogenomics Database (ctdbase.org) to derive a network of previously reported chemical-biological interactions. Associations within the network can be weighted by factors like the number of detected chemicals associated with a given biological target/effect or the median centered concentration of chemical. Then, using a statistical approach termed reverse causal reasoning (Catlett et al. 2013), the extent to which observed responses are consistent with results that might be inferred or expected based on evidence captured in the network model can be evaluated. A strength of this approach is that given the diversity of data sources these on-line databases draw from, chemical coverage can be quite extensive.

However, while the approach is much more rapid than systematic literature review and is amenable to automation, it has limitations due to the type of information that is collected and aggregated in the source databases (Schroeder et al. 2017). For example, chemical-gene/ chemical-protein/ or chemical-pathway interactions curated from the literature often originate from widely varying study designs that may employ different species, doses and durations of exposure, routes of exposure, analytical methods with differing sensitivities, etc. Thus, the chemical-biological associations captured in the database may not be applicable for an exposure scenario relevant to environmental monitoring (e.g., for an aquatic organism, exposed to an environmentally-relevant concentration via a relevant route of exposure). Likewise, some of these databases curate chemical-biological interactions from “omics” studies that may include relatively high numbers of false positives compared those derived from more targeted studies, again raising questions as to the suitability of the information. Finally, for any literature-based approach, selection bias is also an issue. Only those interactions that were studied can be identified. Therefore, highly studied chemicals tend to have large numbers of identified chemical-biological interactions, while less studied compounds may only have one or two identified, even though they may, in fact, interact with additional targets. Thus, the chemical-pathway interaction network approach has several limitations that need to be considered and accounted for when using this approach.

E.3.3.2 Exposure:Activity Ratios (EARs)

A second evidence-based strategy for associating chemicals with biological activity that we developed as part of Action Plan I takes advantage of a more standardized source of chemical-biological interaction data. This approach relies on data generated through U.S. EPA’s ToxCast program (Kavlock et al. 2012), which has screened several thousand chemicals using a battery of high throughput in vitro assays. A major advantage of the ToxCast data set is each assay is conducted in a consistent and standardized manner. Likewise, the resulting data are processed using an automated data analysis pipeline (Filer et al. 2017). Together, this allows for a high degree of comparability across chemicals. Consequently, even if in vitro effect concentrations cannot be readily translated into equivalent in vivo concentrations, relative potency in a given assay can be meaningfully compared among chemicals. This facilitates a semi-quantitative approach for inferring potential associations between chemicals and biological effect(s) that can account for both the variable potency with which different chemicals may act on a biological target/pathway and the varying concentrations of individual chemicals in a sample. For our pilot work, we calculated an Exposure:Activity Ratio (EAR; the concentration of chemical$_i$ detected in a sample divided by the effect concentration of that chemical in assay$_{j;}$ Blackwell et al. 2017). However, the inverse, a bioactivity-to-exposure ratio (BER; effect concentration of a chemical$_i$ in assay$_j$ divided by concentration of chemical$_i$ detected in a sample) can also be used (Wetmore et al. 2015). Either approach can be used to estimate the relative likelihood that a given chemical is responsible for an observed biological response; at least based on simplified assumptions that relative potency in vivo is approximately proportional to relative potency measured in vitro. Certainly, there are cases where that assumption does not hold, for example for chemicals tested in vitro that are rapidly metabolized in vivo. Nonetheless, as a line of evidence to support a potential chemical-biological effect association the approach generally provides greater certainty than the interaction-network approach described above.

Another important advantage of the EAR-based approach is that it can be readily applied to mixture assessments. The EARs calculated for each chemical acting on a specific assay target can be summed to provide a relative estimate of the probable impact of the mixture of chemicals on that target. Thus, in cases where chemical monitoring/analysis is coupled with direct testing of a sample-derived mixture in one or more of the targeted or multi-factorial assays employed by the ToxCast program, it is possible to estimate whether the known chemical composition of a sample (based on analytically detected compounds) can reasonably account for the level of bioactivity observed in the assay, or whether undetected constituents are likely contributing to the activity observed (Blackwell et al. 2018). Likewise, if the observed biological activity measured in a ToxCast assay matches that expected based on a summed EAR, it can reasonably be inferred that other chemicals detected, that were not active individually in a particular assay, are unlikely to be driving the biological response observed. This approach is very analogous to the comparison of expected (based on chemical analyses) versus observed (based on bioassay) estrogenic activity described in section E.2.3 While there are limitations, the EAR approach using ToxCast data was shown to have considerable utility for evidence-based association of chemicals with biological effects.

Given the promising nature of the EAR method, one aim of the pilot research under Action Plan I was to develop computational tools to facilitate the EAR analysis. In concept, the calculations are very straight-forward, involving simple division and summation (in the case of a summed EAR for a mixture). However, if one applies that simple task to a matrix of tens to hundreds of detected chemicals and several hundred ToxCast assay endpoints, the number of calculations quickly multiplies to thousands or even tens of thousands of individual ratios. Consequently, a series of R-based scripts and an associated graphical interface was developed to streamline the computational analysis and visualize results. Initial prototypes of these R-based tools developed under Action Plan I have allowed the basic EAR approach to be scaled to the point that technical challenges associated with implementation could be identified and addressed. Challenges included, how to deal with non-detected chemicals in the dataset, how to best utilize ToxCast data quality flags when calculating EARs, which of several effect concentration estimates from the ToxCast pipeline to use, and others, laying the scientific and technical groundwork for both use under Action Plan II, and transfer of the approach to other stakeholders faced with interpretation of chemical monitoring data.

E.3.3.3 Prospective Application of Evidence-Based Approaches

While the evidence-based approaches described above were developed to aid the association of specific chemicals with observed biological effects, they can also be used in a prospective manner (Schroeder et al. 2017). In this case, the goal is to infer or predict potential biological effects based on a list of measured chemicals and prior knowledge/evidence of chemical-biological interactions. This can be a useful method for inferring possible biological effects of a sample in cases where no biological measurements are available. Because the prospective application represents an inference-based approach that is subject to error, it is ideal to follow-up with targeted, effects-based monitoring. Nonetheless, the evidence-based methods can serve an important role in providing testable hypotheses that inform the design of subsequent monitoring studies.

Several examples of prospective application of the evidence-based approaches for inferring possible biological effects from a list of detected chemicals were developed as part of the research either conducted under Action Plan I or ultimately resulting from that research. For example, Cavallin et al. (2016) used a chemical-gene interaction network derived from a list of CECs detected in a waste-water effluent discharged to the St. Louis River AOC to develop hypotheses regarding a set of genes whose expression in liver might be influenced by chemicals detected in the sample. The resulting hypotheses were tested and significant concentration-dependent effects detected for four of the seven genes investigated. These genes were involved in key biological pathways related to endocrine function and xenobiotic metabolism. Li et al. (2016) used a combination of the EAR and chemical-gene interaction network approaches to prioritize several biological targets and associated pathways that may warrant follow-up investigation within selected areas of the Lower Green Bay/Fox River and Milwaukee Estuary AOCs. Additionally, chemical monitoring results from Action Plan I, along with prospective application of the EAR and chemical-biological interaction network approaches continue to be applied in selecting endpoints for targeted effects-based monitoring under Action Plan II.

E.3.4. Key Findings/Progress – Associating Detected Chemicals with Biological Effects

- **Partial least squares regression was a useful approach for prioritizing chemicals for further investigation.** However, because the approach is based on empirical co-variance rather than mechanistic dependence, the statistical approach does not provide definitive evidence of a causal relationship between exposure to a given chemical and biological effect. Conversely, a lack of co-variance is viewed as evidence that a contaminant is of lesser biological relevance and thus less worthy of additional resources. While statistical confidence is improved with greater numbers of samples on which to determine co-variance, the approach has been successfully used, as part of an overall weight of evidence, with as few as seven samples for which a matrix of chemical concentrations and biological response data are available (Ekman et al. 2018).

- **Chemical pathway-interaction networks provide a qualitative, evidence-based, means to infer potential chemical-biological associations.** The method has prospective utility for hypothesis generation and prioritization, particularly in cases where biological effects information from other data sources is limited and qualitative inference with substantial recognized uncertainty is appropriate. When applying, however, it is critical to recognize that using associations curated from non-standardized experimental designs and/or analysis methods introduces large amounts of uncertainty for sample- and site-specific applications. Given limitations in the quality and consistency of the publicly available association data, the predictive sophistication is low. Therefore the method is most appropriately applied retrospectively, as one line of evidence to complement other approaches. It should not be viewed as definitive evidence that a given chemical is causing an observed effect.

- **The EAR approach, which is analogous to traditional hazard quotient-based approaches, is an intuitive, easy to implement, and quantitatively more sophisticated approach than the chemical-pathway interaction networks.** Given its potential for both retrospective applications in identifying chemical-biological associations and prospective applications for prioritizing chemicals, sites, and/or biological effects for further investigation/monitoring, the EAR approach was targeted for further development and application under GLRI action plan II.

- **Complementary use of multiple approaches is ideal.** Since none of the chemical-biological association methods are definitive, they are best applied as part of a weight-of-evidence based approach. In general, both the statistical and evidence-based approaches described should be employed along with other complementary data or follow-up hypothesis testing to establish greater confidence in the putative chemical-biological associations.

E.4. Linking Biological Activities to Adverse Apical Effects in Wildlife

The approaches described above focus largely on the ability to either measure or infer potential pathway-based biological effects that may be associated with exposure to CECs present in Great Lakes samples. However, even if a specific chemical, sample, or site can be linked to a pathway-based biological response, it can be difficult for scientists, resource managers, and other stakeholders to understand the significance and implications of those biological activities. Resource managers are concerned with sustainable populations and ecosystem function, not with enzyme inhibition, transcription factor activation, gene expression, hormone concentrations, etc. Just as one may have to consult a medical professional to understand the meaning of a diagnostic test at a health care facility, scientists, resource managers, and other stakeholders need access to specialized knowledge and translational tools to understand the implications of pathway-based data for ecosystem health. Consequently, several additional efforts undertaken as part of Action Plan I were aimed at developing some of the needed translational tools and frameworks.

E.4.1. The Adverse Outcome Pathway (AOP) Framework and Effects-Based Monitoring

Adverse outcome pathways are a conceptual framework designed to organize knowledge and evidence concerning the linkage between specific perturbations of a biological system at the biochemical, molecular, or cellular level and corresponding adverse outcomes (typically measured at the individual level) that can occur as a result (Ankley et al. 2010). While the basic concept of understanding chemical modes of action to inform chemical safety assessment has a long history, the AOP framework provides a systematic, transparent, and internationally harmonized approach to address this challenge (Villeneuve et al. 2014; http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm). The framework is viewed as a key translational tool that can enhance the interpretation and utility of pathway-based data for a wide range of chemical assessment applications, including environmental surveillance and monitoring. Consequently, both development and application of the AOP framework was included in the research conducted under Action Plan I. Major objectives in association with Action Plan I were to (1) aid development of a searchable and accessible knowledge base of AOP information that could be used to understand potential hazards associated with pathway-based biological activities elicited by mixtures of contaminants from the Great Lakes; (2) use existing AOPs to identify targeted endpoints for use in effects-based monitoring; and (3) use biological effects surveillance on Great Lakes samples to help prioritize novel AOP development that may be needed to better understand the potential impacts of CECs on Great Lakes fish and wildlife.

E.4.2. Coupling Population Models with Pathway-Based Data

While AOPs are intended to support extrapolation of molecular, biochemical, and/or cellular responses to potential effects on individual survival, growth, or reproduction, resource managers are often most concerned with how impacts on the individual may ultimately influence population sustainability and associated ecosystem services. Population-level impacts cannot be readily tested, particularly in prospective assessments. Consequently, models that can take data collected at lower levels of biological organization (e.g., at the individual, or sub-individual level) and simulate the potential population level consequences of a stressor, can be important tools. They can be used prospectively to understand the potential significance of observed biological effects or to infer benchmark doses for stressors that affect a given pathway (e.g., Conolly et al. 2017). Likewise, they can be used retrospectively to determine how much conditions must improve, based on a pathway-based biological indicator, before populations might be expected to recover.

As part of GLRI Action Plan I, species-specific, density-dependent population projection models were developed and case studies conducted to demonstrate how these models could inform decision-making related to potential effects of complex mixtures in fish. Miller et al. (2013) used a model parameterized for white sucker (*Catostomus commersoni*) to estimate the level of impact associated with exposure to a pulp and paper mill effluent on an existing population of the species in Jackfish Bay, Lake Superior. The model, was then applied, in conjunction with relevant AOPs, to project on-going impacts and subsequent recovery following stressor mitigation (Miller et al. 2015).

E.4.3. Extrapolating Pathway-Based Data across Species

The effects-based surveillance and monitoring approaches developed and demonstrated under GLRI Action Plan I (see section E.2) rely on mammalian cell lines or model aquatic organisms that are amenable to caging in Great Lakes tributaries. Likewise, much of the data upon which chemical-biological associations are derived (see section E.3) are based on a small number of model organisms. However, Great Lakes resource managers and stakeholders are concerned with effects on a broad diversity of resident organisms, not necessarily the well-studied laboratory model species that dominate the toxicological literature and testing. Consequently, to appropriately extrapolate and apply pathway-based data across species, it is important to understand which species may be susceptible, or potentially insensitive, to single CECs or mixtures containing CECs.

As part of GLRI Action Plan I, a strategy for leveraging the growing database of protein sequence data for a wide range of species, available through the National Center for Biotechnology Information (NCBI), was used to infer potential susceptibility to specific chemical-biological interactions was developed (LaLone et al. 2013). That strategy was incorporated into a general framework for estimating the relative hazards of human and veterinary pharmaceuticals, many of which are being detected in Great Lakes surface waters (see Appendices A, D), to aquatic organisms (LaLone et al. 2014). Subsequently, the strategy has been implemented as a publicly accessible web-based tool (LaLone et al. 2016) which can be used to evaluate the degree of similarity of nearly any protein target across thousands of species represented in the NCBI database. The tool facilitates analysis at three levels of resolution, ranging from amino acid sequence similarity across the entire length of a protein (level 1), to similarity within specific functional domains, which tend to be evolutionarily more conserved across species (level 2), to conservation of individual amino acid residues that may be important for interactions with specific classes of chemicals or may be known to confer either sensitivity or resistance to chemical perturbation (level 3; LaLone et al. 2016; Doering et al. 2018). These analyses provide both an important line of evidence for understanding the appropriate scope and limitations associated with extrapolation of pathway-based data. They also help define taxonomic domains of applicability for AOPs that involve perturbation or modulation of specific protein targets.

E.4.4 Key Findings/Progress – Development of Translational Tools and Frameworks for Linking Pathway-Based Measurements of Adverse Effects

- **The AOP-wiki (aopwiki.org) was publicly released in September 2014.** The AOP-wiki provides searchable, public access to AOP information that is being developed across the international scientific community. Several AOPs germane to GLRI Action Plan I were used to inform the structure and content of information captured in the AOP-wiki and associated content pages. Under Action Plan II, information assembled in the AOP-wiki will be used to help infer potential ecological hazards and/or endpoint selection for targeted monitoring of complex mixtures of CECs present in Great Lakes samples.

- **AOPs informed selection of in vivo endpoints for in situ effects-based monitoring under Action Plan I.** With selection of estrogen receptor and androgen receptor-mediated pathways as priorities for targeted monitoring under Action Plan I, AOPs related to those pathways (e.g., https://aopwiki.org/aops/23; https://aopwiki.org/aops/30; https://aopwiki.org/aops/29) were used to select the endpoints that were analyzed in fish that were caged in Great Lakes tributaries, as well as in follow-up studies. The general lack of significant effect on key events aligned with these AOPs agreed well with the low levels of total estrogenic and androgenic activity detected in Great Lakes tributary samples we studied (see section E.2.3).

- **PPARg and GR-related pathways were prioritized for AOP development.** Among the most prominent biological activities identified via bioactivity surveillance using the multi-factorial Attagene assays (section E.2.3), ecologically-relevant AOPs were already available for ER and Aryl hydrocarbon receptor (AhR) activation. Pregnane X receptor (PXR) and nuclear factor (erythroid-derived 2)-like 2 Nrf2-related activities primarily link to induction of hepatic metabolism and oxidative stress response and were viewed as too general to support AOP development. Of the remaining activities, glucocorticoid (GR)- and peroxisome proliferator activated receptor gamma (PPARg)-related were among the next most frequently detected biological activities for which plausible links to adverse outcomes. Putative AOPs based on these plausible links are being exploited as a basis for hypothesis-driven testing and evaluation under Action Plan II.

- **A density dependent population model, coupled to an AOP, was used to infer potential effects on population growth status using readily collected pathway-based biomonitoring data.** An association between measured plasma testosterone concentrations and white sucker fecundity was developed. That relationship was used to forecast the degree of recovery in measured testosterone titers, compared to a reference location, that would be associated with different levels of anticipated population recovery, expressed as a percentage of carrying capacity (Miller et al. 2015).

- **Development of a publicly accessible web-based tool for rapidly evaluating the occurrence and similarity of orthologous proteins across taxonomic diversity was developed.** The web-based tool titled Sequence Alignment to Predict Across Species Sensitivity (SeqAPASS; seqapass.epa.gov) was released in January, 2016. While initially developed under GLRI Action Plan I, multiple EPA programs including the Office of Water and Office of Pesticide Programs as well as regional, state, and academic stakeholders have interest in utilizing SeqAPASS, leading to impact far beyond the initial intended application under the GLRI.

E.5. Application Case Studies

Much of our work under GLRI Action Plan I was explicitly focused on development and evaluation of methods, tools, and conceptual frameworks for evaluating biological effects of CECs in the Great Lakes. In the process of developing and testing these approaches, case studies that demonstrate how these approaches can be integrated to answer specific questions and prioritize subsequent monitoring and/or management activities were conducted. Several examples that demonstrate different applications are provided below.

E.5.1. Integrated Application of Pathway-Based Approaches for Source Attribution

As part of Action Plan I, Davis et al. (2013) conducted a case study in which pathway-based biological effects monitoring approaches were used to evaluate potential sources of estrogenic contaminants in the St. Louis River AOC. T47D-kbluc assays conducted on samples collected around the St. Louis River estuary clearly identified the Western Lake Superior Sanitary District (WLSSD) effluent discharge as a primary source of estrogenic activity. However, approximately 40% of the influent to WLSSD originates from a single pulp and paper mill, while the remainder is from domestic and light industrial/commercial sources. Effects-based monitoring approaches were deployed before, during, and after a scheduled shut-down of the pulp and paper mill operation to evaluate the contribution of that source to the overall estrogenic activity of the WLSSD effluent. Characterization of total estrogenic activity in the receiving water demonstrated a 45-50% reduction in total estrogenic activity during the shut-down period (e.g., 1.7±0.3 ng E2-EQ/L pre-shut down to 0.8 ± 0.4 ng E2-EQ/L during the shut-down). Similarly, metabolite profiles in male fathead minnows caged at locations within 10 and 200 m of the discharge were statistically distinct from laboratory controls during the pre-shutdown period, indistinct from lab controls during the shutdown, and diverged again when the plant came back on-line. Results indicated the pulp and paper source contributed a portion, but not all, of the estrogenic activity detected in the receiving water. Overall, the case study demonstrated the utility of both in vitro and in vivo effects-based monitoring approaches for both source attribution as well as the potential of these approaches for monitoring recovery following remediation or management actions.

E.5.2. A Site Assessment Workflow Moving from Pathway-Based Monitoring to AOP-Informed Hypothesis Testing and Hazard Verification

While source attribution is important to inform appropriate management actions to mitigate the potential biological impacts of CECs, it is also important to elucidate whether pathway-based biological changes are likely to lead to adverse effects in exposed organisms. Where available, AOPs can provide qualitative understanding of the potential hazards that a specific biological perturbation may elicit. However, quantitative understanding of most AOPs is not necessarily sufficient to determine definitively whether adverse outcome(s) are likely under a given set of exposure conditions. In this case, follow-up investigation may be needed to determine whether management actions are necessary. A case study conducted by Cavallin et al. (2016) demonstrated how the AOP framework could guide design of a hypothesis-driven approach for evaluating whether expected adverse outcomes are occurring in response to exposure to a complex mixture. Briefly, fathead minnows were exposed for 21 d to treated wastewater effluent as well as dilutions designed to reflect approximate concentrations of effluent in the receiving water at different distances from the point of discharge. Molecular, biochemical, and apical (i.e., reproductive performance) endpoints aligned with key events along an AOP were evaluated. While adverse effects on reproduction were observed, those effects were significant only for the undiluted effluent. Dilutions similar to those expected in the receiving water showed no effect. Further, comparison of the weekly reproductive output with fluctuating concentrations of CECs and estrogenic activity indicated that while exposure to estrogenic compounds was occurring, they were probably not the sole cause of impaired reproduction. Overall the case study demonstrated how various lines of chemical and biological monitoring data can be integrated, in the context of the AOP framework, to guide evaluation of in vivo hazards and develop evidence supporting or rejecting specific CECs as causative agents.

E.5.3. Effects-Based Monitoring Leading to Reassessment of the Significance of a Common Environmental Contaminant

In a third case study, novel observations from in vivo effects-based monitoring led to new insights into the potential environmental significance of a common CEC. Specifically, in a series of caged fish monitoring studies conducted in 2010-2013 elevated concentrations of 17β-estradiol were observed in male fish caged near waste water discharges. Initially, these measurements were regarded as interesting, but perhaps just anomalous. However, after observing the same pattern across different years as well as different sites, it became more evident that the effect was real. Ultimately, this led to a hypothesis that exogenous estrone, an estrogenic hormone commonly found in wastewater effluents but known to be considerably less potent than other natural and synthetic estrogens like 17β-estradiol and ethynyl estradiol, was being converted to the more potent 17β-estradiol. Ankley et al. (2017), report on a series of laboratory studies that demonstrated that this conversion was indeed occurring and could account for the elevated 17β-estradiol concentrations observed in fish caged at field sites. These data yielded the significant finding that in vitro bioassays may underestimate the in vivo estrogenic activity associated with environmental exposures to estrone. Additionally, chemical monitoring studies may want to assume a relative potency equivalent to that of 17β-estradiol when calculating total E2-EQ. Overall, these results help to inform future monitoring focused on potential effects of estrogenic CECs.

E.5.4. Pathway-Based Surveillance to Prioritize Future Monitoring

In another case study conducted in the lower Green Bay/ Fox River and Milwaukee Estuary AOCs (Li et al. 2016), only minor effects on targeted endpoints were observed. While the effects could be used to prioritize specific locations in the AOCs for further monitoring, they were not severe enough to elicit alarm. To extend the scope beyond the targeted analyses, evidence-based approaches for associating detected chemicals with biological activities (see section E.3) were applied in a prospective manner. The analyses identified napthalene and diethylpthalate and their potential interactions with targets involved in neuronal signaling (cholinergic muscarinic receptor 3 and monoamine oxidase B) as potential priorities for future monitoring at selected sites within the AOCs. Overall, the study highlights how chemical monitoring data can be coupled with existing sources of pathway-based chemical-biological interaction knowledge to optimize deployment of subsequent monitoring resources on the chemicals, biological activities, species, and sites most likely to be of concern.

E.6. Management Implications and Future Directions

Our efforts under GLRI Action Plan I were primarily focused on development, demonstration and evaluation of tools and approaches for applying pathway-based data for environmental surveillance and monitoring. The end goal was to provide practical tools that resource managers can deploy to better understand the ecological significance of CECs in the Great Lakes and to help identify where management action may be needed. Within the field of chemical safety assessment, the use of new approach methods and pathway-based data is an exploding area of research and development, but scientists and risk assessors are still learning the best ways to put these methods into practice as well as their practical limitations. In this respect, it is understood that many of the approaches piloted under GLRI Action Plan I will take some time to mature to the point of routine use. Nonetheless, work conducted in 2010-2014 demonstrates the diversity of potential applications and the value added. The following are products and strategies resulting from this research that are suitable for immediate use by resource managers.

- **Practical and cost-effective caging systems and composite sampling devices for in situ effects-based monitoring.** The caging systems and composite sampling devices developed under Action Plan I proved to be both flexible and easy to deploy. They were successfully used at a diversity of Great Lakes sites, with limited sample loss or system failure. Additionally, through extensive comparisons among a range of controls (e.g., culture controls, shipping controls, time-zero controls, fish holding controls), it was found that transport and handling had minimal influence on pathway-based endpoints following a 4-day field exposure and that a laboratory held reference was generally adequate to support informative comparisons among different field locations (unpublished data). Consequently, a practical infrastructure for obtaining samples required for effects-based analyses with fish and cell-based assays, and for aligning those analyses with representative water samples is available for use by Great Lakes resource managers.

- **The major drivers of estrogenic activity measured in select Great Lakes tributary samples collected 2010-2013 are known.** While most concentrations were below those expected to cause adverse biological effects, where exceedances occur, reducing concentrations of natural and synthetic steroidal estrogens as well as alkylphenols and bisphenol A would likely be effective in reducing the overall level of estrogenic contamination in the system. With regard to estrogenic contaminants, both the major sources and activity levels (or concentrations) of concern are well defined compared to those of other classes of CECs. Identification of the potential conversion of estrone to a more potent estrogen, 17β-estradiol, in vivo further enhances our ability to identify environmental estrogens of greatest concern at specific sites. With some additional targeted development, a cell bioassay-based monitoring strategy would likely be tractable for monitoring and management of estrogen-mediated exposure and effects in the Great Lakes basin.

- **An Exposure:Activity Ratio (EAR) approach, conceptually similar to traditional hazard quotients, provides an effective means to integrate chemical-specific concentration information with relative biological effect potencies in pathway-based assays to identify priority chemicals, sites, and/or biological endpoints/effects for further monitoring and investigation.** A computational infrastructure to facilitate this approach was developed and is transferrable to Great Lakes resource managers and other scientists. The method is currently deployable as a screening and prioritization tool. However, EARs are not directly translatable to anticipated probability or severity of in vivo outcomes and should not, presently, be used for risk assessment. Translation of priority pathway-based activities to potential in vivo hazards is dependent on further development of the AOP knowledgebase.

- **Metabolomic analysis of fish skin mucus offers a non-destructive, yet information rich, biomonitoring tool that can be used with either resident or caged fish, potentially including threatened or endangered species.** Pilot testing demonstrated the ability to identify hundreds of metabolites in skin mucus collected from fathead minnows, the ability to discriminate sexes based on mucus metabolite profiles, and the ability to detect biotransformation products of xenobiotic contaminants in this matrix. The mucus sample collection methods are straight-forward and easily implemented with minimal training. Consequently, sample collection is a tractable method even for citizen science. Further, the approach can be employed in a non-destructive manner, meaning that samples can potentially be collected and analyzed from even threatened and endangered species. However, current bottlenecks in the approach include the sophisticated and expensive analytical instrumentation required for the analyses and the complex and time-consuming nature of the data analysis. Development of standardized analysis pipelines and interpretive guides could help foster wider use of the approach in monitoring.

- **Commercially available multi-factorial assays were an effective biological effects surveillance tool.** The Attagene, multi-factorial assays examined as part of Action Plan I were successful in detecting known activities that were confirmed through orthogonal methods. They also identified novel bioactivities that had not been previously examined in Great Lakes surface waters. The ability to screen for activity against approximately two dozen biological targets per assay offsets the increased per sample cost for preliminary surveillance applications. Targeted cell bioassay kits are commercially available for many of the targets probed in the multi-factorial assays, making more sample intensive, targeted follow-up monitoring tractable. At present, the major limitation of the approach is the limited ability to link pathway-perturbations detected in these assays to specific apical hazards in Great Lakes wildlife. Conceptually, this need is being addressed by the development of the AOP framework and associated knowledgebase (aopwiki.org). However, at present, AOPs are only defined for a relative handful of the activities that can be screened. Consequently, broader use of these approaches by Great Lakes resource managers is likely dependent on continued development of the AOP-knowledgebase. Pilot work conducted under Action Plan I helped define priority targets for AOP development based on those biological activities most frequently observed for Great Lakes samples.

- **The SeqAPASS tool, initially developed under GLRI action plan I, has broad application in helping to define the taxonomic relevance of pathway-based data based on conservation, or lack thereof, of protein sequences.** As a web-based, publicly accessible tool, SeqAPASS is immediately transferrable to Great Lakes resource managers. A user guide and training materials are available. However, at present, detailed understanding of exactly how similar protein sequence or structure must be to indicate probable susceptibility or insensitivity compared to a reference species is not yet well defined. Consequently, current applications of SeqAPASS results are qualitative. Additional case studies are needed to better define the confidence with which intrinsic susceptibility, or lack thereof, can be inferred from the SeqAPASS results.

E.6.1. Future Development

At present, nearly all the methods and approaches piloted under Action Plan I require some level of specialized knowledge, instrumentation, or expertise to implement and interpret. Thus, there is a learning curve and logistical barriers, such as access to appropriate instrumentation or development of commercial sources and standardized analysis pipelines that must be overcome. Nonetheless, research conducted under Action Plan I demonstrates that pathway-based approaches can provide significant value for environmental surveillance and monitoring of CECs, especially in cases where more traditional toxicity benchmarks are lacking. Like most scientific tools, these approaches are best employed as part of an integrated weight of evidence approach. However, appropriate application of these tools can help focus monitoring and testing resources on the most probable hazards, most likely causative agents, and the most severely impacted sites. Moving into Action Plan II, the aim is to utilize these approaches in an integrated manner in additional case studies that address specific questions regarding the occurrence and potential effects of CECs at selected sites within the Great Lakes Basin. The case studies are intended to provide immediate, actionable information for Great Lakes stakeholders and managers, while continuing to refine and enhance the utility of pathway-based approaches as routine tools in the resource manager's tool box.

E.7. Disclaimer

E.8. Acknowledgements

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Appendix F

Transcriptional Effects-Based Monitoring of Contaminants of Emerging Concern in Great Lakes Tributaries: Biological Effects and Chemical Specific Impacts

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F.1. Problem Statement and Scope

Increasing numbers of chemicals and other contaminants are detected in waters, sediment and animals in Great Lakes watershed many of which have little to no toxicological or water quality information is available. Given that these contaminants appear as complex mixtures, available information for individual chemicals may under estimate the effects of contaminant exposure in Great Lakes Tributaries. This makes it difficult for managers and other decision makers to identify which contaminants represent threats to the use of their resources, watersheds or waterways. Here we discuss approaches using effects-based monitoring with transcriptomics, AOPs and water quality assessments to identify the presence and deleterious effects of CECs so that they can be appropriately managed. As part of the Great Lakes Restoration Initiative (GLRI) Action Plan I, our goals were to develop methods and tools using untargeted transcriptomics to (1) Detect biological activities associated with mixtures of CECs in Great Lakes Tributaries, (2) Identify what contaminants could be potentially driving adverse effects or impairments associated with exposure to Great Lakes tributaries, and (3) Link transcriptional changes as a result of exposure in Great Lakes tributaries to apical adverse effects and impairments.

F.2. Detecting Biological Activities Associated with Mixtures of CECs in Great Lakes Tributaries using Transcriptomics

F.2.1. Strategy – In Situ Exposure of Model Organisms

In this appendix, we are principally concerned with determining the potential of CECs in Great Lakes tributaries to cause harmful effects in fish living in the water. However, it is difficult to know what chemical or other stressors native fish might have experienced, when they were exposed and where they were exposed. To overcome this challenge, our strategy is to use laboratory raised fathead minnow placed in cages and then deployed into water bodies for 2, 4 or 8 days as a surrogate for native fish (see Appendix E Kahl et al. 2014). Caged fish were used to examine the effects of CECs at 14 different sites and three different tributaries (Figure F.1). Fathead minnows are easily obtained small fish commonly used in ecotoxicology that are widely distributed in freshwater lakes and streams across much of North America (Ankley and Villeneuve 2006). Using laboratory reared fish in cages has several advantages including control of the exposure period and location, simplicity, and low cost of deployment that can be used by managers. As discussed in Appendix E, this strategy can also be used with other fish species.

F.2.2. Strategy – Laboratory Exposure of Model Organisms

The impact of many CECs on gene expression model organisms such as Fathead minnow are often limited or unknown. We used the strategy of laboratory exposures of fish to understand the potential contribution of individual CECs to the overall effects of mixtures of CECs on fish. Laboratory exposures provide a high level of control over the experimental conditions including known length of exposure, well characterized chemistry, and minimization of other stressors that might confound results.

F.2.3. Endpoints and Analyses

The biological endpoints that we use are changes in gene expression measured using transcriptomics. Our goal in using untargeted transcriptomics, or global gene expression analysis, was to capture as many changes or adverse effects as possible in exposed fish rather than focus on one or a few specific changes such as a change in vitellogenin protein. Transcriptomics is the simultaneous measurement of messenger RNAs from large numbers of different genes in a cell and is used to examine gene expression changes (step 4 below). Since we are using tools to capture changes in a very broad range of genes rather than a small number of specific genes, it is considered an untargeted approach and can capture many different biological effects. To detect effects of chemical exposure we looked at gene expression two different organs after fish are exposed: Ovaries of females were tested because ovaries are reproductive organs and the site of estrogen synthesis. Livers of males were tested as metabolism and toxicity of CECs such as PAHS or pharmaceuticals can often be seen in livers. Male livers are also interesting to look at since estrogenic effects are more easily observed in male livers.

Box F.1. The way chemicals can cause changes in gene expression that lead to toxicity or adverse effects can be summed up as:

1. A chemical enters biological system.
2. A chemical interacts with a receptor – typically some type of protein.
3. This receptor becomes activated or inactivated by chemical interaction activating or deactivating signals within cells.
4. Activation or deactivation of signals leads to changes in gene expression, which are changes in the amounts of specific messenger RNAs within the cells.
5. Some of these changes in RNAs are translated into changes in the amount and type of proteins that are generated within the cell
6. Changes in the amount and type of proteins results in new signals that cause changes to cells
7. These changes can include causing some cells to divide and produce more cells (tumors develop when cells divide out of control, making too many cells), activate or inactivate the immune system, cause cells to die (which can also activate the immune system), cause cells to change how they use energy which changes the amount of energy molecules available for the organism to use, or impair reproduction.

Since expressed genes do not always directly translate to changes in proteins or, ultimately, adverse effects, we use the expression of a collection of genes belonging to different biological pathways (e.g., a sequence of enzymatic steps resulting in production of a chemical or physical change) to indicate potential activities and functions that have changed, or been impaired, in exposed fish. To do this, we examine how many genes involved in different biological functions or pathways changed relative to controls (gene enrichment or enriched biological pathways). We also used chemical gene regulator relationships for the same genes in humans to understand what chemicals might be present based on gene expression patterns (Kramer et al., 2014). In addition to gene expression, we used physiological and biochemical endpoints for the caged fish including plasma hormone (17β-estradiol and testosterone) and vitellogenin levels and gene expression levels of the PAH responsive cyp1a1 (see Appendix E for details). We compared gene expression in fish tissues to types and levels of CECs detected in water samples obtained via grab sampling. Water was tested for 134 organic compounds indicative of industrial, domestic, or agricultural wastewaters, including a suite of 48 pharmaceuticals (Lee et al. 2012).

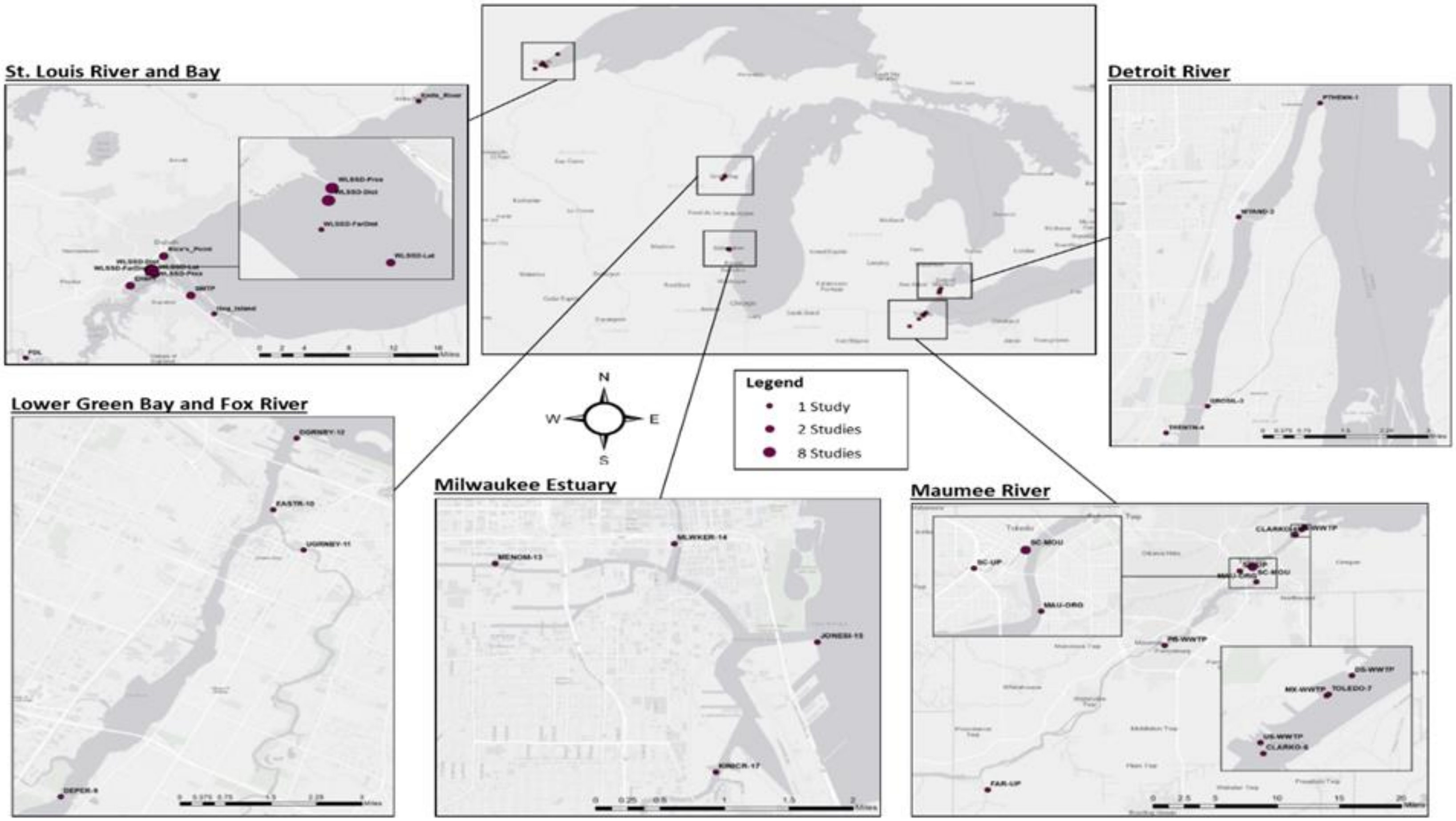

Figure F.1. Locations of case studies conducted as part of Action Plan I and corresponding study sites. Size of each marker reflects the number of caged fish exposures conducted at each site. Map surface layer credits: ESRI, HERE, Garmin, OpenStreetMap Contributors, and the GIS user community. Map created by Kendra Dean (10/24/18). This figure from Appendix E. Final figure under development.

F.2.4. Case Study: Effects-Based Monitoring of Waste Water Treatment Plant Effluent at Increasing Distances from Point of Discharge

Male fathead minnows were deployed at varying distances from a WWTP effluent outfall to determine if biological effects associated with the effluent diminished with distance from the point of discharge (Garcia-Reyero, unpublished). Gene expression was examined in livers of male exposed for 4d at each site. The impact of exposure site on gene expression decreased with distance from the effluent discharge consistent with a reduction in the number of chemicals detected in water samples farther from the discharge site. Males exposed closest to the discharge site had enriched biological pathways similar to those induced by 17β-estradiol, while males at the distant site (250m) had fewer pathways similar to 17β-estradiol. The similarity of male fish deployed near the outfall to males exposed to 17β-estradiol was consistent with increased plasma 17β-estradiol in males at that site. Enrichment of pathways in males deployed distant from the discharge site indicated the presence of stressors different from those near the discharge site. Analysis of gene expression indicated the potential gene regulator effects of several dibenzofurans, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs). Overall the estrogenic effect of effluent reduced with distance from the discharge site. At farther distances, other CECs, such as PAHs and PCBs, likely drove the transcriptional effects seen in deployed fish.

F.2.5. Key Findings/Progress - Detecting Biological Activities with Transcriptomics

- Transcriptional-effects based monitoring can be used to survey tributaries for chemical effects on wildlife including estrogenic and effects caused by other CECs.

- Transcriptomics can be used to identify potential chemicals causing effects even if they were not looked for in analytical tests of the exposure water.

- The impact of specific CECs in mixtures and surface water can be assessed by comparing laboratory exposures of a target CEC to mixture effects.

F.3. Associating Detected Chemicals with Biological Effects Using Transcriptomics and Evidence-Based Approaches

The goal of this section was to determine if changes in gene expression in exposed fish can be related to the concentration of specific chemicals detected in the water they were exposed to. Many different chemicals and CECs can be detected in surface waters especially those into which waste water treatment plants discharge. It would be of great use to identify, or reduce the number of, contaminants that could potentially drive adverse effects so that managers and other decision makers can prioritize efforts and best utilize limited resources.

F.3.1. Statistical Approaches for Associating Chemicals with Transcriptomics and Biological Effects

Here we used the knowledge of how chemicals may cause biological effects by activation, or deactivation, of signals by chemicals can lead to changes in gene expression. In this approach we counted the number of genes whose expression level patterns were highly similar to (correlated with) the concentration levels of each chemical detected in surrounding water (Perkins et al., 2017). We then looked at which chemicals had the largest number of genes whose expression correlated with chemical concentration. Exposure to increasing concentrations of chemical has generally been found to result in increasing numbers of changed genes (e.g. Deng et al 2011; Webster et al 2015) indicating that chemicals with high numbers of correlating genes are likely to have had greater biological impact than those with low numbers of correlating genes. While several factors can change this relationship (e.g. length of exposure, potency, mechanism of action and thresholds of toxicity), the number of genes affected by a chemical provides an easily measured indicator of the impact of a chemical that is suitable for screening and prioritization.

F.3.2. Evidence-Based Approaches for Associating Chemicals with Biological Effects

Evidence-based approaches utilize existing evidence, or data, that a chemical causes biological effects to extrapolate what effect that chemical would have on wildlife exposed to it in surface water. The evidence is generally based on published literature, toxicological assays (e.g. ToxCast https://www.epa.gov/chemical-research/toxcast-dashboard), chemical gene/protein relationships or other health hazard data. Here we utilized two different evidence-based approaches, one that uses evidence of a chemical's known impact on gene expression, and a second that uses known chemical effects on a fish reproductive biomarker, vitellogenin and bioassay data related to tumor formation.

F.3.2.1. Single Chemical Gene Expression Effects

Exposure of a fish to an individual CEC can establish what kind of genes and biological pathways are changed in response to that chemical. This can also be used to establish a gene signature characteristic of exposure to the chemical. Known signatures and biological impacts of individual CECs have the potential to be used to determine if that CEC is causing biological effects when present in a mixture (see 8.3.4).

F.3.2.2. Causal Networks Linking Gene Expression and Chemicals

Knowledge on what genes are expressed in response to a specific chemical exposure can be useful in identifying chemical(s) that an animal may have been exposed to. Causal networks linking drug and chemical interactions to gene expression and apical effects in humans that enables prediction of potential upstream regulators of gene expression can be useful in identifying conserved chemical gene interactions conserved across species (Kramer et al., 2014). Vertebrates have many biological and regulatory pathways in common, so causal networks such as the Ingenuity Knowledge Base (QIAGEN Bioinformatics, Redwood City, CA) can be used to identify chemicals potentially impacting exposed fathead minnows.

F.3.2.3. Hazard Quotients

A rapid approach to estimate the potential of contaminated water to cause adverse effects on wildlife is to divide the exposure amount (concentration in water) by a reference concentration (a known concentration where the chemical can cause toxicity or activity in an assay). One advantage of this approach is that results from cell-based assays representing biological pathways or outcomes of interest (e.g. estrogen receptor activation, cell proliferation) can be used to establish toxicity reference values based on the point of departure from control assays. A hazard quotient approach was used as it is a well establish approach that allows incorporation of uncertainty values for extrapolating from human cell-based assays to fish. Risk assessors have long used hazard quotients to described the potential of different media to cause toxic effects (EPA 1989, EPA 2009, 2018). A hazard quotient (HQ) is derived by dividing the exposure concentration by a toxicity reference value. When an HQ>1 is present, enough chemical is present to potentially cause an effect. Since we are using a toxicity reference value based on human in vitro data, the toxicity reference value was divided by an uncertainty factor of 100 to account for extrapolation of species (human to fish) and for cell assays to animal (in vitro to in vivo). A hazard Index is the sum of HQs for chemicals having similar modes of action.

F.3.3. Case Study: Transcriptomics of Bisphenol A Laboratory Exposures to Assess Bisphenol A Contribution to Estrogenicity in Waste Water Treatment Plant Effluent

A major concern for CECs is the potential to cause reproductive effects by increasing estrogenicity of surface waters. Chemical monitoring only provides a partial picture of potential risk posed by complex mixtures of contaminants in aquatic systems. Here we used transcriptomics to examine the impacts of effluent on fathead minnows in the laboratory in comparison to a CEC (Bisphenol A) thought to cause estrogenic effects in the effluent (Garcia-Reyero unpublished). Estrogenic effects have been observed in male fish exposed to treated wastewater that discharges to the St Louis Bay. Bisphenol A (BPA), a known estrogenic chemical, was among the contaminants detected. To determine whether BPA was a major contributor to estrogenic effects, male and female adult fathead minnow were exposed in the laboratory to effluent collected directly from the discharge or BPA. Exposure to 50% effluent, but not BPA, had an estrogenic effect, increased levels of vitellogenin, on male fish. Both BPA and effluent exposures caused significant changes in liver transcriptomics. However, the gene signature of BPA derived from laboratory exposed fish only partially overlapped with the effects on transcriptomics caused by 50% effluent. The increase of vitellogenin by effluent, but not by BPA, and the partial overlap of the gene signatures of BPA and effluent indicates that BPA was not likey to be a major cause of estrogenicity in the effluent.

F.3.4. Case Study: Integrated Application of Transcriptomics and Evidence-Based Approaches Assessing the Estrogenic Impact of CECs

We applied a combination of HQ and statistical approaches with caged female fish to identify potential CECs causing estrogenic effects near Waste Water Treatment plants

(WWTP) in the St louis Bay area (Perkins et al., 2017). Analysis of the water chemistry near discharge sites identified 4 chemicals (naphthalene, 4-nonylphenol, BPA, estrone) at concentrations known to cause any biological effect (HQ>1). Three of these (naphthalene, 4-nonylphenol and BPA) were the most highly ranked/impactful chemicals by covariation with gene expression in ovaries, but only BPA also had an HQ>1 for vitellogenin production in male fathead minnows (Figure F.2). Causal network analysis of transcriptomics of ovaries from exposed females indicated the effects of several chemicals associated with hospital waste consistent with WWTP having untreated hospital waste delivered to its system. Overall, the combination of HQ and statistical association of detected chemicals with transcriptomics and causal network analysis provides evidence that BPA is a significant contributor to estrogenic effects in the St Louis Bay. However, it also indicates that other CECs may potentially be causing biological effects such as PAHs and chemicals in hospital waste.

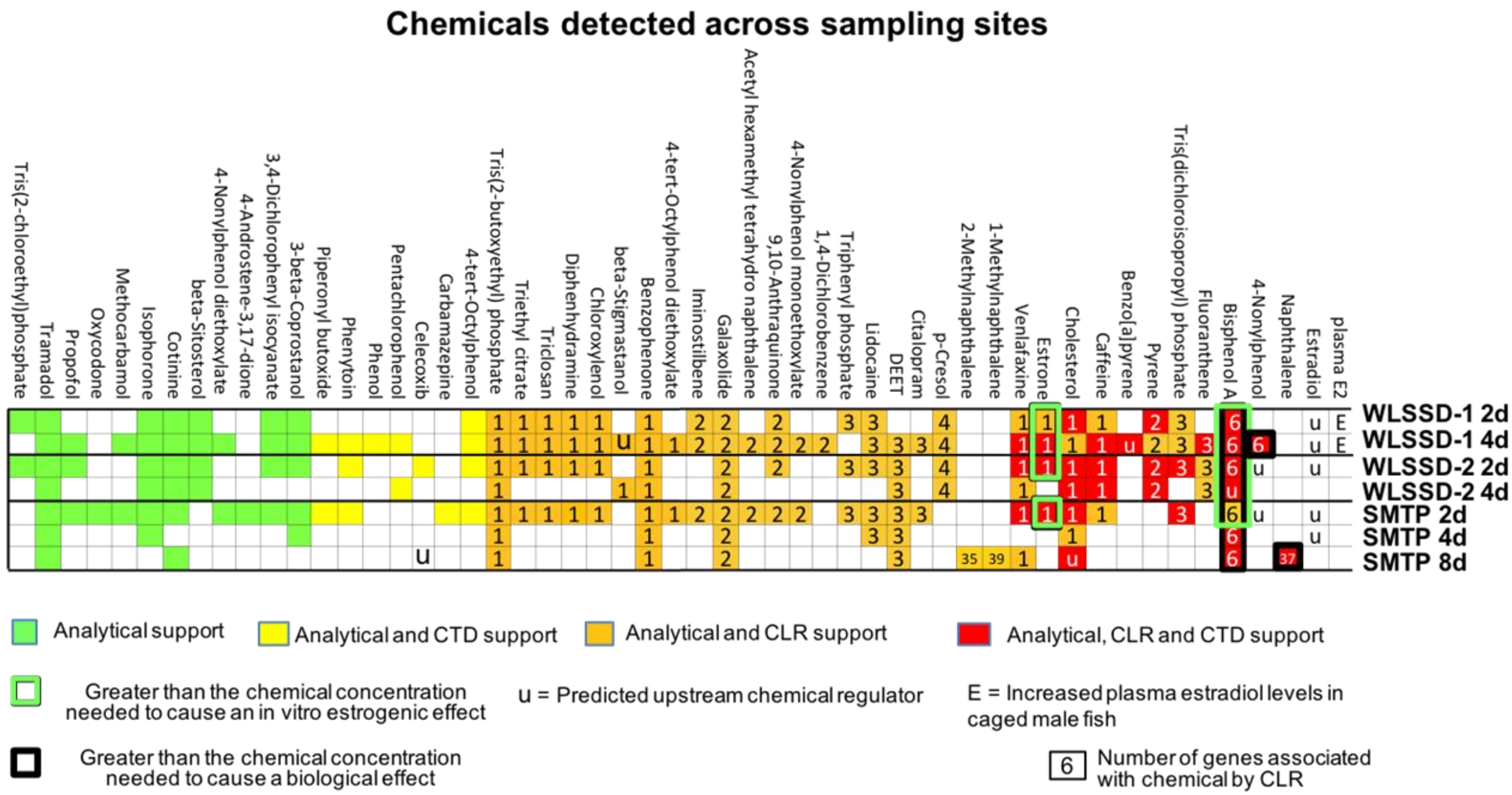

Figure F.2. Prioritization of chemicals detected near waste water treatment plants in St Louis bay. Chemicals with more evidence supporting biological impacts are on the right and chemicals with the least supporting evidence are on the left. Evidence of chemical impact include the number of differentially expressed genes that covary with a chemical (CLR), the number of differentially expressed genes associated with a chemical in the Comparative Toxicogenomics Database (CTD), presence detected by analytical chemistry, and hazard quotient for biological effect or estrogenic effect. Note that not all chemicals had information available in CTD.

F.3.5. Case Study: Determining CECs Associated with Transcriptomics Effects in Great Lakes Tributaries

The Maumee and Detroit rivers have a number of different inputs in the rivers including combined sewage overflow sites, agricultural sources, and urban sources such as WWTP effluent. Both rivers have had tumor formation in fish identified as a beneficial use impairment (Blazer et al. 2014; https://www.epa.gov/great-lakes-aocs/about-maumee-river-aoc; https://www.epa.gov/great-lakes-aocs/about-detroit-river-aoc). We used HQ and statistical approaches with transcriptomics and water chemistry to identify chemicals detected in the mixture of CECs that represent potential threats to fish health (Perkins unpublished). HQ were developed for estrogenicity and tumor formation based on in vitro assay data. Analysis of water chemistry identified four chemicals at sufficient levels to cause estrogenicity (HQ>1) with two at much higher HQ than the rest (17-beta-Estradiol>>> Estrone>> BPA >4-Androstene-3,17-dione> 4-Nonylphenol). However, estrogenic effects in males was found at only two sites and did not correlate with HI for estrogenicity. Two chemicals were identified at sufficient levels to potentially cause tumor formation (HQ fluoranthene >> tris (2-chloroethyl) phosphate). PAHs had the most impact across all sites (agricultural and urban) when measured by transcriptomics associated with specific chemicals, followed by pharmaceuticals, pesticides and alkylphenol ethoxylates (Figure F.3). On an individual chemical level (Figure F.4), phenanthrene, fluoranthene, and pyrene were associated with the highest number of gene expression changes. These data indicate that PAHs have significant impacts on fish, with fluoranthene a significant concern due to its high HQ and association with a large number of gene expression changes.

			WWTP discharge			
	SWANC	CLARKO	TOLEDO	WYAND	GROSIL	TRENTN
Alkylphenol ethoxylates	6	0	18	21	8	22
Antimicrobial	1	0	5	10	3	6
Aromatics	6	7	6	8	6	8
Flame retardant	4	5	7	7	2	4
Flavors, Fragrances	1	0	6	11	7	8
Herbicides	6	12	12	3	4	3
Hormones	2	2	4	16	6	15
Industrial chemicals	4	2	12	12	7	12
Sterols	4	2	8	9	3	7
PAHs	49	46	45	77	45	75
Pesticides	8	7	28	28	16	26
Pharmaceuticals	14	7	66	45	21	46
Plasticizer/Solvent	2	2	10	3	1	6

Figure F.3. Ranking of chemicals in Maumee and Detroit rivers using the sum of covarying differentially expressed genes in caged fish exposed at sites upstream of, and nearby, WWTP discharge sites. The total number of differentially expressed genes covarying with a chemical class are listed in each box with higher values shaded a darker red. Boxes with zero represent chemicals with no significant covarying differentially expressed genes.

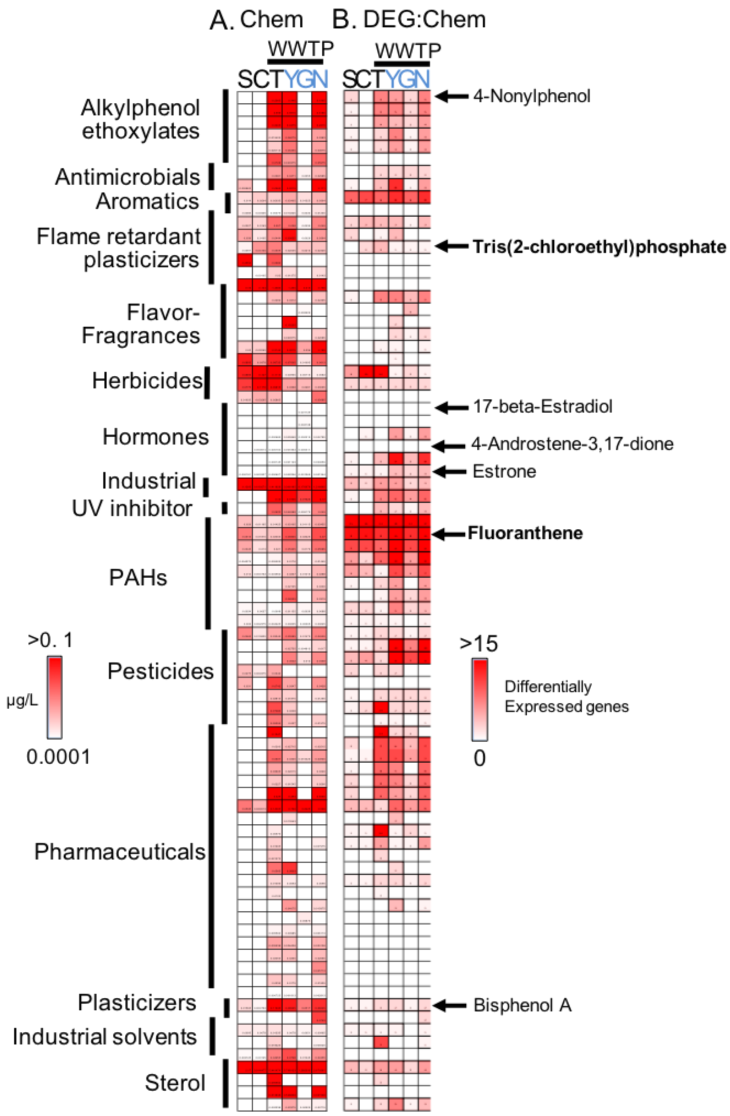

Figure F.4. Prioritization of individual chemicals detected near waste water treatment plants in the Maumee and Detroit Rivers. Heat maps of the average surface water chemistry and gene expression correlated to average surface water chemistry across deployment sites in the Maumee and Detroit Rivers. A. Heat map of average chemistry concentration in surface water during 4 days exposure (Chem). Classes of chemicals are listed are identified on the left. Blank boxes= concentrations below detection limits. Black SCT=sites on Maumee river, Blue YGN=sites on Detroit river. WWTP=Waste Water Treatment Plant outfalls. S=Swan Creek, C= Clark Oil site, T=Toledo WWTP outfall, Y=Wyandotte WWTP outfall, G=Grosse Ile WWTP outfall, N=Toledo WWTP outfall. B. The number of differentially expressed genes that were correlated with average chemistry at deployment sites (DEG:Chem). Blank boxes indicate no significant association between genes and chemistry. Chemicals on the right were identified as being present at concentrations sufficient to cause estrogenicity (normal type) or tumors (bold type).

F.3.6 Key Findings/Progress – Associating Detected Chemicals with Biological Effects Using Transcriptomics and Evidence-Based Approaches

- Statistical association of water chemistry with gene expression from exposed caged fish can be used to develop a biological impact ranking based upon the number of genes that covary with chemicals in surface water. This can be used to prioritize management actions to reduce costs and lower the number of CECs that must be managed.

- The combination of evidence-based approaches with transcriptomics analysis provided more weight of evidence that specific chemicals in CEC mixtures cause biological and adverse effects.

- PAHs appear to be present at many sites at high concentrations and have significant biological impacts on exposed fish. This indicates that PAHs are potential threats to wildlife in the Great Lakes.

- Gene expression changes can be used to identify potential exposure to chemical not tested for such as hospital waste chemicals.

F.4. Linking Biological Activities to Adverse Apical Effects in Wildlife

Effects-based monitoring can detect changes in fish as a result of being exposed to CEC mixtures. However, these effects need to be related to adverse apical effects in wildlife to determine if the tributary is adversely impacted and to identify what CECs might potentially cause these effects. Transcriptomic changes can be linked to apical effects because gene expression changes can lead to changes in cells, tissues and health of an animal (Box F.1). The AOP provides a framework that describes how a molecular initiating event can lead to activation of key events that cause and adverse outcome (Ankley et al 2010). Using the AOP framework we can develop pathways in which gene expression changes can be linked to apical effects.

F.4.1. The Adverse Outcome Pathway (AOP) Framework with Transcriptomics Effects-Based Monitoring

Since an AOP is a series of events with one leading to another, key events leading an outcome can be measured to predict the likelihood of an adverse event occurring (Perkins et al., 2019a). This is especially useful when using untargeted monitoring approaches such as transcriptomics as an AOP serves as a framework to organize information and test hypotheses as to whether evidence exist to support the activation on an AOP. Here we have developed an AOP network describing the events that lead from sustained liver cyp1a activation and/or steatosis activation causing liver damage that results in proliferative regeneration and subsequent tumor formation (Figure F.5). The AOP network uses gene expression as indicators of molecular events in the network which lets one use transcriptomic analysis of exposed animals to assess how much of the pathway might be activated. This permits linkage of transcriptomic impacts to tumor formation, a common biological use impairment (BUI) in Great Lakes Tributaries.

F.4.2. Coupling Organ Damage Models with Transcriptomics Data

While most attention has focused on adverse outcomes that impact reproduction, liver damage in fish can result in tumor formation can result in tumor formation, metabolic impairments or other diseases. Tumor formation can lead to impacts on resources and sportfishing and therefore is considered a BUI. We developed a network of AOPs that describe how chemicals can cause liver toxicity by impairing fatty acid metabolism or steatosis (Burgoon et al., 2019 unpublished, Perkins et al., 2019b). We combined the steatosis AOP network with gene expression to create a steatosis AOP Bayesian Network model (AOPBN) to quantitatively model the transcriptomic effects. The steatosis AOPBN is available in the US Army's Bayesian Inference for Substance and Chemical Toxicity risk assessment software tool (Burgoon et al 2019 in review; https://github.com/DataSciBurgoon/bisct). The steatosis model calculates the probability of a chemical causing steatosis given the activation or inactivation of genes in the network. The model describes signaling, fatty acid metabolism and transport pathways important in steatosis using the activity of key proteins and measurements of fatty acid influx, efflux, lipogenesis, and fatty acid beta oxidation. Here, gene expression was used as an indicator of increased or decreased levels of protein and activity. Since sustained steatosis over long periods results in repeated cellular injuries, it can lead to regenerative proliferation and eventually tumor formation.

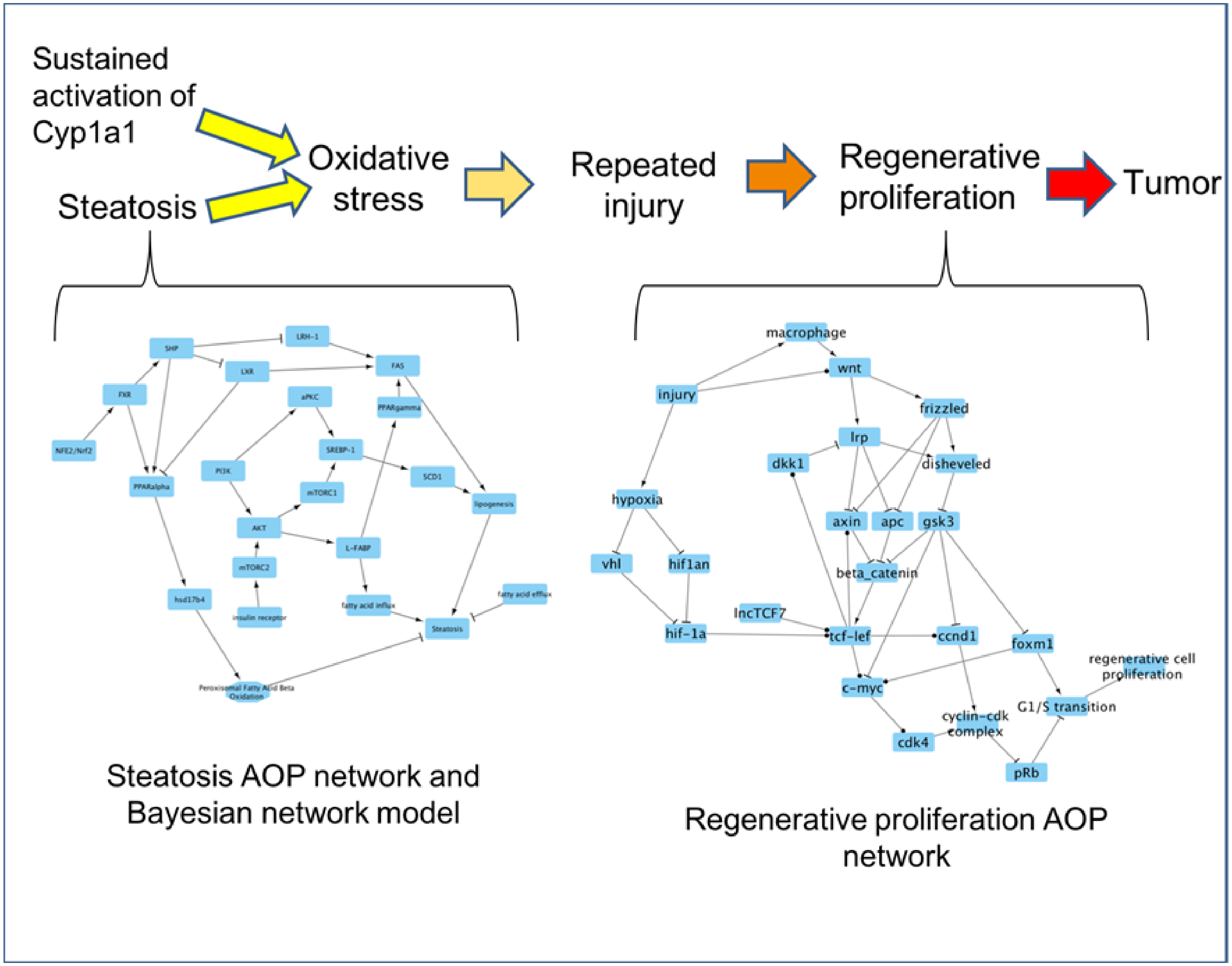

Figure F.5. AOP network representing sustained cytochrome p450 activity or steatosis creates oxidative stress resulting in repeated liver tissue injury, leading to regenerative proliferation, tumor formation and carcinogenesis.

F.4.3. Case Study: Transcriptional Effect-Based Monitoring of Potential for CEC Mixtures to Cause Tumor Development

The Maumee and Detroit Rivers have input from agricultural, industrial and urban sources. Both Maumee and Detroit rivers have experienced degradation of fish and wildlife populations and fish tumors or other deformities (https://www.epa.gov/great-lakes-aocs/about-maumee-river-aoc; https://www.epa.gov/great-lakes-aocs/about-detroit-river-aoc). Surveys of native fish have indicated tumor formation as an adverse outcome in these rivers (Blazer et al. 2014). We examined how CECs and transcriptomics can be linked to adverse outcomes with effects-based monitoring of CECs present in different areas near the outlets of the rivers (Perkins unpublished). To do this we examined the hypothesis that deployment of fathead minnows near WWTP discharge sites in the Maumee and Detroit Rivers would activate genes in the AOP network leading to tumor formation and carcinogenesis via steroidogenesis and regenerative proliferation (Figure F.5). A high number of genes related to tumor formation were activated in livers of exposed fish (Figure F.6). Analysis of gene expression results supporting the AOP indicated sustained activation of cyp1a1 and oxidative stress, steatosis activation at all sites, and activation of key genes controlling regenerative proliferation. This is consistent with the association of PAHs as the most impactful chemicals on gene expression, the high fluoranthene HQ for tumor formation, activation of tumor pathway related genes, and elevated levels of cyp1a and oxidative stress related gene expression consistent with exposure to PAHs in native fish in the Detroit river (Braham et al., 2017).

F.4.3.1 Key Findings/Progress – Development of Translational Tools and Frameworks for Linking transcriptional Measurements to Adverse Effects in Wildlife

- A combination of AOPs, HQ, and association approaches can be used to support the assessment of the contribution of individual chemicals to adverse effects seen due to exposure to CEC mixtures.

- AOP networks and models combined with transcriptomics can be used to assess activation of key events leading to adverse effects seen in wildlife such as tumor formation as a result of CEC exposure.

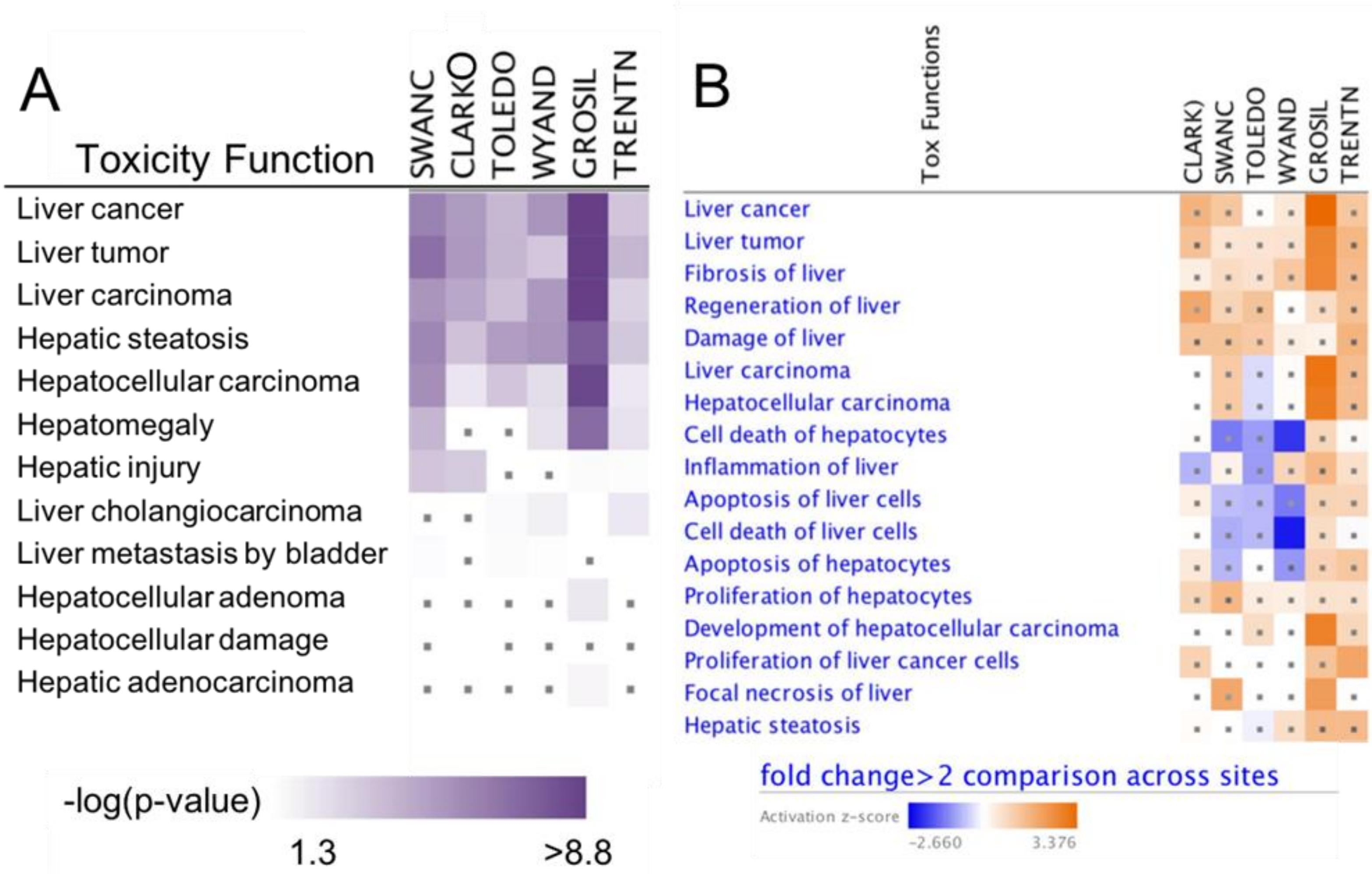

Figure F.6. Heatmap comparing liver toxicity functions enriched in differentially expressed genes from caged fathead minnows exposed along the Maumee and Detroit rivers. Dots denote values below significance. A. Significance of enrichment for genes associated with toxicological functions. More significant functions are represented by darker purple. B. Activation of liver toxicity functions across deployment sites. Orange is activated, blue is inhibited.

F.5 Management Implications and Future Directions

In this section we described how gene expression data can be used by risk managers to identify potential adverse effects in waters of the Great Lakes tributaries. We described two different types of gene expression platforms: caged fish and laboratory exposed fish. We have demonstrated that transcriptomics combined with these systems can be used to identify CECs that are likely to cause harmful effects so that managers can focus efforts on smaller list of CECs. The benefits of using these platforms are that predictions of potential chronic toxicity can be made based on relatively short exposure times. This is especially helpful when determining if management actions have been successful for mitigating irreversible toxicities, such as tumor formation or structural abnormalities. Due to the nature of irreversible toxicity, it would be possible to find fish and other organisms with tumors and structural abnormalities even though the water quality has improved. The use of caged fish or laboratory exposures enables the use of newly hatched and reared organisms would not develop the irreversible toxicity if no chemical is present.

Transcriptomics effect-based monitoring is most effectively used to link CECs to adverse outcomes when used in combination with evidence-based approaches such as Hazard Quotients, water chemistry, and AOPs representing adverse outcomes of concern.

Currently, our models perform well for tumor formation and liver toxicity, including fatty liver (steatosis). We are extending our models and validating our approaches for additional toxicities that may be of interest to risk managers. Currently, we recommend that our models may have the greatest impact on those welfare effects that are associated with tumors in fish. These welfare effects include many of the ecosystem services associated with sport fishing, recreation, and cultural uses of water bodies, and the related economic impacts.

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Contaminants of Emerging Concern in the Great Lakes: GLRI Integrated Phase II Group Progress Report

Contaminants of Emerging Concern in the Great Lakes: GLRI Integrated Phase II Group Progress Report

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Cover photo: Detroit River, Micigan. Photo Credit: Kimani Kimbrough

This page: Superior Falls at the mouth of the Montreal River on the border of Wisconsin and Michigan. Photo credit: Austin Baldwin.

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Sampling the Manitowoc River in Wisconsin.
Photo credit: Austin Baldwin.

Executive Summary

Under Action Plan II (2015-2019) of the Great Lakes Restoration Initiative (GLRI), Federal and Academic partners continued an investigation that began under Action Plan I (2010-2014) into the presence and distribution of contaminants of emerging concern (CECs) in the Great Lakes, and their potential impacts on fish and wildlife. The term CECs is applied to a broad range of chemicals that are currently present in the environment due to their historical or ongoing use. Data are currently lacking to determine whether fish, wildlife, or humans are being exposed to CECs or if negative health or environmental effects are expected if exposure to CECs occurs. Pharmaceuticals, personal care products, flame retardants, some current-use pesticides, polycyclic aromatic hydrocarbons, and poly- and perfluorinated chemicals are often identified as CECs, but currently there is no definitive or comprehensive list.

This progress report seeks to provide an update on the results of the activities undertaken in the 2016 field season (and other completed work to-date), which built on the results of the Phase I work conducted under GLRI Action Plan I. Specifically, Phase I was focused on the development of methods and gathering of CEC data to guide the design and help achieve the objectives of Phase II. The activities presented in this report contributed towards fulfilling the GLRI Action Plan II Objective 1.2.2: Identify emerging contaminants and assess impacts on Great Lakes fish and wildlife. Three specific goals were further developed from Objective 1.2.2:

1. Characterize and evaluate the extent to which CECs might threaten fish and wildlife populations relative to other chemical stressors present in the Great Lakes.

2. Pilot and develop state-of-the-art surveillance techniques for biological effects from CECs in the Great Lakes basin.

3. Develop information and tools for resource managers to better manage and address potential CEC threats to fish and wildlife populations.

A three-pronged approach was used to implement these goals which included:

1. Implementing a **surveillance program** to determine which CECs occurred with the greatest frequency and abundance across the Great Lakes basin to identify seasonal and spatial patterns of occurrence.

2. Conducting **Integrated Assessment Case Studies (IACS)** focusing on a particular waterbody and/or suite of CECs and conducting an analysis of the effects of CECs on organisms. The 2016 IACS focused on pesticides in the Maumee River, Ohio.

3. Evaluating the toxicity of **priority contaminant mixtures** (determined by the work conducted in Phase I) on fish and native mussels in a laboratory setting.

Manitowoc River in Wisconsin.
Photo credit: Austin Baldwin.

Key Findings

- **Pesticides were found in water at all sampled sites,** with the greatest concentrations occurring in May-July for herbicides. Complex chemical mixtures were common, and an average of 18 pesticide compounds, including pesticides and pesticide degradation products, were detected in each sample.
  - **Many pesticides were detected in the Maumee River water samples.** The watershed includes inputs associated with agricultural practices and urban influences such as runoff from residential, commercial, and park lands. The Maumee River had the most pesticide and pesticide degradates detected of the 16 Great Lakes watersheds sampled. Seventy compounds were detected of the 231 analyzed throughout the study with a maximum of 57 compounds detected in one individual sample.
- **Polycyclic aromatic hydrocarbons (PAHs) were a class of contaminants detected throughout the basin in water and occasionally biota** dreissenid mussels and aquatic insects). A multiple lines-of-evidence approach indicated that coal-tar-sealed pavement was the most likely source of PAHs in sediment at 80% of the 71 locations sampled, and vehicle emissions were the primary source at 10% of sites.
- **Observed concentrations of CECs would not be expected to produce lethal effects in fish and wildlife species, but some CECs were detected in aquatic life.** These results indicated potential impacts to the species of fish (e.g., lake sturgeon, sunfish, and fathead minnows) and mussels (pocketbook, spike, fatmucket, zebra, and quagga mussels) examined as part of this effort. These possible effects include anatomical changes (e.g., feminization of males), impacts on the nervous system, declining offspring production, and developmental delays. The possibility of impacts on these types of endpoints warrants further investigation.
  - Results from caged fish (sunfish, fathead minnow) studies indicated some molecular and biochemical changes in exposed animals, but it is unclear the degree to which this was due to CECs nor what the consequences of these changes might be in terms of impacts on overall fish populations. Inconclusive results were found surrounding developmental delays in fish, which were seen in some years and at some sites but not consistently.
  - Initial metabolomics results for dressinid mussels found patterns which showed differences between sites. Work is still in progress to understand the relationship between CECs and dressinid metabolomics data.
  - Native mussel bio-effects assessments did not find relationships between environmental CEC exposure and glycogen and gamete counts in wild caught native mussels, but CECs were present in tissues indicating other assessments may be warranted to determine if CECs impact native mussel biology.

Milwaukee River, Wisconsin. Photo credit: Kimani Kimbrough.

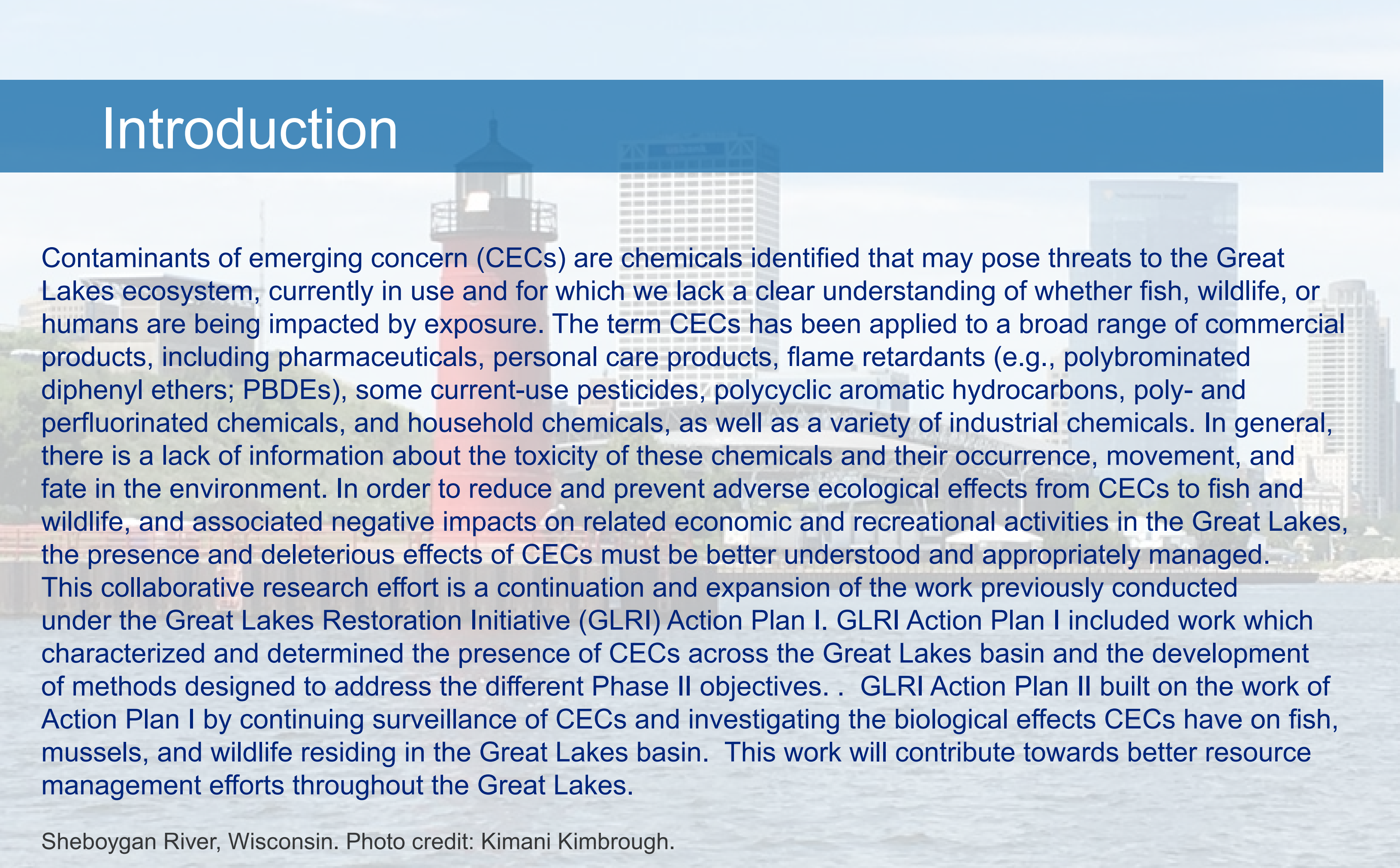
- Laboratory exposures with formulated CEC mixtures designed to mimic the field with fish and native mussels did not impact survival but yielded decreased number of offspring produced in fish. Native mussel glycogen, fatty acid, sperm production, and behavior were not affected by exposure to CEC mixtures in the laboratory, but offspring viability decreased.

- **Pesticides and herbicides were rarely detected in tree swallow tissues, with two exceptions.** The insect repellent ingredient DEET, or diethyltoluamide, was detected in nearly all liver tissue samples, and desethylatrazine, a byproduct of the herbicide atrazine, was detected in virtually all tree swallow tissues including in swallows nesting at remote WI lakes. These compounds were not expected to be found in tree swallows because of their chemical properties and modes of action, so follow-up studies are warranted to learn more about the metabolic and ecosystem pathways leading to this exposure.

Manitowoc River in Wisconsin.
Photo credit: Austin Baldwin.

Introduction

Contaminants of emerging concern (CECs) are chemicals identified that may pose threats to the Great Lakes ecosystem, currently in use and for which we lack a clear understanding of whether fish, wildlife, or humans are being impacted by exposure. The term CECs has been applied to a broad range of commercial products, including pharmaceuticals, personal care products, flame retardants (e.g., polybrominated diphenyl ethers; PBDEs), some current-use pesticides, polycyclic aromatic hydrocarbons, poly- and perfluorinated chemicals, and household chemicals, as well as a variety of industrial chemicals. In general, there is a lack of information about the toxicity of these chemicals and their occurrence, movement, and fate in the environment. In order to reduce and prevent adverse ecological effects from CECs to fish and wildlife, and associated negative impacts on related economic and recreational activities in the Great Lakes, the presence and deleterious effects of CECs must be better understood and appropriately managed.
This collaborative research effort is a continuation and expansion of the work previously conducted under the Great Lakes Restoration Initiative (GLRI) Action Plan I. GLRI Action Plan I included work which characterized and determined the presence of CECs across the Great Lakes basin and the development of methods designed to address the different Phase II objectives. . GLRI Action Plan II built on the work of Action Plan I by continuing surveillance of CECs and investigating the biological effects CECs have on fish, mussels, and wildlife residing in the Great Lakes basin. This work will contribute towards better resource management efforts throughout the Great Lakes.



Sheboygan River, Wisconsin. Photo credit: Kimani Kimbrough.

A three-pronged approach was used to implement research goals for GLRI Action Plan II which included:

1. Implementing a **surveillance program** to determine which CECs occurred with the greatest frequency and abundance across the Great Lakes basin, to identify seasonal changes in patterns of occurrence.

2. Conducting **Integrated Assessment Case Studies (IACS)** focusing on a particular waterbody and/or suite of CECs and conducting an analysis of the effects of CECs on organisms. The 2016 IACS, which is summarized in this report, focused on the Maumee River/watershed, Ohio, and evaluated several chemicals, with an emphasis on pesticides.

3. Evaluating the toxicity of **priority contaminant mixtures** (determined by the work conducted in Phase I) on fish and native and invasive mussels in a laboratory setting.

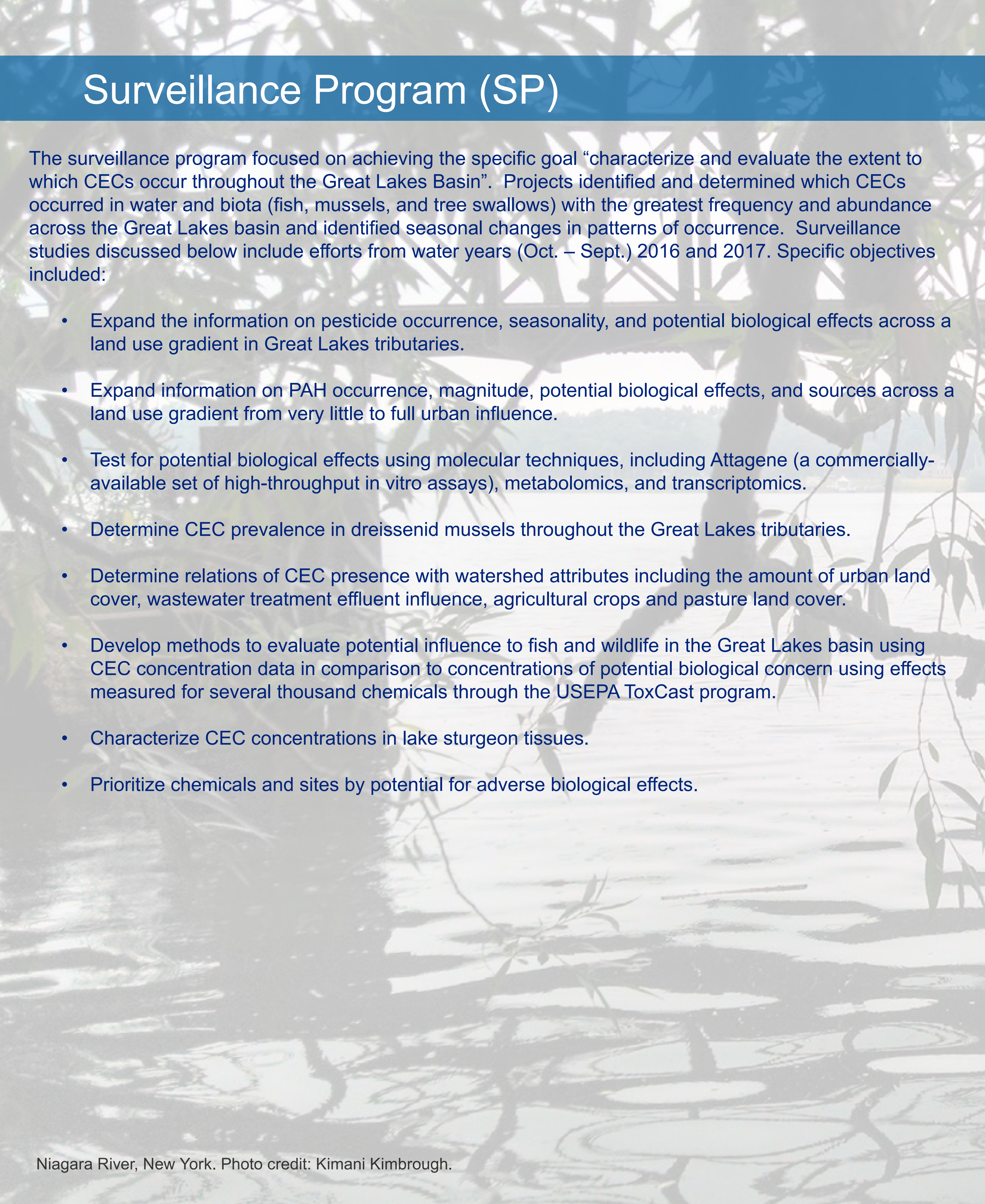
This research effort was comprised of individual and collaborative projects from multiple federal agencies and academic institutions, and was overseen by the U.S. Environmental Protection Agency (EPA), Great Lakes National Program Office. Partners include the U.S. Geological Survey, National Oceanic and Atmospheric Administration, U.S. Army Corps of Engineers, U.S. Fish and Wildlife Service, Saint Cloud State University, and the U.S. EPA Office of Research and Development.

This progress report details results of the research completed during the 2016 field season and completed works to date.

Surveillance Program (SP)

The surveillance program focused on achieving the specific goal “characterize and evaluate the extent to which CECs occur throughout the Great Lakes Basin”. Projects identified and determined which CECs occurred in water and biota (fish, mussels, and tree swallows) with the greatest frequency and abundance across the Great Lakes basin and identified seasonal changes in patterns of occurrence. Surveillance studies discussed below include efforts from water years (Oct. – Sept.) 2016 and 2017. Specific objectives included:

- Expand the information on pesticide occurrence, seasonality, and potential biological effects across a land use gradient in Great Lakes tributaries.
- Expand information on PAH occurrence, magnitude, potential biological effects, and sources across a land use gradient from very little to full urban influence.
- Test for potential biological effects using molecular techniques, including Attagene (a commercially-available set of high-throughput in vitro assays), metabolomics, and transcriptomics.
- Determine CEC prevalence in dreissenid mussels throughout the Great Lakes tributaries.
- Determine relations of CEC presence with watershed attributes including the amount of urban land cover, wastewater treatment effluent influence, agricultural crops and pasture land cover.
- Develop methods to evaluate potential influence to fish and wildlife in the Great Lakes basin using CEC concentration data in comparison to concentrations of potential biological concern using effects measured for several thousand chemicals through the USEPA ToxCast program.
- Characterize CEC concentrations in lake sturgeon tissues.
- Prioritize chemicals and sites by potential for adverse biological effects.



Niagara River, New York. Photo credit: Kimani Kimbrough.

Key Findings:

- Monthly water samples were collected during variable flow conditions from 16 Great Lakes tributaries during water year 2016 (October 2015 and September 2016; Figure 1). Agricultural land use varied from 0.1-79% of the drainage area for these watersheds. Pesticides were detected at all sites. At least one pesticide or degradation product was detected in 190 of the 198 pesticide samples collected, and 104 compounds were detected in at least one sample out of the 231 parent compounds and degradation products analyzed.[4 ŧ]

  - o Pesticides were detected in each month of the year with the greatest concentrations occurring in May-July for herbicides.

  - o The presence of complex chemical mixtures was common; samples had an average of 18 pesticide-related compounds (i.e., parents or metabolites) detected per sample and 30 or more compounds were detected in 15% of samples.

  - o Metolachlor, 2,4-D, diuron, and atrazine were the chemicals of greatest concern given their widespread occurrence (detected at >11 sites) and potential for adverse biological effects (exceedance of bioeffect thresholds at >12 sites and in >35% of samples). Concentrations were compared to bioeffect thresholds including EPA Aquatic life benchmarks and endpoints within the ToxCast high-throughput screening database. Among the 16 tributaries studied, the Maumee River basin had frequent detection of pesticides; exceedances of bioeffect thresholds occurred for all 18 Maumee River samples, including exceedances for 15 compounds. The Maumee River also had the most chemicals detected across the duration of the study (70) and in a given sample (57).

[ŧ] Superscript numbering following each *Key Findings* bullet corresponds to the principal investigator conducting the study and generating the key finding. Numbers identifying associated principal investigator can be found below the author list.

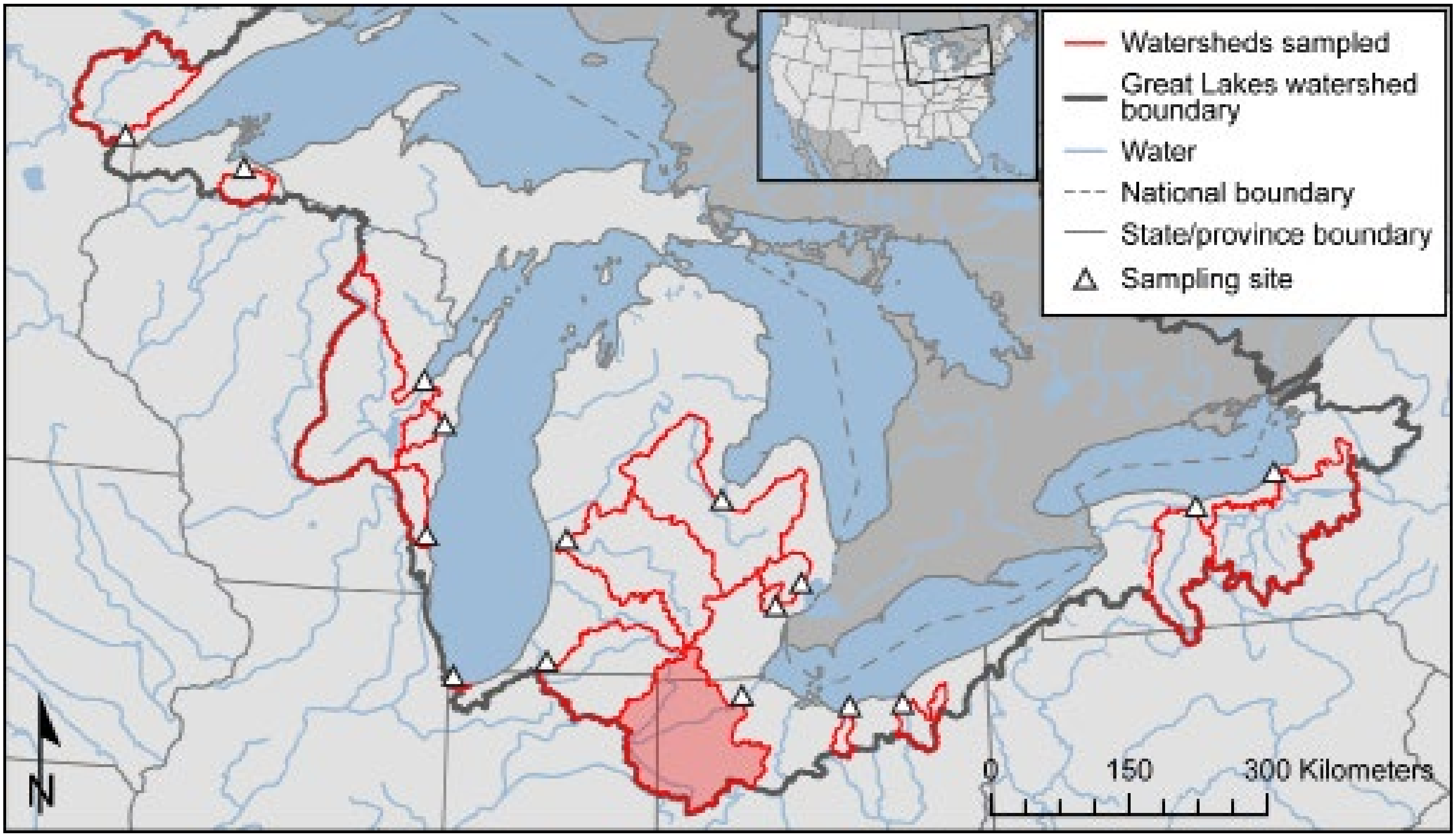


Figure 1: 167 Tributaries surveyed for the presence of pesticides from Oct. 2015 – September 2016. The Maumee River basin had a high detection of pesticides and the most chemicals detected.

- Pharmaceutical and personal care products (PPCPs) were detected at all dreissenid mussel sites and were highest at urban sites near municipal wastewater treatment plant (WWTP) discharges. The most common compounds detected were sertraline (an antidepressant), nonylphenol and nonylphenol ethoxylates (surfactants).[6]

- Statistical models were developed to predict the probability of CEC occurrence in water and sediment across the Great Lakes Basin. Water and sediment data, along with 21 watershed characteristics, from 24 US Great Lakes tributaries were used in the models. Watershed characteristics included land use, number of permitted point sources, and distance to point sources all of which were important predictors of CEC occurrence. Developed land use and distance to point sources were most often important predictors of CEC occurrence. Of the 24 sites sampled, sites with the greatest predicted occurrence of CECs included the Fox River, Milwaukee River, North Shore Channel, Little Calumet River, Cuyahoga River, and Tinker's Creek (Keisling et al. 2019).Results from the predictive models can be used to assess vulnerability of Great Lakes tributaries to CEC occurrence to guide future research and/or resource management decisions.[1]

- Polycyclic aromatic hydrocarbons (PAHs) were the dominant contaminant class of CECs in dreissenid mussel tissues and found throughout the basin. Levels were highest in river harbors and bays with high percentage of impervious surfaces but also found in nearshore and offshore lakes zones (Kimbrough et al., 2019; Figure 2).[6]

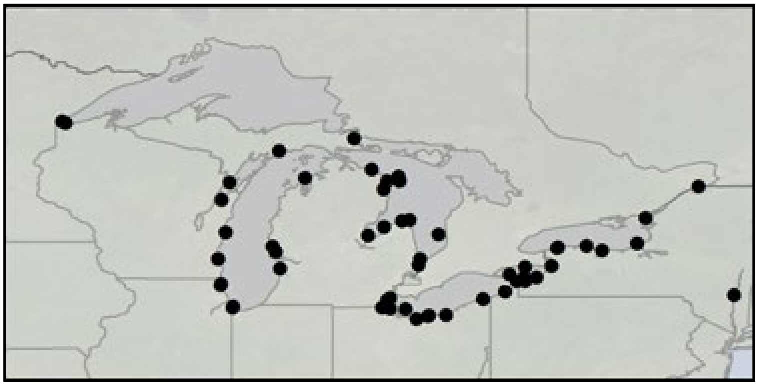


Figure 2: Great Lakes PAH dreissenid mussel locations occur throughout the basin. Some of the locations have multiple sites.

Rouge River, Michigan. Photo credit: W. Edward Johnson.

- Streambed sediments were sampled and analyzed for PAHs at 71 locations across 26 Great Lakes tributaries in 2017 (Figure 3). The results were compared to Threshold Effect Concentrations (TEC; 1,610 µg/kg for ΣPAH16) and Probable Effect Concentration (PEC; 22,800 µg/kg for ΣPAH16) defined as consensus-based freshwater sediment quality guidelines (Ingersoll et al., 2001). TEC and PEC are used as indicators to measure the potential for adverse biological effects. The TEC was exceeded at 62% of sampling locations and the PEC was exceeded at 41% of sampling locations. Based on source apportionment conducted using multiple lines-of-evidence, coal-tar-sealed pavement was the most likely source of PAHs in sediment at 80% of the 71 locations sampled, vehicle emissions were the primary source at 10% of sites, and a primary source could not be determined for 10% of the sites.[4]
- PPCPs and PBDEs were detected in lake sturgeon serum and gamete samples from populations at four sites located in the lower Great Lakes (Detroit River, Lower Niagara River, Upper Niagara River, and St. Lawrence River). Four PPCPs (bentropine, DEET, hydrocortisone, and amitriptyline) were found in at least 25% of adult lake sturgeon serum samples across all four study sites, and three PPCPs (sertraline, DEET, and 10-hyrdoxy-amitriptyline) were found in at least 25% of lake sturgeon gamete samples from the St. Lawrence River study site. Twenty, out of 40, polybrominated diphenyl ethers (PBDEs) were found in every serum sample at every site. A total of 14 PBDEs were found in all gamete samples (Banda et al. 2020, in review).[1]

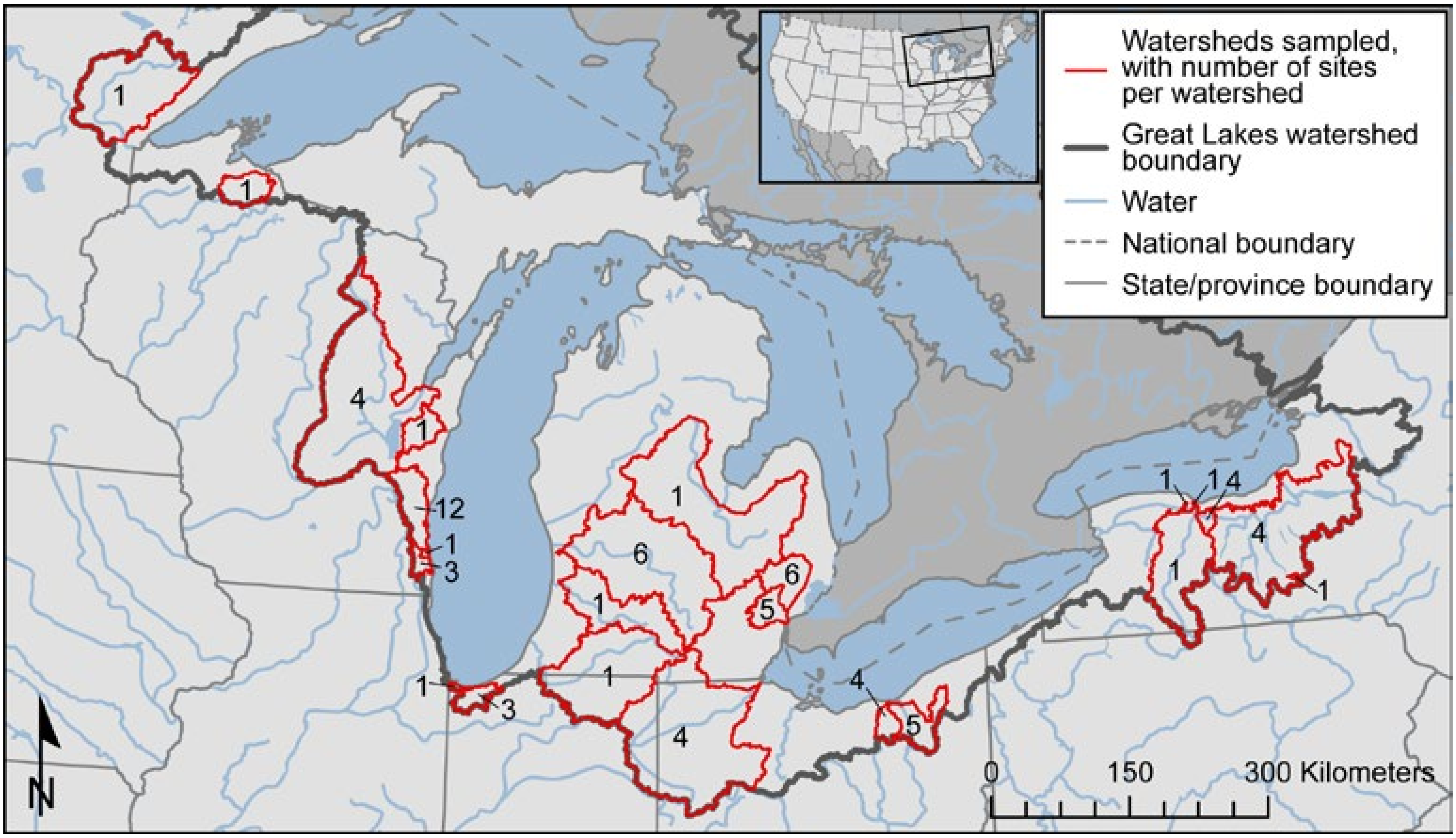


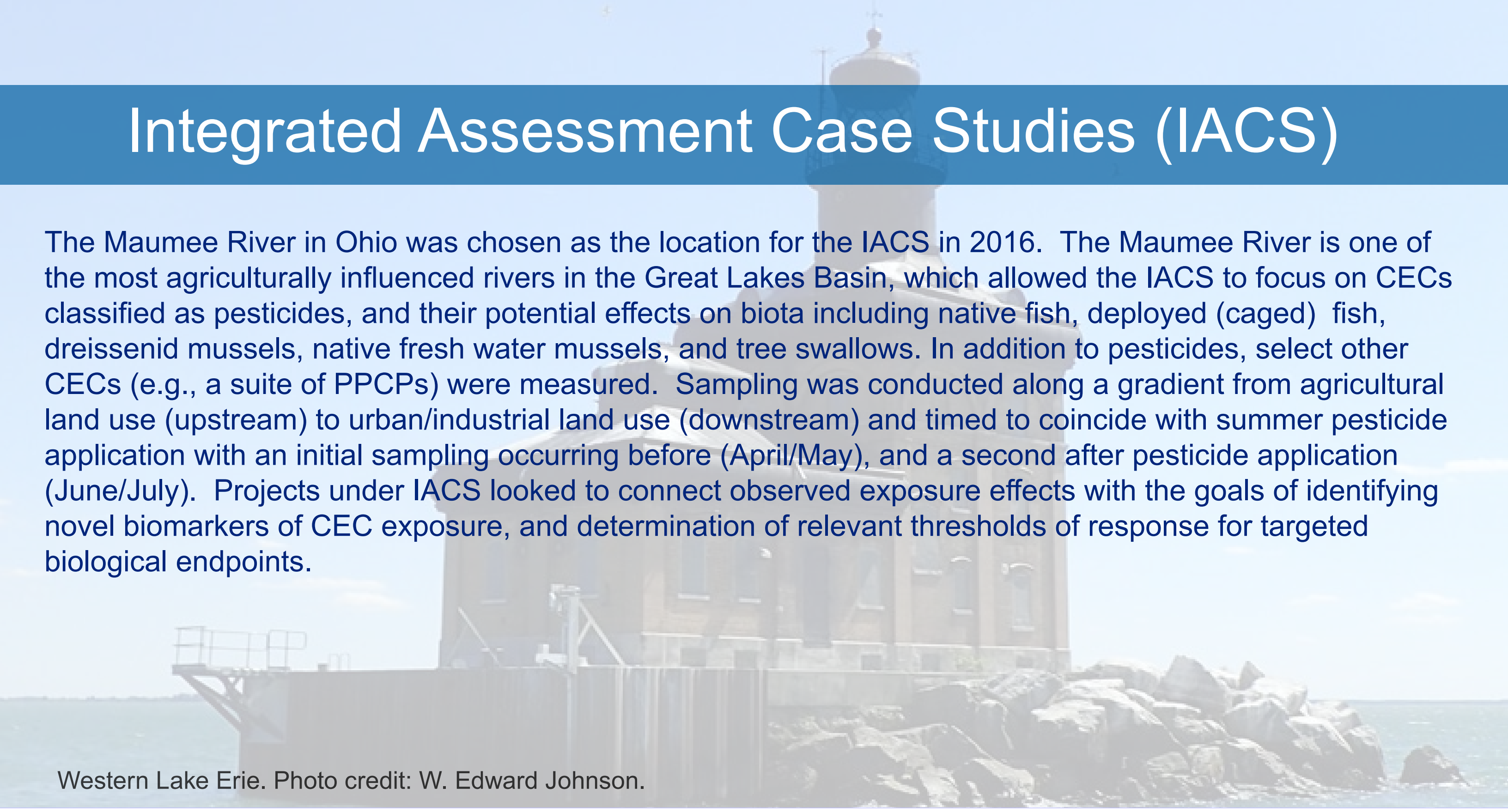
Figure 3: Map of the Great Lakes Basin and watersheds sampled for PAHs in sediment for during 2017. Numbers indicate the number of sampling sites within each watershed

- The software package toxEval was developed to facilitate analysis and visualization of potential biological effects for complex chemical concentration data sets that represent multiple chemicals, sampling locations, and samples per location (DeCicco et al. 2018). These techniques were used to evaluate a data set from Phase I of the GLRI that included 709 water samples from 57 sites that included analysis of 67 organic compounds.[2,4]
    - Comparison using ToxCast high-throughput screening results (https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data) and established water quality benchmarks identified 12 chemicals with the greatest potential for biological effects at multiple sites including 4-nonylphenol, bisphenol A, metolachlor, atrazine, DEET, caffeine, tris(2-butoxyethyl) phosphate, tributyl phosphate, triphenyl phosphate, benzo(a)pyrene, fluoranthene, and benzophenone (Corsi et al. 2019).
    - Evaluation of chemical mixtures through examination of adverse outcome pathway networks commonly predicted effects on reproduction and mitochondrial function.

Maumee River, Ohio Photo credit: W. Edward Johnson.

Integrated Assessment Case Studies (IACS)

The Maumee River in Ohio was chosen as the location for the IACS in 2016. The Maumee River is one of the most agriculturally influenced rivers in the Great Lakes Basin, which allowed the IACS to focus on CECs classified as pesticides, and their potential effects on biota including native fish, deployed (caged) fish, dreissenid mussels, native fresh water mussels, and tree swallows. In addition to pesticides, select other CECs (e.g., a suite of PPCPs) were measured. Sampling was conducted along a gradient from agricultural land use (upstream) to urban/industrial land use (downstream) and timed to coincide with summer pesticide application with an initial sampling occurring before (April/May), and a second after pesticide application (June/July). Projects under IACS looked to connect observed exposure effects with the goals of identifying novel biomarkers of CEC exposure, and determination of relevant thresholds of response for targeted biological endpoints.



Western Lake Erie. Photo credit: W. Edward Johnson.

Key Findings:

- Chemistry and biology results for the Maumee River along the gradient of sites indicate a system potentially heavily influenced by land use (both agricultural and urban land use). These inputs reflect various degrees of non-point source contamination and input from specific point-source discharges, predominantly WWTPs.[2]
    - There is a distinct seasonality relative to potential agricultural impacts on the system, with marked changes in concentrations of some nutrients and herbicides associated with crop production cycles. In some instances (e.g., metalochlor and atrazine) observed seasonal elevations in surface water concentrations in June/July versus April/May which were sufficient, based on existing effects data for possible impacts on biological components of the system, such as aquatic plants. Conversely, non-pesticidal contaminants (i.e., PPCPs) at sites predominantly impacted by WWTPs tended not to exhibit large seasonal variations.
- Both resident and caged sunfish from seven sites in 2016 exhibited biological stress responses that may be attributable to CECs present in Maumee River water and sediment.[1,7]
    - Greater responses were seen at the downstream (urban) sites, and greater changes in resident fish compared to the caged fish.   CECs were found in every sunfish tissue sample analyzed in this study. Chronic exposure of agricultural and urban contaminants may alter sunfish anatomy and physiology, which could lead to population declines and altered ecosystem functioning (Cipoletti et al. 2020, in review).

- 21-day exposures of fathead minnows at embryonic, larval and adult life stages, in 2016 and 2017 to in-situ water samples from the same seven Maumee River sites where sunfish were assessed, resulted in biological effects that differed by life stage and by year.[1,7]
    - o Fecundity was the most sensitive variable measured in adults. Embryonic morphological development delays were seen in 2016 but not 2017. Contaminants were detected in every tissue sample, with six pesticides and eight pharmaceuticals detected in at least half of tissue samples. No clear upstream (agricultural) to downstream (urban) gradient was seen because many of the CECs measured were ubiquitous, occurring at multiple sites (Cipoletti et al. 2019; Figure 4).
- Dreissenid mussels exposed to Maumee River, accumulated measurable concentrations of current use pesticides common to corn, soybean and wheat agriculture including herbicides (metolachlor and atrazine, trifluralin, and atrazine transformation product desethylatrazine), and the insecticide chlorpyrifos, and its transformation product (chlorpyrifos oxon) (Kimbrough et al. 2018).[6]

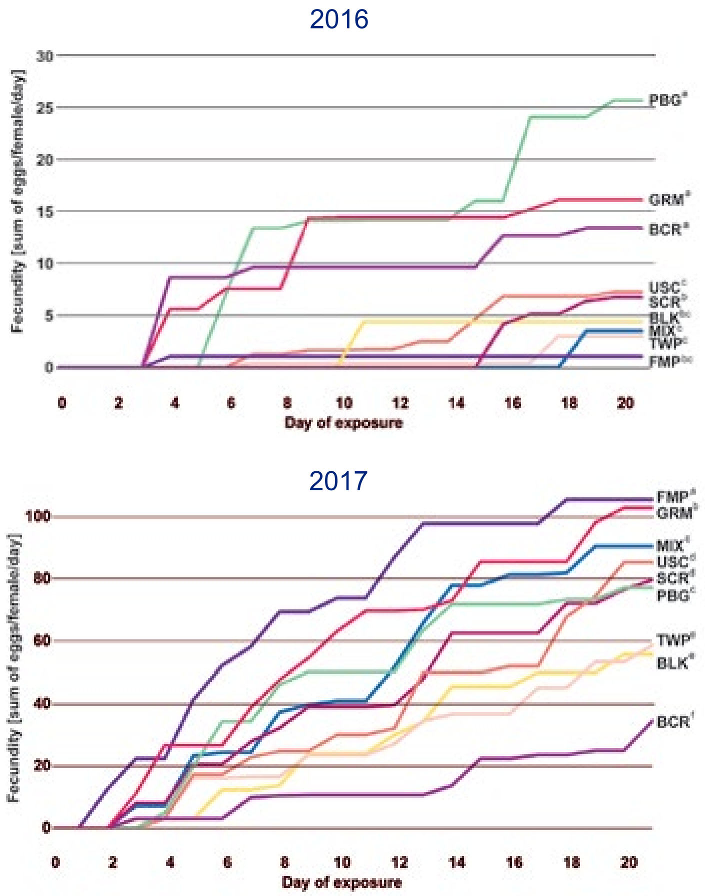


Figure 4: IACS fathead minnow exposure fecundity results. Each line corresponds to the site where water collections took place. Superscript letters following site code indicate significant differences in total fecundity if the letters are different and non-significant differences if the letters are the same. In both years some sites had significantly higher fecundity than the controls.

- Multiple lines of evidence indicate generalized physiological responses in fish to chemical stressors at sites throughout the Maumee watershed.[2]
    - o For example, enzymes related to the metexample, enzymes related to the metabolism of chemicals like pesticides and pharmaceuticals were elevated in fathead minnows caged for 4-days at several of the study sites. The nature of these responses could indicate adaptive/compensatory responses to the chemical exposures and, potentially, adverse impacts on population-relevant endpoints such as survival and growth.
    - o Overall, there was little evidence in the caged fish responses consistent with endocrine-disrupting chemicals like estrogens.

- PPCPs (n = 140) were rarely detected in tree swallow tissue or sediment samples from 6 sites along the Maumee River in 2016 and 2017.[5]

    - o Tissues analyzed included egg, liver, and diet samples all of which were pooled (n = 5 individuals per pool) by site. The same tissues were also sampled at one or two remote lakes in north central Wisconsin for comparative purposes. Of the 34 PPCPs detected in at least one sample, 26 were detected in at least two samples. The most frequently detected PPCP was DEET which was detected in nearly all liver tissue and sediment samples, but in only ~30% of egg samples. Iopamidol, a frequently used contrast agent by medical professionals, was detected in diet and liver samples, but not in egg or sediment samples. Interestingly, both DEET and Iopamidol were detected in samples from the remote lakes, as well as, from the more urbanized and populated Maumee River.

- Non-organochlorine pesticides (n = 34), which included triazine herbicides, fungicides, and organophosphate and carbamate insecticides, were rarely detected in tree swallow samples except for desethylatrazine, a metabolite of atrazine.[5]

    - o Desethylatrazine was detected in nearly all swallow egg, nestling carcass, and diet samples. It was only detected in the sediments at one of six sites. The parent compound, atrazine, was detected in diet samples at all Maumee River sites, but only at the most upstream sites in swallow tissues and sediment. Ametryn, another triazine herbicide, was detected, but in only one composite egg and one composite nestling sample over the 2-year study period. Neither the PPCPs nor these pesticides were expected to be detected in avian tissues because of their chemical composition and mode of action.

- Preliminary results for targeted metabolomics in dreissenid mussels exposed to the Maumee and Detroit Rivers, indicate differences in metabolite profiles associated with the specific location of sites relative to non-point source contamination and proximity to WWTPs.[6]

    - o Because responses to environmental variables may be dependent on life stage, sex, size or age, longitudinal studies require a deeper understanding of dreissenid mussel physiology over a season. Methodological (caging) influences may be important to acknowledge in the validation of dreissenid mussels as bioindicators in priority areas.


Constructing tree swallow nest boxes. Photo credit: USGS

- Preliminary anticholinesterase (AChE) biomarker response results in dreissenid mussels caged in the Maumee River showed severe inhibition, indicating the potential for significant neurotoxicity associated with pesticide exposures. Additionally, perturbation of the biomarker responses related to oxidative stress (glutathione and lipid peroxidation) was observed at some sites. [6]

    - Multiyear studies with dreissenid mussels facilitated the identification of "normal ranges" of biomarkers based on an extensive database, providing greater potential for diagnostic approaches that do not necessarily require a specific reference site as part of the monitoring strategy. Therefore more biomarker studies suggest the potential for wide-spread cellular and physiological impacts throughout the watershed that may be related to pesticide exposures.

- Twenty-one day exposure of native freshwater adult mussels in the Maumee River watershed (2017: Sp. plain pocketbook) and Milwaukee River watershed (2017 and 2018: Spp. plain pocketbook, fat mucket, and spike) to in-situ water did not result in significant differences in biological responses along the exposure gradients. However, temporal differences were seen in sperm production and CEC presence in Milwaukee. Limited sample numbers and high variability did not allow trends to be elucidated for the endpoints (behaviors, fatty acids, glycogen, teste development, and sperm density) and species assessed (Rzodkiewicz et al. 2019, in review).[1]

- Native freshwater mussel health assessment was completed in the Maumee River in 2016. Preliminary results revealed although mussel CEC tissue concentrations significantly differed by site, glycogen concentrations and egg and sperm counts showed no relationship to CEC concentrations. This indicates glycogen and gamete counts are not useful indicators when assessing CEC exposure. Native freshwater mussels bio-accumulate some CECs which could impact health and lead to population impairments.[1]

- Preliminary analyses suggest that Great Lakes tributaries with similar overall chemistry profiles did not have the same changes in gene expression related to hepatocyte toxicity in cell lines (including liver cancer pathways), suggesting overall chemistry profiles are not predictive of potential liver toxicity.[3]

- Preliminary analyses suggest that no developmental abnormalities were observed in zebrafish embryos exposed to surface waters from the Genesee River, Saginaw River, Indiana Harbor and the Oswego River.[3]

- Preliminary analyses suggest that concentrated pollutants at levels greater than those seen in the environment at sites within the Kinnickinnic River and Menomonee River, may have impacts on zebrafish embryo survival and zebrafish embryos with developmental abnormalities may be observed.[3]

Some of the species found during the native freshwater mussel health assessment (fatmucket and rayed bean). Photo credit: Daelyn Woolnough

Priority Contaminant Mixtures (PCM)

Much of the known toxicity data on CECs is for single chemical analyses, which is not representative of environmental conditions. Complex mixtures of CECs are present in the environment from a variety of sources such as wastewater treatment plant effluent, combined sewer overflows, and runoff. Projects first worked towards defining chemicals and their relevant environmental concentrations from the surveillance program data. Phase I surface water data were assessed to determine which chemicals and at what concentrations are most common across the Great Lakes Basin. Two diverse mixtures emerged; chemicals predominantly found in agricultural landscapes (atrazine, DEET, TBEP, bromacil, estrone, BPA, nonylphenol), chemicals predominantly found in urban landscapes (sulfamethoxazole, fexofenadine, desvenlafaxine, metformin, DEET, TBEP, estrone, HHCB, Methyl-1H-benzotriazole, BPA, nonylphenol). The results of this work were used to make environmentally relevant chemical mixtures that could be used in a laboratory setting to conduct exposure toxicity assessments (Elliott et al. 2018).

Fathead minnows (a model small fish species) were exposed to those defined chemical mixtures over multiple generations to characterize biological endpoints and to help better understand if CECs are the source of observed effects in the IACS. Native freshwater mussels were exposed to the same chemical mixtures, during peak female reproductive periods and monitored through larval attachment on host fish, concluding when juveniles dropped detached from fish for a total of 100 day study
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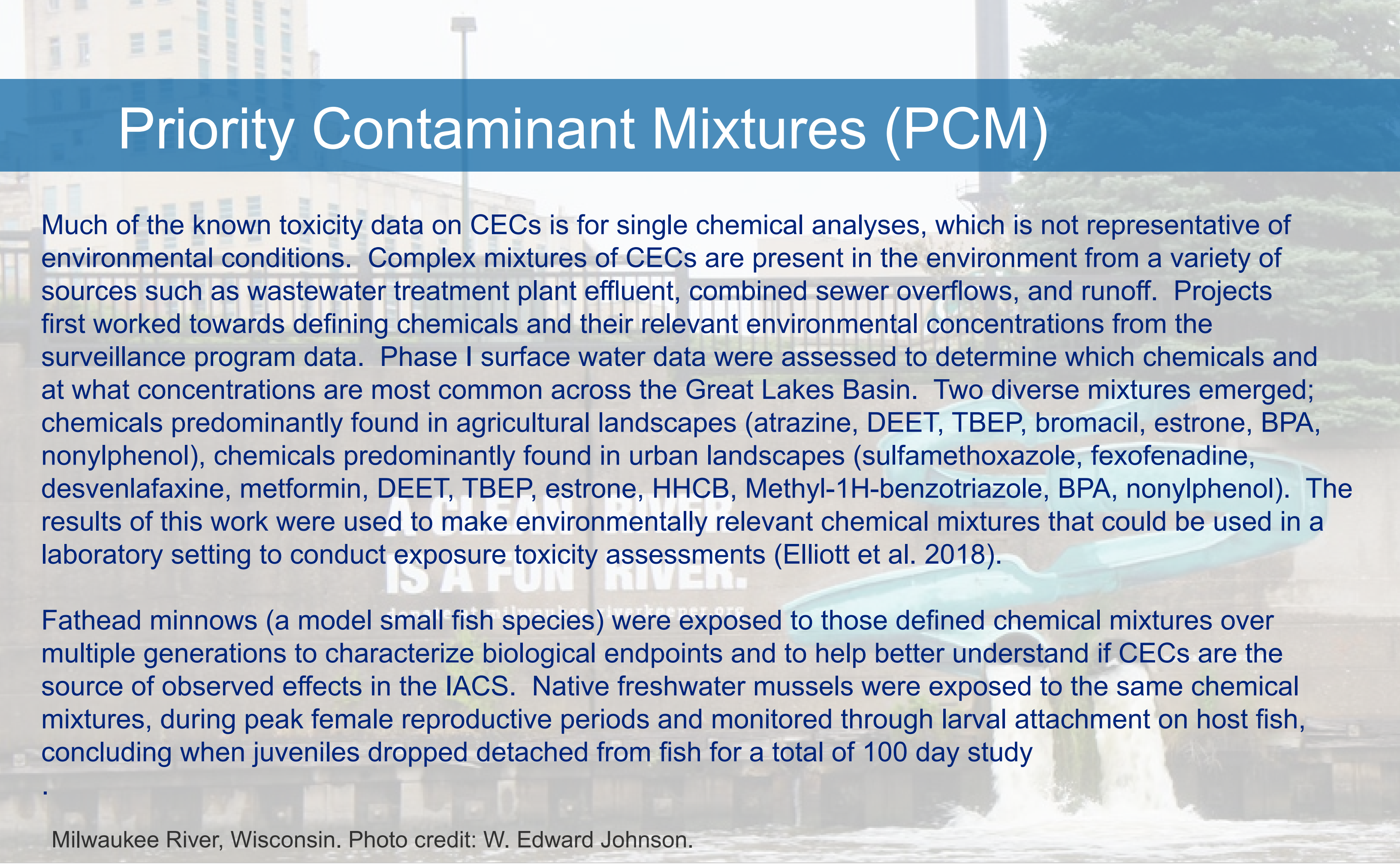
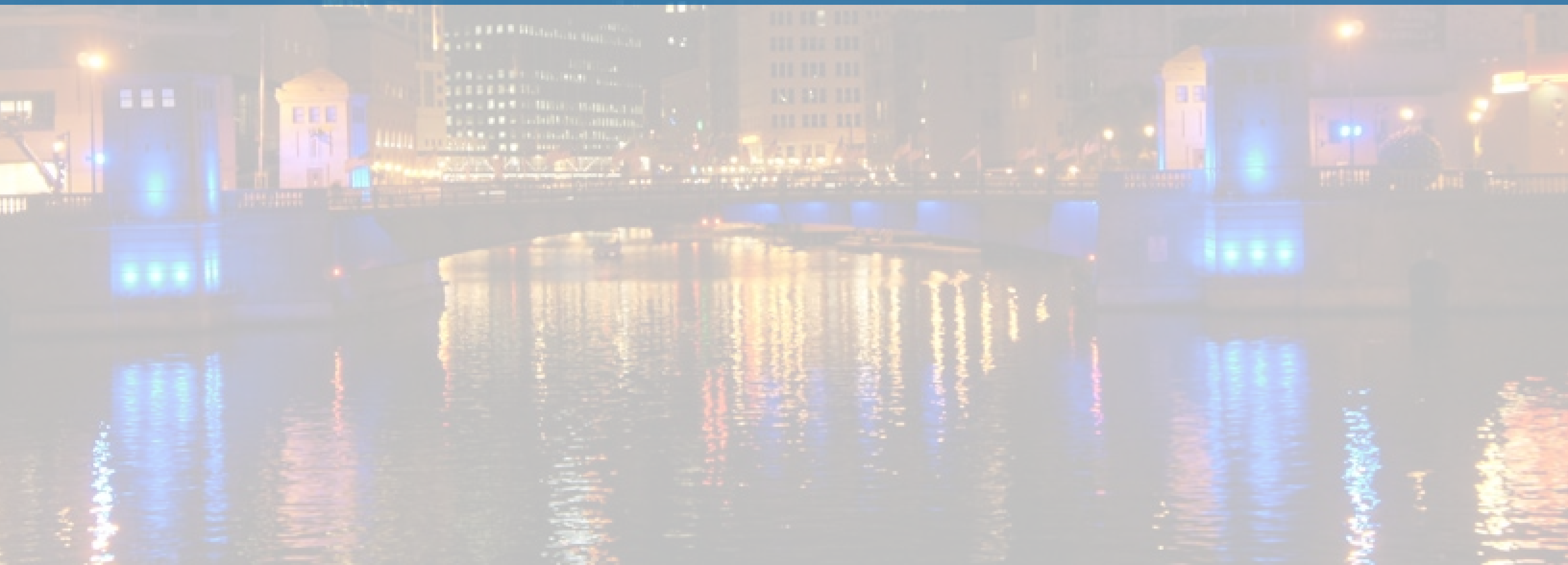


Milwaukee River, Wisconsin. Photo credit: W. Edward Johnson.

Key Findings:

- When fathead minnows were exposed in 2016 across three generations to a statistically derived mixture of CECs commonly occurring in Great Lakes tributaries associated with urban land-use (Elliott et al. 2018), physiological and reproductive changes were noted. In the first generation exposed to the CEC mixtures, an egg-yolk protein was present in males at concentrations usually seen only in female fish. However, this effect was suppressed in the subsequent exposure generation. Female fathead minnows exposed in the second generation to higher concentrations of urban CECs, experienced declining fertility. (Lina Wang, 2017. MS Thesis, St. Cloud State University).[1,7]
- Adult native mussel health assessments of glycogen, fatty acids, and behavior were not found to be sensitive to the evaluated CEC mixes and concentrations. Additionally, CEC life cycle exposures were performed on native plain pocketbook early mussel life stages (i.e., embryos in vivo and post spawn-parasitic glochidia on exposed largemouth bass host fish) to assess population relevant endpoints. Trials in 2017 and 2018 suggest sensitivity of early native mussel life stages to both agricultural and urban exposures with delay of transformation from glochidia to free living juveniles. (Gill, Rappold, and Rzodkiewicz, 2019. MS Theses, Central Michigan University).[1]

Products Associated With Key Findings In This Progress Report



Milwaukee River, Wisconsin. Photo credit: Kimani Kimbrough.

Surveillance Program

Completed Reports and Publications

All data collected as part of the surveillance program are available:
USGS Water Data for the Nation. 1 October 2019. https://waterdata.usgs.gov/nwis.

Banda, J.A., D.J. Gefell, V. An, A. Bellamy, Z. Biesinger, J. Boase, J. Chiotti, D. Gorsky, T. Robinson, S. Schlueter, J. Withers, S. L. Hummel. 2020. Contaminants of emerging concern: Body burden characterization in lake sturgeon. Manuscript in review.

Baldwin, A.K., Corsi, S.R., Oliver, S.K., Lenaker, P.L., Nott, M.A., Mills, M.A., Norris, G.A., Paatero, P., 2020. Primary sources of PAHs to Great Lakes tributaries using multiple lines-of-evidence, Environmental Toxicology and Chemistry, in press.

Corsi, S.R., L.A. De Cicco, D.L. Villeneuve, B.R. Blackwell, K.A. Fay, G.T. Ankley, A.K. Baldwin. 2019. Prioritizing chemicals of ecological concern in Great Lakes tributaries using high-throughput screening data and adverse outcome pathways. Science of The Total Environment 686, 995–1009. https://doi.org/10.1016/j.scitotenv.2019.05.457

De Cicco, L., S.R. Corsi, D.L. Villeneuve, B.R. Blackwell, G.T. Ankley. 2018. toxEval: Evaluation of measured concentration data using the ToxCast high-throughput screening database or a user-defined set of concentration benchmarks. R package version 1.0.0., https://code.usgs.gov/water/toxEval, <doi:10.5066/P906UQ5I>

Keisling, R. L., S. M. Elliott, L. E. Kammel, S.J. Choy, S. L. Hummel. 2019. Predicting the occurrence of chemicals of emerging concern in surface water and sediment across the U.S. portion of the Great Lakes Basin. Science of the Total Environment. 651:838-850. https://doi.org/10.1016/j.scitotenv.2018.09.201.

Kimbrough, K., W. E. Johnson, A. Jacob, M. Edwards, E. Davenport. 2018. Great Lakes Mussel Watch: Assessment of Contaminants of Emerging Concern. Silver Spring, MD. NOAA Technical Memorandum NOS NCCOS 249, 66 pp. https://repository.library.noaa.gov/view/noaa/19484.

Product Topics in Development

Basin wide assessment of pharmaceuticals in dreissenid mussels based on habitat type (river, nearshore, offshore).

Basin wide assessment of pesticides in dreissenid mussels based on habitat type (river, nearshore, offshore).

Using caged and in situ dreissenid mussels to characterize chemical contamination in agricultural and urban watersheds.

Legacy and contaminants of emerging concern (CECs) in tree swallows along an agricultural to industrial gradient: Maumee River, OH.

An assessment and classification of pharmaceuticals and personal care products (PPCPs) along the Great Lakes Basin Coastal Zone: Relationship to mixed land-use and point sources.

A multi-matrix assessment of pesticides environmental occurrence and their link to pollution gradients in the Maumee and Ottawa River watersheds and riverine systems.

A predictive analysis of chemicals of emerging concern (CECs) occurrence and distribution in the Great Lakes Basin coastal riverine systems and adjacent mixed land-use watersheds.

The distribution, seasonality, and potential biological effects of pesticides in Great Lakes tributaries.

Rouge River, Wisconsin. Photo credit: Kimani Kimbrough.

Integrated Assessment Case Studies

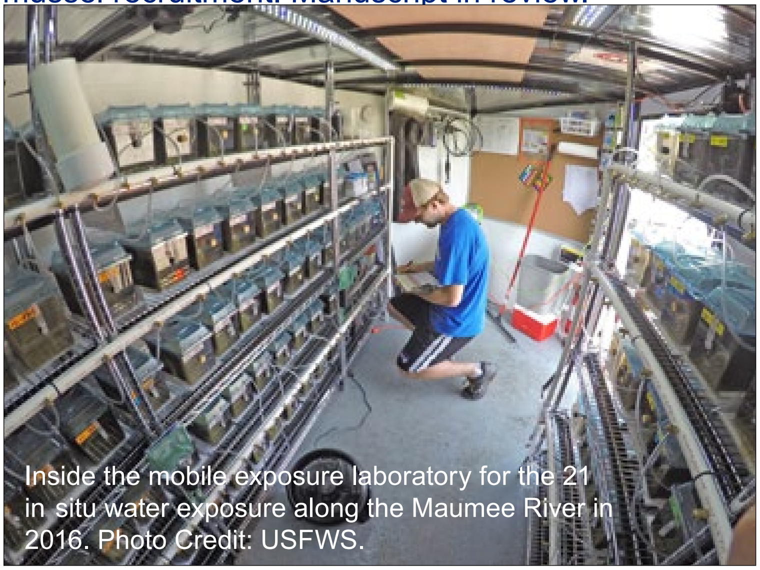
Completed Reports and Publications

Cipoletti, N., Z. G. Jorgenson, J. A. Banda, S. L. Hummel, S. Kohno, H. L. Schoenfuss. 2019. Land use contributions to adverse biological effects in a complex agricultural and urban watershed: A case study of the Maumee River. Environmental Toxicology and Chemistry. 38:1035-1051. https://doi.org/10.1002/etc.4409.

Cipoletti, N., Z. G. Jorgenson, J. A. Banda, S. L. Hummel, H. L. Schoenfuss. 2020. Impacts of Agricultural and Urban Land Use in the Maumee River Watershed on the Anatomy and Physiology of Caged and Resident Sunfish (Lepomis spp.) Manuscript in review.

Kimbrough, K., A. Jacob, E. Davenport, W.E. Johnson, M. Edwards. 2019. Characterization of Polycyclic Aromatic Hydrocarbon in the Great Lakes Basin using Driessenid Mussels. Manuscript in review.

Rzodkiewicz, L.D., M. Annis, S. L. Hummel, D. A. Woolnough. 2019. Contaminants of emerging concern may pose prezygotic barriers to freshwater mussel recruitment. Manuscript in review.


Inside the mobile exposure laboratory for the 21 in situ water exposure along the Maumee River in 2016. Photo Credit: USFWS.

Product Topics in Development

Using dreissenid mussels for targeted and untargeted metabolomics in place-based environmental health assessment of rivers with agricultural and urban influence.

Basin wide assessment of targeted and untargeted metabolomics in established populations of dreissenid mussels based on habitat type (river, nearshore, offshore).

Using cellular biomarkers in dreissenid mussels for place-based environmental health assessment of rivers with agricultural and urban influence.

Impacts of Agricultural and Urban Land Use in the Maumee River Watershed on the Anatomy and Physiology of Caged and Resident Sunfish (Lepomis spp.).

A case study of native freshwater mussel health in the Maumee River.

Assessments of CECs, including PPCP and non-organochlorine pesticides, in tree swallows nesting along the Maumee River, 2016 and 2017.

Assessment of targeted metabolomics in tree swallows based on habitat type and exposure to a range of CECs.

Novel pathway-based approaches for assessing biological hazards of complex mixtures of contaminants: Application to an integrated assessment of the Maumee River.

Priority Contaminant Mixtures

Completed Reports and Publications

Cipoletti, N. 2018. Complex agricultural mixtures: assessing effects on aquatic species (Pimphales promelas and Leomis spp.) through short-term field and multi-generational laboratory exposures. M.Sc. Thesis. St Cloud State University, St Cloud, Minnesota.

Elliott, S. M., M. E. Brigham, R. L. Kiesling, H. L. Schoenfuss. 2018. Environmentally relevant chemical mixtures of concern in waters of the United States tributaries to the Great Lakes. Integrated Environmental Assessment and Management. 9999:1-10. https://doi.org/10.1371/journal.pone.0182868.

Gill, S. 2019. Effects of a mixture of contaminants of emerging concern found in agricultural waterways on the freshwater mussel Lampsilis cardium and host fish Micropterus salmoides. M.Sc. Thesis. Central Michigan University, Mount Pleasant, Michigan.

Rappold, J.C. 2019. Effects of a mixture of urban contaminants of emerging concern on Lampsilis cardium in a laboratory setting and waste water treatment plant discharge influence on field deployed Amblema plicata from the Great Lakes Region. M.Sc. Thesis. Central Michigan University, Mount Pleasant, Michigan.

Rzodkiewicz, L.D. 2019. Contaminants of emerging concern exposure may alter unionid reproductive success. M.Sc. Thesis. Central Michigan University, Mount Pleasant, Michigan.

Wang, L. 2017. Three generational exposure of Pimphales promelas to an urban contaminants of emerging concern mixture. M.Sc. Thesis. St Cloud State University, St Cloud, Minnesota.

Product Topics in Development

Laboratory effects assessment of Agricultural and Urban CEC mixture exposure to fathead minnows over multiple generations .

Laboratory effects assessment of Agricultural and Urban CEC mixture exposures to native freshwater mussels from glochidia to larval stages and their host fish.

Native freshwater mussel microbiome changes with exposure to CEC mixtures.

Native freshwater mussel transformation rate modeling after exposure to CEC mixtures.

Understanding the Ecological Consequences of Ubiquitous Contaminants of Emerging Concern in the Laurentian Great Lakes Watershed: A Continuum of Evidence from the Laboratory to the Environment.

Maumee River 2016 Photo credit: W. Edward Johnson.

Tributary of the Ontonagon River, Michigan.
Photo credit: Barbara Corsi