Environmental Science & Technology

pubs.acs.org/est

Article

Untargeted Metabolomics Analyses and Contaminant Chemistry of Dreissenid Mussels at the Maumee River Area of Concern in the Great Lakes

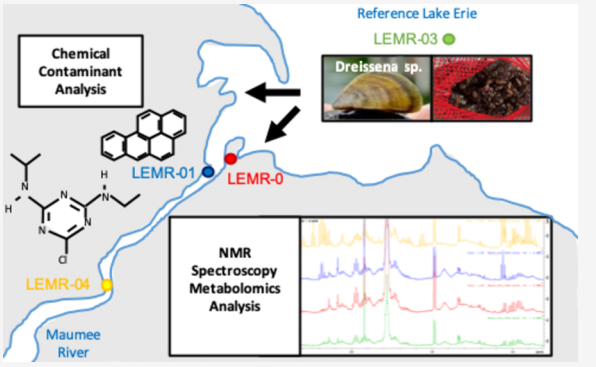
Elena Legrand,* Amanda L. Bayless, Daniel W. Bearden, Fabio Casu, Michael Edwards, Annie Jacob, W. Edward Johnson, and Tracey B. Schock*

Cite This: https://doi.org/10.1021/acs.est.3c00812

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Bivalves serve as an ideal ecological indicator; hence, their use by the NOAA Mussel Watch Program to monitor environmental health. This study aimed to expand the baseline knowledge of using metabolic end points in environmental monitoring by investigating the dreissenid mussel metabolome in the field. Dreissenids were caged at four locations along the Maumee River for 30 days. The mussel metabolome was measured using nuclear magnetic resonance spectroscopy, and mussel tissue chemical contaminants were analyzed using gas or liquid chromatography coupled with mass spectrometry. All Maumee River sites had a distinct mussel metabolome compared to the reference site and revealed changes in the energy metabolism and amino acids. Data also highlighted the importance of considering seasonality or handling effects on the metabolome at the time of sampling. The furthest upstream site presented a specific mussel tissue chemical signature of pesticides (atrazine and metolachlor), while a downstream site, located at Toledo's wastewater treatment plant, was characterized by polycyclic aromatic hydrocarbons and other organic contaminants. Further research into the dreissenid mussel's natural metabolic cycle and metabolic response to specific anthropogenic stressors is necessary before successful implementation of metabolomics in a biomonitoring program.



KEYWORDS: *biomonitoring, ecotoxicology, NMR, metabolomics, mollusc*

INTRODUCTION

Bioindicator species have been widely used to assess the ecological health of aquatic ecosystems. These organisms bioaccumulate contaminants, and their responses to changes in their environment (combining biotic and abiotic factors) are measured (e.g., physiological markers) to provide a snapshot of the environmental health for a determined time. Due to their wide geographic distribution, sessile nature, and filter-feeding strategy, mussels make favorable ecological indicators. Marine mussels *Mytilus* sp. are commonly used in biomonitoring programs worldwide.[1−3] As the equivalent of *Mytilus* in the marine environment, *Dreissena* sp. has been used as a bioindicator species in freshwater ecosystems.[4] For example, *Dreissena polymorpha* was used as a bioindicator species in the Saint Lawrence River, Canada;[5] in Lake Maggiore, Italy;[6] and in four French rivers.[7]

In the United States, the National Oceanic and Atmospheric Administration (NOAA) Mussel Watch Program (MWP) began monitoring the Great Lakes in 1992[8,9] when *Dreissena* sp. had become widely distributed across the basin following their discovery in 1988.[10] The Great Lakes Restoration Initiative (GLRI) was launched in 2010 to accelerate efforts to protect and restore Areas of Concern (AOCs) in the Great Lakes.

Biomonitoring and restoration initiatives have historically measured and reported the status and temporal trends of chemical contaminants in bivalve tissue, fish tissue, sediment, and water at AOCs nationwide, including the Great Lakes.[9,11,12] More recently, the MWP initiated the utilization of sublethal biological end points as indicators of ecosystem health in the presence of the routinely measured chemical contaminants. One example is the measurement of oxidatively induced genotoxicity in the dreissenid mussels.[13]

While not yet formally implemented into monitoring programs, high-throughput 'omics' technologies, such as genomics, transcriptomics, proteomics, and metabolomics, have the potential to characterize and quantify our under-

Received: April 30, 2023
Revised: October 30, 2023
Accepted: October 31, 2023

standing of organismal fitness from a molecular perspective. Among the 'omics', metabolomics reveals the phenotypic function through the study of low-molecular-weight (<1500 Da) metabolites in a biological sample (whole body, tissues, biofluids). Environmental metabolomics specifically focuses on detecting ecosystem health signatures via the system-wide biochemical changes of specific indicator species in response to environmental exposures.[14,15] As a result, a bioindicator metabolome may act as a proxy for the ecosystem health. Over the years, environmental metabolomics has successfully been applied to aquatic ecosystems and has differentiated impacted areas from nonimpacted ones. In particular, environmental metabolomics discriminated between different stressors in the context of multistressor exposures, thus showcasing its potential as a tool in the biomonitoring tool kit.[16] In addition to the perspective of defining certain ecosystem conditions (e.g., profiles of xenobiotic exposures), the identification of metabolic signatures may lead to elucidating contaminant mechanisms of action or become a valuable resource to monitor remediation efforts and risk assessment strategies via multitime point metabolic pathway evaluation.

In freshwater aquatic ecosystems, environmental metabolomics using dreissenid mussels is emerging in ecotoxicological evaluations. MWP used NMR-based metabolomics in a biomonitoring validation study in the Milwaukee River in Milwaukee, Wisconsin, which successfully distinguished dreissenid metabolic profiles from a contaminated and a reference site.[17] A recent study supported the potential of this monitoring strategy by identifying *D. polymorpha* metabolic profile differences between upstream and downstream sites in the vicinity of wastewater treatment plants (WWTPs) in the watershed of Meuse (France and Belgium).[18]

In efforts to build on these two *in situ* freshwater assessments and broaden the body of knowledge, the present study was conducted in the Great Lakes area along the Maumee River, Ohio, in Lake Erie to evaluate the ecosystem condition using mussels collected from a reference site and redeployed in cages for one month at sites of interest. In addition to the application of metabolomics analysis in this Maumee River study, complementary comprehensive chemical analyses of mussel tissues for legacy contaminants and chemicals of emerging concern were measured to describe the environmental condition contributing to the measured dreissenid metabolic signatures. When appropriate, contaminant concentrations were compared to data from previous years. Contaminants included polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), pharmaceuticals and personal care products (PPCPs), pesticides, and alkylphenols. These successive metabolomics research projects aim to progress metabolomics as a tool to evaluate the environmental health of global water systems, including the Great Lakes.

MATERIALS AND METHODS

Sampling Sites and Collection Procedures. The study evaluated four sites, three in the lower Maumee River and a reference site in Lake Erie (Figure S1). The reference site (REFERENCE-LEMR-03; Table S1) was located in Lake Erie, 13 km northeast of the Maumee River mouth at the small artificial island built in 1901 to support the Toledo Lighthouse. Due to the distance from the mouth of the river and the expected lower contamination burden relative to the other sites, this site was designated as a reference as we recognize that there are no truly pristine sites within the study area. The three river sites are located within an anthropogenically (agriculture, industrial, urban) impacted ecosystem and are described as follow: RIVER MOUTH-LEMR-0 (river-km 0), located at the river mouth between the dredged shipping channel and the southeast corner of Grassy Island; WWTP OUTFALL-LEMR-01 (river-km 1.9), located at the Toledo WWTP outfall; and UP RIVER-LEMR-04 (river-km 10), located along the eastern riverbank between the Anthony Wayne Bridge (Clayton St) and the Norfolk Southern railroad bridge.

Divers used stainless-steel scrapers to remove *Dreissena* sp. (approximately 22 ± 2 mm in length) from their attachment to rocks and sheet piles that protect the island lighthouse (REFERENCE-LEMR-03, May 23, 2016 (T0)). Mussels were then placed in a nylon mesh dive bag, gently shaken underwater to remove debris, and immediately placed in coolers with aerated site water upon surfacing. The mussels (approximately 300 to 500) were then placed in cages (torpedo minnow trap, Frabill, Plano, Illinois) and deployed the same day as collection on metal moorings approximately 0.5 m above the river/lake bottom at three locations along the river (Figure S1) and at the reference site (4 sites total). The cages were retrieved four weeks later, June 20 to June 22, 2016 (T1), and the mussels were divided into two sets for metabolomics and chemistry analysis.

Mussels collected for the metabolomics analysis (20−24/site) were collected from REFERENCE-LEMR- 03 at both time points to evaluate metabolic change after 30 days and included both *in situ* and caged mussels for a handling comparison (Table S1). Caged mussels were collected from the river sites at 30 days (Table S1) to assess the ecosystem health. Mussels were rinsed with site water to remove debris, wiped, bagged, and snap frozen in liquid nitrogen. The mussels were shipped via overnight carrier in a cryogenic dry shipper (about −150 °C) and stored at −80 °C upon receipt at the Hollings Marine Laboratory in Charleston, SC, until analysis. Mussels collected for chemical analysis (200/site to 400/site) were rinsed with site water to remove debris, placed in freezer bags, packed on water ice, and shipped to laboratories within 2 days.

Untargeted Metabolomics. *Sample Processing.* Individual frozen mussel tissues were removed from the shells by gently cracking the shell with a stainless-steel wrench in a liquid nitrogen cryocart (MVE CryoCart, Princeton Cryogenics, Inc.) to maintain frozen sample integrity during processing. Small remaining pieces of shell were gently removed using precleaned surgical scalpels. Ice found in the shell was kept to avoid any loss of tissue. Each individual mussel was then homogenized using a cryogenic ball-mill homogenizer (Cryomill, Retsch Inc.) with either a 25 or 35 mL grinding jar and 15 or 20 mm balls, respectively, for 2 min at 25 Hz. The homogenized tissue was placed in preweighed 2 mL cryovials using the Cryogenic Workstation. The individual experimental mussel samples were lyophilized (Heto Cooling Trap CT 110, ATR, Laurel, MD) in 2 mL cryovials for approximately 15 h, and 10 mg ± 1 mg of dry tissue was aliquoted into bead-beating tubes. The dried samples were stored at −80 °C before extraction.

Quality Control Materials. Quality assurance and quality control are critical parts of a metabolomics study assuring sample integrity is preserved, instrumentation is optimally performing, sample preparation and analysis are precise, and only high-quality data are analyzed in an overarching aim to communicate that the data are of high quality and reliable. For NMR-based metabolomics studies, a 2 mmol sucrose in a 10% D_2O sealed standard sample was used as a system suitability test

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(SST) before the analysis of experimental samples.[19] Control materials used to evaluate technical precision included a process blank, an in-house pooled mussel control material (MCM), an NIST standard reference material (SRM 2974a - Organics in freeze-dried mussel tissue), and a technical replicate added to each extraction batch. MCM was prepared using 81 additional mussel samples collected during the study. All individual MCM mussels were removed from the shell, homogenized, and lyophilized following the same protocol as for the experimental samples. The resulting dried homogenates were pooled and stored at −80 °C until extraction and analysis.

Metabolite Extraction. A total of 144 experimental samples were extracted in batches along with the QC materials described above using a chloroform:methanol:water technique modified from Wu et al.[20] The extraction protocol was derived from a previous study by Watanabe et al.[17] and is fully described in the Supporting Information (S.I. Methodology). Briefly, ice-cold polar solvent mix and frozen dried tissue samples were homogenized using the bead-beating homogenizer Precellys 24. The homogenates were then added to a cold solvent mix for a final solvent volume ratio of 2 chloroform: 2 methanol: 1.8 water. After incubation and centrifugation, the upper polar phase was dried by a vacuum centrifuge (Eppendorf) for 2 h at room temperature. The dried polar extracts were rehydrated with 600 μL of NMR buffer containing 100 mmol/L phosphate buffer, pH 7.3, 1 mmol/L TMSP (3-trimethylsilyl 2,2,3,3-d_4 propionate, CAS 24493-21-8), and 1 mg/mL NaN_3 (sodium azide, CAS 26627-22-8) prepared in D_2O, and 550 μL of each sample was then transferred into 5 mm NMR tubes (Bruker).

NMR Spectroscopy. All NMR spectra of mussel samples were collected at 298 K (calibrated) on a 700 MHz Bruker Avance II (Bruker Biospin) NMR spectrometer equipped with a 5 mm triple-resonance, z-gradient TCI cryoprobe. NMR spectra were acquired using an Icon-NMR (Bruker Biospin). An initial 10 min temperature equilibration period was followed by automated shimming with on-axis and off-axis shims, automated probe tuning/matching, and pulse calibration on each sample. One-dimensional (1D) ^{1}H NMR spectra were acquired with water suppression using a three-pulse sequence based on a standard one-dimensional (1D) NOESY pulse sequence (*noesygppr1d*). Two-dimensional (2D) edited ^{13}C heteronuclear single quantum coherence (HSQC) spectra with adiabatic ^{13}C decoupling (*hsqcedetgpsisp2.2*) were acquired on 12 selected samples (2 to 4 samples per sampling location) for metabolite identification. One and two-dimensional NMR experiments are similar to the ones previously described in Watanabe et al[17] and they are fully described in the Supporting Information (S.I. Methodology).

Statistical Analysis. Processed NMR spectra were binned using NMRProcFlow v1.2,[21] where an adaptive, intelligent binning procedure was carried out from 10 to 0.5 ppm using a signal-to-noise threshold of 3:1[22] and a resolution factor of 0.5. Residual water (4.84−4.76 ppm) and experimental contaminants (acetate 1.92 ppm) detected in the blanks were manually removed. Data were then imported to MetaboAnalyst 5.0[23] where spectra were normalized to total spectral intensity, and bins were mean-centered and Pareto-scaled before statistical analysis. Differences in the metabolome between sites, time points, and handling methods were visualized using principal component analysis (PCA). Differences between sites were further investigated by partial least-squares discriminant analysis (PLS-DA; MetaboAnalyst[23]). Pairwise comparisons between the river sites and the reference site were performed by PLS-DA. The resonances presenting a variable importance in projection (VIP) > 1.5 on the average of five components were selected as significant resonances leading to the metabolite identification. Since a database representative of mollusc is not available, identified metabolites that were driving the difference between sites were implemented in the pathway analysis against the zebrafish (*Danio rerio*) database instead in MetaboAnalyst.[23]

Metabolite Identification. Metabolites that distinguish dreissenid mussel metabolism between sites were assigned based on 1D ^{1}H and 2D ^{1}H, and ^{13}C-HSQC NMR experiments. Peaks were assigned by comparing the chemical shifts and spin−spin couplings with reference spectra found in databases such as the Human Metabolome Database (HMDB),[24] an in-house compiled database; Chenomx NMR Suite profiling software (Chenomx Inc. version 8.5); and a recent in-depth zebra mussel metabolome annotation.[18] Our annotations are consistent with other published assignments for dreissenid mussels.[17,25,26]

Chemical Analysis. *Mussel Chemistry.* Mussel tissue samples were analyzed for PAHs, PCBs, and PBDEs by TDI-Brooks International, Inc., and for PPCPs, pesticides, and akylphenols by SGS AXYS Analytical Services Ltd. (Canada). Detailed analytical methods for organic contaminant analysis in mussel tissue can be found in Kimbrough et al. for PCBs and in Kimbrough et al. for PDBEs.[27,28] Briefly, 50−100 mussels per site were shucked and homogenized. Aliquots of homogenized samples were chemically dried using Hydromatrix and extracted in dichloromethane using a Dionex Accelerated Solvent Extractor. The extracts were purified by using alumina/silica gel chromatography columns. The resultant eluent was further purified by using a gel permeation column coupled to a high-performance liquid chromatograph. The volume of the resultant eluent was reduced and analyzed for selected PAHs, PCBs, and PBDEs.

PAHs, including alkylated homologues, were analyzed using a gas chromatograph−mass spectrometer (GC-MS) (Agilent Technologies 5890-II and 5972-MSD) using an HP-5MS column (Agilent Technologies: 60 m × 0.25 mm ID and 0.25 μm film thickness) in selected ion mode (SIM). PBDEs were analyzed using the same GC-MS coupled to a DB-XLB column (Agilent Technologies: 30 m × 0.25 mm ID and 0.10 μm film thickness) in SIM, and the data were acquired with electron impact ionization. PCBs were analyzed using a gas chromatograph with an electron capture detector and two capillary columns (DB-5: 30 m × 0.25 mm ID and 25 μm film thickness; DB-17HT: 30 m × 0.25 mm ID and 0.15 μm film thickness).

Proprietary methods used by SGS AXYS for the measurement of PPCPs (MLA-075 R06.01), pesticides (MLA-035 R07.02 and MLA-037 R05) and alkyphenols (MLA-080 R02.04) are available by contacting the laboratory and are based on EPA methods 1964 and 1699.[29,30] The compounds that were analyzed are listed in the Supporting Information (Tables S2−S7). Reported concentrations were blank subtracted. Reported concentrations for PAHs, PCBs, and PBDEs were greater than the adjusted method detection limit (MDL). Alkylphenols, PPCPs, and pesticides with reported concentrations were greater than three times the MDL.

RESULTS

Metabolomic Analysis. *Quality Control.* The system suitability test of the NMR showed that the instrument was performing within expected specifications (data provided in the SI) allowing for the analysis of experimental samples. Data produced from metabolite extraction and metabolite measure-

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

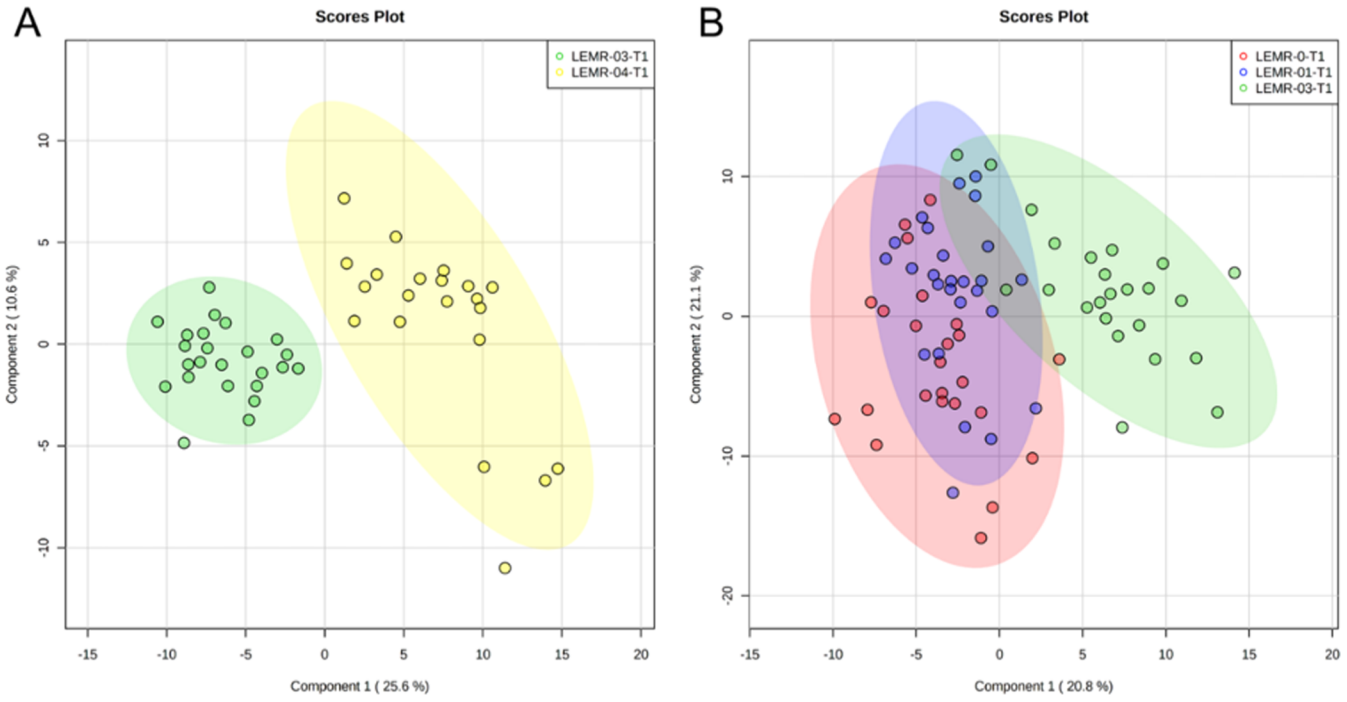


Figure 1. Partial least-squares discriminant analysis scores plot of the ^{1}H NMR spectra of dreissenid mussels at T1 between (A) the upstream site UP RIVER-LEMR-04 and the reference site REFERENCE-LEMR-03 and (B) downstream sites RIVER MOUTH-LEMR-0 and WWTP OUTFALL-LEMR-01 and the reference site REFERENCE-LEMR-03. For figure visibility purposes, only the sampling location site code names (LEMR-0 to 04) are displayed on the figure.

ment have a high degree of reproducibility as shown by the low median spectral relative standard deviation (RSD) for both quality control materials, MCM (3.87%), and NIST SRM 2974a (2.58%) (Figure S2), providing confidence in the data for analysis.

Site Comparison of Caged Mussels. The untargeted metabolomic profiles postcaging (T1) showed distinct differences in the dreissenid mussel metabolomes between the reference site (REFERENCE-LEMR-03) and the farthest upstream site (UP RIVER-LEMR-04; Figure S3, PC2 explained variance 17.4%), with a total explained variance in PC1 and PC2 of 45.8%. The PCA also illustrated that WWTP OUTFALL-LEMR-01 and RIVER MOUTH-LEMR-0 mussels presented similar metabolic profiles, which differ from the reference site REFERENCE-LEMR-03 (Figure S3).

The variation in the mussel metabolome across sites was further investigated by PLS-DA and PCA, which illustrated a clear distinction between the reference site (REFERENCE-LEMR-03) and the most upstream site (UP RIVER-LEMR-04; Figures 1A and S4A). The analysis by PLS-DA also confirmed that the mussel metabolomes at RIVER MOUTH-LEMR-0 and WWTP OUTFALL-LEMR-01 were similar (data overlap) and collectively differed from the downstream reference site, REFERENCE-LEMR-03 (Figures 1B and S4B−D). Both PLS-DA models were validated by the good performance values for the 10-fold cross-validation in the comparison between UP RIVER-LEMR-04 and REFERENCE-LEMR-03 (R^2 = 0.97 and Q^2 = 0.90) and in the comparison between RIVER MOUTH-LEMR-0, WWTP OUTFALL-LEMR-01, and REFERENCE-LEMR-03 (R^2 = 0.91 and Q^2 = 0.76). Both models had an estimated empirical p-value < 0.01 by 100 permutation tests.

According to the PLS-DA, 29 ^{1}H NMR resonances (VIP score > 1.5) distinguished REFERENCE-LEMR-03 from UP RIVER-LEMR-04 at T1 (Figure S5A, Table S8) and 25 resonances differed between REFERENCE-LEMR-03, WWTP OUTFALL-LEMR-01, and RIVER MOUTH-LEMR-0 at T1 (Figure S5B, Table S9). The resonance at 2.41 ppm (bin B2_4072, succinate) had the highest VIP score in both comparisons (Figure S5) with an average of 12.93 (REFERENCE-LEMR-03 vs UP RIVER-LEMR-04, Table S8) and 12.80 (REFERENCE-LEMR-03 vs WWTP OUTFALL-LEMR-01 vs RIVER MOUTH-LEMR-0, Table S9). A total of 11 resonances commonly had a VIP > 1.5 in both comparisons.

Metabolite annotation resulted in the putative identification (level 2)[31] of 13 and 8 metabolites contributing to the difference in the metabolome between mussels caged at REFERENCE-LEMR-03 and UP RIVER-LEMR-04, and between mussels caged at REFERENCE-LEMR-03, WWTP OUTFALL-LEMR-01, and RIVER MOUTH-LEMR-0, respectively (Table 1). The metabolite annotations were applied to a study of the biochemical pathways contributing to metabolic differences between sites.

Although not statistically significant (−log(p-value) < 1, pathway impact < 0.1), amino acid metabolism and energy pathways represent differences between the river sites and the reference site of the study REFERENCE-LEMR-03 (UP RIVER-LEMR-04, Figure S6A.; WWTP OUTFALL-LEMR-01 and RIVER MOUTH-LEMR-0; Figure S6B). Many similar pathways were impacted in each site comparison. However, the starch and sucrose metabolism pathways were only impacted between UP RIVER-LEMR-04 and REFERENCE-LEMR-03, and had the greatest pathway impact value, suggesting involvement in the metabolic differences between the upstream site and the reference site.

Analysis of Sampling Time and Handling Methods. Mussel metabolomes were compared between two different sampling times (T0, May 2016; T1, June 2016) and two different handling methods (caged, *in situ*) at the reference site. The dreissenid

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Table 1. Putative Metabolite Identification (Level 2) of the ^{1}H NMR Resonances That Contributed to the Maumee River Site Differences[a]

RIVER MOUTH-LEMR-0	WWTP OUTFALL-LEMR-01	UP RIVER-LEMR-04	Annotation	^{1}H (ppm)	^{13}C (ppm)	Multiplicity
+	+	+	leucine	0.95	14.02	d
		+	valine	1.05	20.79	d
+	+	+	threonine/lactate	1.33	22.43	d
+	+	+	alanine	1.49	19.04	d
−	−	−	putrescine	1.78	26.64	m
−	−		NA	1.97	25.98	m
+	+	+	succinate	2.41	37.06	s
−	−		NA	2.45	34.96	m
−	−	−	putrescine	3.05	41.61	m
−	−	−	malonate	3.11	55.5	s
+	+		NA	3.48		
		+	threonine/valine	3.59	63.20	d
		−	maltose	3.67	79.78	t
		−	maltose	3.70	75.7	t
		−	maltose/glucose	3.83	63.33	dd
		−	maltose/glucose	3.87	63.30	dd
		−	maltose	3.91	63.52	dd
		−	maltose	3.97	76.23	t
		−	adenosine monophosphate	4.02	66.41	m
−	−		adenosine monophosphate	4.41	86.8	m
−	−		adenosine monophosphate	4.52	73.53	m
		+	glucose + maltose + glucose-6-phosphate	5.24	94.89	d
-	-		adenosine monophosphate	8.62	143.09	s

[a]Increased or decreased metabolite concentrations were based on the PLS-DA result (VIP > 1.5) (Figure S5) and are indicated by "+" and "−" in comparison to the reference site (REFERENCE-LEMR-03). Metabolites without a concentration designation change were nonsignificant for the respective site. Nonannotated (NA) resonances were unable to be identified. 1H Multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet, and dd = doublet of doublets.

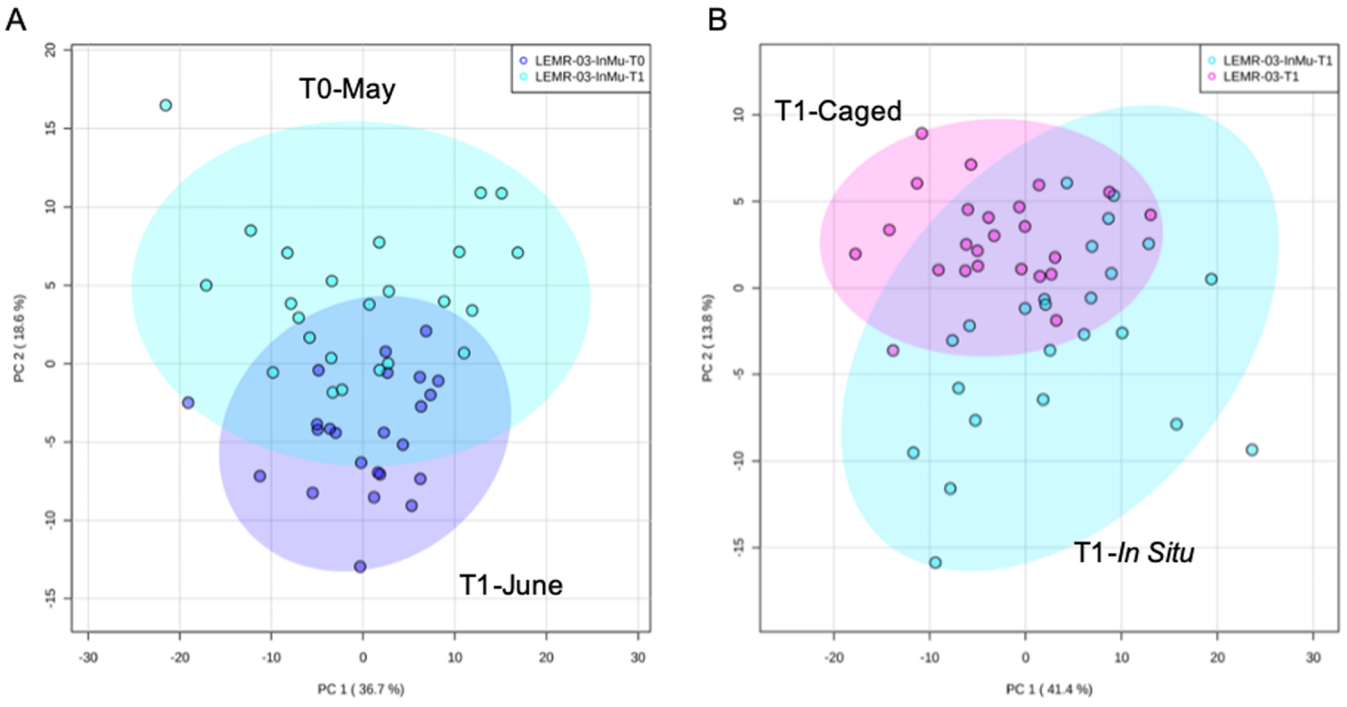


Figure 2. Principal component analysis scores plot of ^{1}H NMR spectra of dreissenid mussels at the REFERENCE-LEMR-03 site in the Maumee River (A) at two different sampling times in situ and (B) response to two different handling techniques. For figure visibility purposes, only the sampling location site code names (LEMR-0 to 04) are displayed on the figure.

mussel metabolome showed subtle differences in the time of sampling, 30 days apart (REFERENCE-LEMR-03-InMu-T0 vs REFERENCE-LEMR-03-InMu-T1; Figure 2A) and method of handling at the same sampling time point (*in situ*: REFER-

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

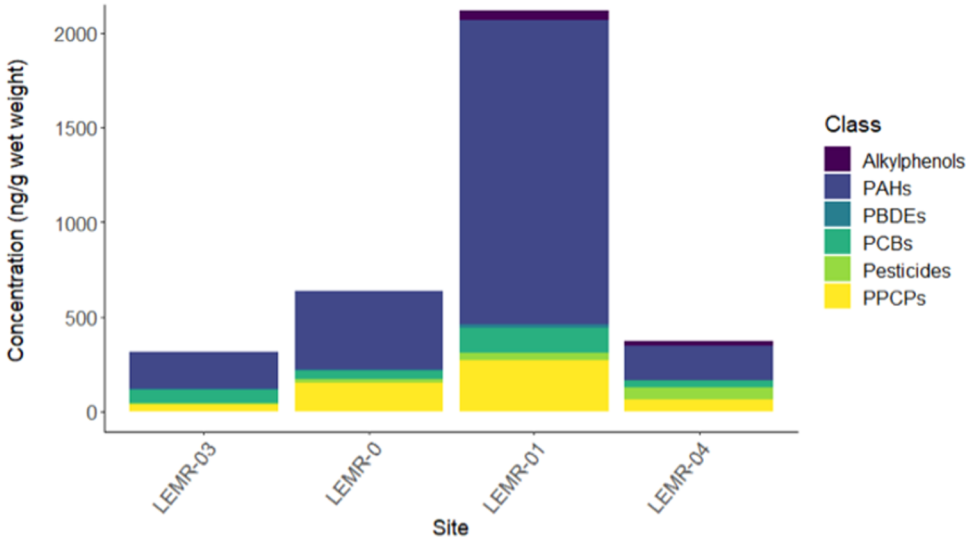


Figure 3. Cumulative concentration of contaminant chemicals (by class) measured in freshwater mussel tissue at each site. Detected analytes were included if greater than the adjusted MDL for PAHs, PCBs, and PBDEs or if greater than three times the MDL for alkylphenols, PPCPs, and pesticides. For figure visibility purposes, only the sampling locations site code names (LEMR-0 to 04) are displayed on the figure. Full sampling names are REFERENCE-LEMR-03, RIVER MOUTH-LEMR-0, WWTP OUTFALL-LEMR-01, and UP RIVER-LEMR-04.

ENCE-LEMR-03-InMu-T1; caged: REFERENCE-LEMR-03-T1; Figure 2B) with a total combined explained variance in PC1 and PC2 of 55.3 and 55.2%, respectively.

Chemical Exposure. *Mussel Chemistry.* Six chemical classes (PAHs, PBDEs, PCBs, alkylphenols, pesticides, and PPCPs) were measured in caged mussels at each site, and the concentrations (>MDLs) were summed to examine the total contaminant load (Figure 3). Concentrations for each individual contaminant are presented in the Supporting Information (Tables S2−S7). A similar number of chemicals per class were detected in the caged mussels at all sites, except for PPCPs which included 12 compounds detected in mussels at WWTP OUTFALL-LEMR-01 versus two at REFERENCE-LEMR-03, and four at both RIVER MOUTH-LEMR-0 and UP RIVER-LEMR-04. PCBs and PAHs had the highest numbers of chemicals identified in the mussel tissue with 55 to 71 and 40 to 55 analytes, respectively. Mussels caged at UP RIVER-LEMR-04 presented the lowest summed analyte concentration, whereas mussels caged at WWTP OUTFALL-LEMR-01 showed the highest summed analyte concentration (Figure 3). PAHs contributed the most to the summed concentration at each site. The highest concentration of all chemical classes, except pesticides, was measured in the mussels caged at WWTP OUTFALL-LEMR-01 (alkylphenols: 53.21 ng/g wet weight (ww), PAHs: 1608 ng/g ww, PBDEs: 17.8 ng/g ww, PCBs: 134 ng/g ww, and PPCPs: 268 ng/g ww). A gradual increase in pesticide concentration was measured in mussels from the reference site REFERENCE-LEMR-03 (8.08 ng/g ww) to the upstream sites RIVER MOUTH-LEMR-0 (17.6 ng/g ww), WWTP OUTFALL-LEMR-01 (38.2 ng/g ww), and UP RIVER-LEMR-04 (58.7 ng/g ww). While 18 pesticide compounds were detected at UP RIVER-LEMR-04, the sum was dominated by two herbicides: atrazine (32.8 ng/g ww) and metolachlor (10.5 ng/g ww). The alkylphenol 4-nonylphenol also presented a higher concentration (12.7 ng/g ww) at UP RIVER-LEMR-04 than other sites.

DISCUSSION

Environmental metabolomics has risen over the past 20 years.[32] However, challenges remain for including this promising ecosystem assessment technique in biomonitoring. This study demonstrated the ability to distinguish dreissenid mussel metabolome profiles between a reference site (REFERENCE-LEMR-03) and sites along the Maumee River (RIVER MOUTH-LEMR-0, WWTP OUTFALL-LEMR-01, UP RIVER-LEMR-04), an anthropogenically (agriculture, industrial, urban) impacted ecosystem that includes a wastewater treatment plant (WWTP) outfall and a series of combined sewer outfalls. The chemicals detected in the mussel tissue from the Maumee River provide context for the observed changes in mussel biochemistry, particularly in amino acid and energy metabolism pathways. Additionally, the subtle variability observed in the dreissenid mussel metabolome at two different sampling time points over a 1 month window and in response to caging methods highlights the need to consider seasonal cycles and handling effects in mussel ecotoxicology studies.

Metabolomics. Effect-based monitoring and surveillance have been implemented in the ecotoxicological tool kit to assess the health of AOCs in the Great Lakes, in addition to traditional chemical monitoring.[33] Stressor discrimination remains challenging in environmental monitoring, though metabolomics is a promising analytical technique to differentiate chemical-dependent effects[16] and previous work successfully discriminated the metabolome of dreissenid mussels between a clean and a polluted site in Lake Michigan.[17] In this study, we observed variation in the freshwater mussel metabolome along the Maumee River (Figures 1, S3, and S4). The greatest metabolomic differences were observed between the Lake Erie reference site (REFERENCE-LEMR-03) and the upstream site (UP RIVER-LEMR-04). Multiple studies have demonstrated upstream−downstream effects in the fish metabolome, including a study with upstream sites characterized by farm and agricultural activities.[34−36]

In the present study, the metabolome did not exhibit clear changes between the three upstream river sites despite the agricultural contamination signature of pesticides at UP RIVER-

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

LEMR-04 and the distinctive WWTP effluent-derived contaminants at downstream sites, WWTP OUTFALL-LEMR-01 and RIVER MOUTH-LEMR-0. WWTP OUTFALL-LEMR-01 had a higher total concentration of PAHs, PCBs, PBDEs, and PPCPs (Figure 3), but the mussel metabolome was very similar to the individuals at RIVER MOUTH-LEMR-0 (Figures 1B, S3, and S4B) where contaminant concentrations were lower. A Maumee River caged sunfish study reported similar results where the biological effects could not always be associated with the total chemical load.[34] In contrast, the metabolome of crayfish tissue was distinct between wastewater and agricultural contamination.[37] Without sediment and water quality data for the present study, it is difficult to distinguish between real-time contaminant exposure, noncontaminant abiotic stressors, and food quality. These may all play a role in the interindividual metabolic variability, resulting in the lack of a metabolic distinction between upstream and downstream sites. Additionally, experimental factors such as caging, length of exposure, and seasonality of mussel metabolism may also obscure upstream and downstream site differences. The present holistic analysis does provide insight into the mussel tissue accumulation of contaminants over 30 days, illuminates specific contaminants of concern for this AOC, and identifies altered metabolic pathways when comparing contaminated river sites to a reference site.

Contaminant Chemistry. Chemical contamination in the Great Lakes region has often been evaluated through the analysis of freshwater mussels.[8,9,38] As part of the MWP efforts under GLRI, the chemical contaminant content of mussel tissue was characterized for the present study. Pesticide cumulative concentrations in mussel tissue gradually increased from reference (REFERENCE-LEMR-03) to upstream, reaching the highest concentration at UP RIVER-LEMR-04 (Figure 3). Historically, large agricultural pesticide loads to Lake Erie have been measured in several tributaries most notably the Maumee.[39,40]

Although current agricultural management practices have reduced runoff of pesticides and nutrients, recent monitoring of contaminant mixtures in 69 tributaries of the Great Lakes found the highest levels of pesticides in the Maumee River.[41] In this study, atrazine and metolachlor, which are mainly used on one of the most prominent crops grown in the Maumee watershed (corn), were the main contributors to the elevated concentration observed in mussel tissue at UP RIVER-LEMR-04 (Table S7). Another 2016 Maumee River study found that atrazine and metolachlor had the highest average water concentration (21.2 and 8.28 μg/L, respectively) of all of the contaminants measured in June,[42] which is above the US EPA's aquatic life benchmark for chronic exposure to metolachlor (1 μg/L).[43]

In addition, herbicide concentrations reported in previous years (2010 and 2014) showed that the Maumee River had the highest maximum exposure−activity ratios (EARs), which is a ratio based on the ToxCast assay and database, of all 69 sites measured in the Great Lakes.[41,44] The mussels are chronically exposed to these pesticides; therefore, physiological impacts are expected at UP RIVER-LEMR-04. Environmentally relevant concentrations of atrazine (0.03 to 3.8 μg/L), which are lower than the previous Maumee River measurements,[42] have been shown to cause genotoxicity, inhibit acetylcholinesterase, and increase detoxification enzymes in the green mussel (*Perna viridis*).[45] These herbicides may also have a significant impact on the mussel's diet and food availability since zebra mussels primarily feed on algae. The lower concentration of atrazine at WWTP OUTFALL-LEMR-01 and absence of both pesticides at RIVER MOUTH-LEMR-0 and REFERENCE-LEMR-03 suggest that atrazine becomes degraded or diluted downstream of UP RIVER-LEMR-04.

In contrast to pesticides, the highest cumulative mussel tissue concentration for PAHs, PCBs, PBDEs, PPCPs, and alkylphenols was found downstream at WWTP OUTFALL-LEMR-01 (Figure 3). WWTP OUTFALL-LEMR-01 is located at Toledo's WWTP outfall, a suspected source of these contaminants. PPCPs have also been measured in the drinking water originating from Lake Erie wastewater treatment effluents.[46] In addition, legacy contaminants were expected in this area since it has been a major industrial port since 1850.[47]

Total PAHs (from 81 measured analytes) in mussel tissue remain high (1.6 μg/g dry weight) compared to Mussel Watch data from 2009 to 2018, which presented 2.79 μg/g dry weight as a high concentration for total PAHs (65 analytes) in the Great Lakes.[38] Similarly, these elevated concentrations were found at the riverine and inshore sites. The PAHs measured at WWTP OUTFALL-LEMR-01 are concerning since caged mussels in PAH contaminated areas have been associated with oxidative stress, detoxification, and DNA damage.[48,49] On the contrary, total PCBs in the mussel tissue were considered low (39.1 ng/g dry weight to 134 ng/g dry weight) and have shown a decreasing trend in mussels and sediment throughout the US.[50] However, they persist in sediments, and our results suggest a potential for bioaccumulation during the 30-day exposure. Their lipophilic nature makes them prone to biomagnification, and subsequent human consumption constitutes a major concern.

In addition to high levels of PAHs, the total PBDE concentrations measured in mussel tissue (15.6 ng/g dry weight to 157.8 ng/g dry weight) were considered medium and high in the Maumee River sites when compared to the rest of the US.[28] The sum of PPCPs in the Maumee River mussel tissue at WWTP OUTFALL-LEMR-01 (268.4 μg/g ww) was within the concentration range found for the unionid mussel (*Lasmigona costata*) in another Great Lakes river, Grand River in ON, Canada (217.8 μg/g ww to 1088.4 μg/g ww).[51] In particular, the antidepressant sertraline contributed to almost half of the total PPCP concentration found at WWTP OUTFALL-LEMR-01. These levels were higher than the concentrations measured in the dreissenid mussel tissue sampled at that approximate location in June 2015 (101 ng/g ww in 2016 vs 61.7 ng/g ww in 2015).[12] In aquatic invertebrates, environmentally relevant concentrations of selective serotonin reuptake inhibitor, such as sertraline, have been shown to impact behavior, defense mechanisms, attachment to substrate, development, and reproduction.[52] Although sertraline was detected at concentrations below the median effect and lethal concentrations (EC_{50} 20 μg/L, LC_{50} 40 μg/L) determined for the juvenile unionid mussel *Lampsilis siliquoidea*[53,54] and at only two of the 69 sites in the Great Lakes during 2010 and 2014 analyses,[41] water concentrations may have increased since 2010.

Alkylphenols, which elicit estrogenic and carcinogenic effects on organisms, can bioaccumulate as well.[55] The sum of phenols measured in mussel tissue reached 53.2 ng/g of ww at WWTP OUTFALL-LEMR-01, but 4-nonylphenol (4-NP) was present at higher levels in mussels from UP RIVER-LEMR-04 (12.7 ng/g ww). A decrease in testosterone has been observed in zebra mussels with 4-NP concentrations 15 times (200 ng/g ww) the levels found in the current study.[56] An important consideration is that the surfactant properties of alkylphenols can exacerbate the toxicity of other types of contaminants on zebra mussels.[57]

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Overall, these results highlight the importance of the toxicity threshold characterization of chemicals of concern for biomonitoring species. With the elevated concentrations observed for two pesticides at UP RIVER-LEMR-04 and the increased levels observed for PPCPs, PAHs, and PBDEs at the downstream sites, impacts on the mussel's metabolic pathways were anticipated.

Impact of Contaminants and Environment on Mussel Metabolism. Stress response to contaminants is commonly observed in environmental metabolomics analyses.[32] In the present study, metabolites related to energy metabolism and amino acid metabolism were impacted in the mussels caged in the Maumee River (Table 1, Figure S6). Energy metabolism was impacted in all sites of the Maumee River when compared to the reference site; however, the tricarboxylic acid (TCA) cycle was more impacted in the comparison between downstream sites WWTP OUTFALL-LEMR-01 and RIVER MOUTH-LEMR-0 (Figure S6). This result might reflect the greater PAH concentration at WWTP OUTFALL-LEMR-01 than at the other sites. In the pearl oyster (*Pinctada martensii*), benzo(a)-pyrene exhibited disruptive effects on energy metabolism and osmotic regulation.[58,59] In addition to PAHs, PPCPs present in the Maumee River could have affected the energy metabolism of freshwater mussels. Several PPCPs (fluoxetine, diphenhydramine, 17α-ethynylestradiol (EE2), and N,N-diethyl-meta-toluamide) have exhibited energy metabolism alteration in the marine eastern oyster (*Crassostrea virginica*) via the disruption of Krebs cycle metabolites, fatty acids, and amino acids.[60]

Several abiotic factors are also known to influence the health of an ecosystem. In the present study, the increase in alanine and succinate (Table 1) could indicate a shift from aerobic to anaerobic metabolism in the freshwater mussel,[61] which suggests that the mussel's environment could be hypoxic. Water quality has been an issue in Lake Erie since the 1990s, and the Maumee River is a known contributor of phosphorus load into Lake Erie.[62] Nutrient discharge, which can lead to eutrophication, is likely derived from the Toledo WWTP at WWTP OUTFALL-LEMR-01 or from agricultural application. Impact on amino acid metabolism has been reported in the blue mussel[63,64] in hypoxic conditions. In particular, Tuffnail et al. (2009) reported an increase in succinate and valine and a decrease in leucine, which were also altered in the present study. Bivalves are resistant to hypoxia. However, unlike their marine counterparts, the freshwater mussel *D. polymorpha* may not be a good oxygen regulator.[65] Therefore, such water conditions could negatively affect the health of this organism. In the context of a monitoring program, we suggest that water quality indicators (e.g., dissolved oxygen level, temperature, and turbidity) should be measured at the collecting sites to fully assess the environmental health of the species of interest.

Metabolomics is a sensitive measurement that reflects environmental changes and variation in diet. The agricultural and urban contamination in the Maumee River has the potential to influence food resources.[66] Here, sucrose and starch metabolism was the most impacted pathway between the reference and upstream sites (Figure S6). Reduced levels of adenosine monophosphate have been associated with food limitation in the freshwater mussel *Amblema plicata*[67] In the present study, purine metabolism and adenosine monophosphate were altered, with a notable decrease in adenosine monophosphate concentration, supporting the hypothesis that food limitation could have contributed to the overall impact on the freshwater mussel health. These results showed that the chemical body burden in the mussel alone represents only a limited evaluation of the health of an ecosystem and that additional complementary measures should be included.

Ideally, biomonitors would provide a link between metabolic effects and specific contaminant or environmental signatures. The only metabolic distinction observed was between the study sites and the reference site, but no significant metabolic changes were revealed among the river sites where distinct contaminant signatures were detected. Here, metabolomic extractions were performed on whole freshwater mussels, which gives a holistic view of organism health. Stressor-dependent effects may be tissue specific,[37] however, and could have been diluted or masked by a whole-body metabolome analysis. To further validate dreissenids as metabolomic bioindicators, whole-organ and organ-specific tissue analyses can be compared at reference and impacted sites. Additionally, validation of the freshwater mussels as metabolomic bioindicators requires a full understanding of both the natural metabolic cycle through seasons and the effect of external factors, such as handling, in order to assess a health state indicative of environmental exposure. While subtle differences were observed when mussels were caged in the present study (Figure 2), the water quality differences between sites may have a greater impact on the mussel metabolome, suggesting that caging mussels may constitute a suitable handling technique for biomonitoring studies. The metabolic variation induced by temporal and seasonal changes should also be monitored closely to determine whether seasonality or reproductive cycles confound field studies similar to the one presented here. This Maumee River study was part of a larger multiagency effort to assess current-use pesticides as a contaminant class and their possible correlation with observable biological effects.

■ ASSOCIATED CONTENT

Data Availability Statement

Metabolomics data have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gkz1019, PMID:31691833) with identifier MTBLS6311. The complete data set can be accessed here https://www.ebi.ac.uk/metabolights/MTBLS6311.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c00812.

Detailed methodology, metabolite extraction, NMR Spectroscopy, and metabolomics quality control; collection sites in the Maumee River (Figure S1); PCA scores plot and spectral RSD 1H spectra of experimental dreissenid mussels and control materials (Figure S2) PCA scores plot of dreissenid mussels at different sites and times (Figures S3−S4); top 30 NMR resonances with VIP scores of different sites (Figure S5); pathway analysis of caged mussels between sites (Figure S6); site and NMR sample information (Table S1); PAHs, PCBs, PBDEs, alkylphenols, PPCPs, and pesticides measured in mussel tissue (Tables S2−S7); and NMR resonance VIP scores in the five components (Tables S8 and S9) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Elena Legrand − *National Institute of Standards and Technology, Hollings Marine Laboratory, Charleston, South Carolina 29412, United States;* Present Address: Stantec

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Consulting Ltd., 300−1331 Clyde Avenue, Ottawa, Ontario K2C 3G4, Canada; orcid.org/0000-0002-0473-2220; Email: elena.legrand@stantec.com

Tracey B. Schock − *National Institute of Standards and Technology, Hollings Marine Laboratory, Charleston, South Carolina 29412, United States*; Email: tracey.schock@nist.gov

Authors

Amanda L. Bayless − *National Institute of Standards and Technology, Hollings Marine Laboratory, Charleston, South Carolina 29412, United States*

Daniel W. Bearden − *National Institute of Standards and Technology, Hollings Marine Laboratory, Charleston, South Carolina 29412, United States;* Present Address: Metabolomics Partners, 1065 Fronie Dr., Nesbit, Mississippi 38651, United States.

Fabio Casu − *National Institute of Standards and Technology, Hollings Marine Laboratory, Charleston, South Carolina 29412, United States*

Michael Edwards − *National Oceanic and Atmospheric Administration, National Centers for Coastal Ocean Science, Silver Spring, Maryland 20910, United States*

Annie Jacob − *Consolidated Safety Services, Fairfax, Virginia 22030, United States;* Present Address: U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., N.W., Washington, DC 20460, United States.

W. Edward Johnson − *National Oceanic and Atmospheric Administration, National Centers for Coastal Ocean Science, Silver Spring, Maryland 20910, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.3c00812

Author Contributions

Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J. Beyond authorship: attribution, contribution, collaboration, and credit. *Learned Publishing* **2015**, *28* (2), 151−155. 10.1087/20150211 Conceptualization: D.W.B., W.E.J.; Methodology: D.W.B., F.C., W.E.J.; Formal Analysis: E.L.; Investigation: D.W.B., F.C., M.E., A.J., W.E.J.; Data Curation: A.L.B., D.W.B., E.L., T.B.S.; Writing − Original Draft: D.W.B., E.L., F.C., A.J., W.E.J., T.B.S.; Writing − Review and Editing: All; Visualization: A.L.B., M.E., E.L.; Supervision, Project Administration and Funding Acquisition: DB, WEJ, TBS.

Funding

Funding for this work was provided by the Great Lakes Restoration Initiative Grant: EPA/GLRI IA# DW-013-92524901. Authority for work: Clean Water Act: (i) P.L 114−113 (Consolidated Appropriations Act, 2016), Title II, Division G, Section 426 (12/18/2015) and (ii) P.L. 114−322 (Water Infrastructure Improvements for the Nation Act) Section 5005 (12/16/2016)

Notes

Any mention of commercial products is to specify adequately the analytical procedures used. It does not imply recommendation or endorsement by NIST, or that the products mentioned are the necessarily the best available for the intended purpose. The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Boat and logistical support were provided by NOAA's Great Lakes Environmental Research Laboratory, US EPA Office of Research and Development (Cincinnati, OH), and Cherie Blair, Maumee Area of Concern lead for the GLRI and Ohio EPA. NOAA scuba diving support was provided by Andrew Mason and Beau Braymer. Student support was provided by Tuan Phan and Char'Mane Robinson. Sample preparation and NMR spectral processing support was provided by Kehau Hagiwara.

■ REFERENCES

(1) Bajt, O.; Ramšak, A.; Milun, V.; Andral, B.; Romanelli, G.; Scarpato, A.; Mitrić, M.; Kupusović, T.; Kljajić, Z.; Angelidis, M.; Çullaj, A.; Galgani, F. Assessing Chemical Contamination in the Coastal Waters of the Adriatic Sea Using Active Mussel Biomonitoring with *Mytilus Galloprovincialis*. *Mar. Pollut. Bull.* **2019**, *141*, 283−298.

(2) Tsangaris, C.; Kormas, K.; Strogyloudi, E.; Hatzianestis, I.; Neofitou, C.; Andral, B.; Galgani, F. Multiple Biomarkers of Pollution Effects in Caged Mussels on the Greek Coastline. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2010**, *151*, 369−378.

(3) Walker, T. R.; Macaskill, D.; Weaver, P. Blue Mussels (*Mytilus Edulis*) as Bioindicators of Stable Water Quality in Sydney Harbour during Remediation of the Sydney Tar Ponds, Nova Scotia, Canada. *Water Qual. Res. J. Canada* **2013**, *48* (4), 358−371.

(4) Binelli, A.; Della Torre, C.; Magni, S.; Parolini, M. Does Zebra Mussel (*Dreissena Polymorpha*) Represent the Freshwater Counterpart of *Mytilus* in Ecotoxicological Studies? A Critical Review. *Environ. Pollut.* **2015**, *196*, 386−403.

(5) de Lafontaine, Y.; Gagné, F.; Blaise, C.; Costan, G.; Gagnon, P.; Chan, H. M. Biomarkers in Zebra Mussels (*Dreissena Polymorpha*) for the Assessment and Monitoring of Water Quality of the St Lawrence River (Canada). *Aquat. Toxicol.* **2000**, *50*, 51−71.

(6) Ricciardi, F.; Binelli, A.; Provini, A. Use of Two Biomarkers (CYP450 and Acetylcholinesterase) in Zebra Mussel for the Biomonitoring of Lake Maggiore (Northern Italy). *Ecotoxicol. Environ. Saf.* **2006**, *63*, 406−412.

(7) Kerambrun, E.; Rioult, D.; Delahaut, L.; Evariste, L.; Pain-Devin, S.; Auffret, M.; Geffard, A.; David, E. Variations in Gene Expression Levels in Four European Zebra Mussel, *Dreissena Polymorpha*, Populations in Relation to Metal Bioaccumulation: A Field Study. *Ecotoxicol. Environ. Saf.* **2016**, *134*, 53−63.

(8) Robertson, A.; Lauenstein, G. G. Distribution of Chlorinated Organic Contaminants in Dreissenid Mussels along the Southern Shores of the Great Lakes. *J. Great Lakes Res.* **1998**, *24* (3), 608−619.

(9) Kimbrough, K. L.; Johnson, W. E.; Jacob, A. P.; Lauenstein, G. G. Contaminant Concentration in Dreissenid Mussels in the Laurentian Great Lakes A Summary of Trends from the Mussel Watch Program. In *Quagga and Zebra Mussels: Biology, Impacts, and Control*, 2nd ed., 2013.

(10) Hebert, P. D. N.; Muncaster, B. W.; Mackie, L. Ecological and Genetic Studies on *Dreissena Polymorpha* (Pallas): A New Mollusc in the Great Lakes. *Can. J. Fish. Aquat. Sci.* **1989**, *46*, 1587−1591.

(11) Apeti, D. A.; Lauenstein, G. G.; Christensen, J. D.; Kimbrough, K.; Johnson, W. E.; Kennedy, M.; Grant, K. G. A Historical Assessment of Coastal Contamination in Birch Harbor, Maine Based on the Analysis of Mussels Collected in the 1940s and the Mussel Watch Program. *Mar. Pollut. Bull.* **2010**, *60* (5), 732−742.

(12) Kimbrough, K.; Johnson, W. E.; Jacob, A.; Edwards, M.; Davenport, E. *Great Lakes Mussel Watch: Assessment of Contaminants of Emerging Concern NOAA National Centers for Coastal Ocean Science Stressor Detection and Impacts Division. Silver Spring, MD. NOAA Technical Memorandum NOS NCCOS 249*, 2018.

(13) Jaruga, P.; Coskun, E.; Kimbrough, K.; Jacob, A.; Johnson, W. E.; Dizdaroglu, M. Biomarkers of Oxidatively Induced DNA Damage in Dreissenid Mussels: A Genotoxicity Assessment Tool for the Laurentian Great Lakes. *Environ. Toxicol.* **2017**, *32* (9), 2144−2153.

(14) Viant, M. R. Recent Developments in Environmental Metabolomics. *Mol. Biosyst.* **2008**, *4* (10), 980−986.

(15) Simpson, A. J.; McNally, D. J.; Simpson, M. J. NMR Spectroscopy in Environmental Research: From Molecular Interactions to Global Processes. *Prog. Nucl. Magn. Reson. Spectrosc.* **2011**, *58*, 97−175.

(16) Pomfret, S. M.; Brua, R. B.; Izral, N. M.; Yates, A. G. Metabolomics for Biomonitoring: An Evaluation of the Metabolome as an Indicator of Aquatic Ecosystem Health. *Environ. Rev.* **2020**, *28* (1), 89−98.
(17) Watanabe, M.; Meyer, K. A.; Jackson, T. M.; Schock, T. B.; Johnson, W. E.; Bearden, D. W. Application of NMR-Based Metabolomics for Environmental Assessment in the Great Lakes Using Zebra Mussel (*Dreissena Polymorpha*). *Metabolomics* **2015**, *11* (5), 1302−1315.
(18) Hani, Y. M. I.; Prud'Homme, S. M.; Nuzillard, J. M.; Bonnard, I.; Robert, C.; Nott, K.; Ronkart, S.; Dedourge-Geffard, O.; Geffard, A. 1H-NMR Metabolomics Profiling of Zebra Mussel (*Dreissena Polymorpha*): A Field-Scale Monitoring Tool in Ecotoxicological Studies. *Environ. Pollut.* 2021, *270*. DOI: 10.1016/j.envpol.2020.116048.
(19) Kirwan, J. A.; Gika, H.; Beger, R. D.; Bearden, D.; Dunn, W. B.; Goodacre, R.; Theodoridis, G.; Witting, M.; Yu, L. R.; Wilson, I. D. Quality Assurance and Quality Control Reporting in Untargeted Metabolic Phenotyping: MQACC Recommendations for Analytical Quality Management. *Metabolomics.* **2022**, *18*, 70.
(20) Wu, H.; Southam, A. D.; Hines, A.; Viant, M. R. High-Throughput Tissue Extraction Protocol for NMR- and MS-Based Metabolomics. *Anal. Biochem.* **2008**, *372*, 204.
(21) Jacob, D.; Deborde, C.; Lefebvre, M.; Maucourt, M.; Moing, A. NMRProcFlow: A Graphical and Interactive Tool Dedicated to 1D Spectra Processing for NMR-Based Metabolomics. *Metabolomics* **2017**, *13*, 1−5, DOI: 10.1007/s11306-017-1178-y.
(22) De Meyer, T.; Sinnaeve, D.; Van Gasse, B.; Tsiporkova, E.; Rietzschel, E. R.; De Buyzere, M. L.; Gillebert, T. C.; Bekaert, S.; Martins, J. C.; Van Criekinge, W. NMR-Based Characterization of Metabolic Alterations in Hypertension Using an Adaptive, Intelligent Binning Algorithm. *Anal. Chem.* **2008**, *80* (10), 3783−3790.
(23) Pang, Z.; Chong, J.; Zhou, G.; De Lima Morais, D. A.; Chang, L.; Barrette, M.; Gauthier, C.; Jacques, P. É.; Li, S.; Xia, J. MetaboAnalyst 5.0: Narrowing the Gap between Raw Spectra and Functional Insights. *Nucleic Acids Res.* **2021**, *49*, W388−W396.
(24) Wishart, D. S.; Tzur, D.; Knox, C.; Eisner, R.; Guo, A. C.; Young, N.; Cheng, D.; Jewell, K.; Arndt, D.; Sawhney, S.; Fung, C.; Nikolai, L.; Lewis, M.; Coutouly, M. A.; Forsythe, I.; Tang, P.; Shrivastava, S.; Jeroncic, K.; Stothard, P.; Amegbey, G.; Block, D.; Hau, D. D.; Wagner, J.; Miniaci, J.; Clements, M.; Gebremedhin, M.; Guo, N.; Zhang, Y.; Duggan, G. E.; MacInnis, G. D.; Weljie, A. M.; Dowlatabadi, R.; Bamforth, F.; Clive, D.; Greiner, R.; Li, L.; Marrie, T.; Sykes, B. D.; Vogel, H. J.; Querengesser, L. HMDB: The Human Metabolome Database. *Nucleic Acids Res.* **2007**, *35*, D521−D526.
(25) Prud'homme, S. M.; Hani, Y. M. I.; Cox, N.; Lippens, G.; Nuzillard, J.-M.; Geffard, A. The Zebra Mussel (Dreissena Polymorpha) as a Model Organism for Ecotoxicological Studies: A Prior 1H NMR Spectrum Interpretation of a Whole Body Extract for Metabolism Monitoring. *Metabolites* **2020**, *10* (6), 256.
(26) Hani, Y. M. I.; Prud'Homme, S. M.; Nuzillard, J.-M.; Bonnard, I.; Robert, C.; Nott, K.; Ronkart, S.; Dedourge-Geffard, O.; Geffard, A. 1H-NMR Metabolomics Profiling of Zebra Mussel (Dreissena Polymorpha): A Field-Scale Monitoring Tool in Ecotoxicological Studies. *Environ. Pollut.* **2021**, *270*. 116048.
(27) Kimbrough, K. L.; Lauenstein, G. G. *Major and Trace Element Analytical Methods of the National Status and Trends Program: 2000−2006. Silver Spring, MD. NOAA Technical Memorandum NOS NCCOS 29*, 2006.
(28) Kimbrough, K. L.; Johnson, W. E.; Lauenstein, G. G.; Christensen, J. D.; Apeti, D. A.. *Mussel Watch Program NOAA National Status & Trends an Assessment of Polybrominated Diphenyl Ethers (PBDEs) in Sediments and Bivalves of the U.S. Coastal Zone. Silver Spring, MD NOAA Technical Memorandum NOS NCCOS 94*, 2009.
(29) US EPA. *EPA Method 1699 2007 Pesticides in Water, Saoil, Sediment, Biosolids, and Tissue by HRGC-HRMS*, 2007.
(30) US EPA. *Method 1694 : Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS*, 2007.
(31) Sumner, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W.-M.; Fiehn, O.; Goodacre, R.; Griffin, J. L.; Hankemeier, T.; Hardy, N.; Harnly, J.; Higashi, R.; Kopka, J.; Lane, A. N.; Lindon, J. C.; Marriott, P.; Nicholls, A. W.; Reily, M. D.; Thaden, J. J.; Viant, M. R. Proposed Minimum Reporting Standards for Chemical Analysis. *Metabolomics* **2007**, *3* (3), 211−221.
(32) Zhang, L.-J.; Qian, L.; Ding, L.-Y.; Wang, L.; Wong, M. H.; Tao, H.-C. Ecological and Toxicological Assessments of Anthropogenic Contaminants Based on Environmental Metabolomics. *Environ. Sci. Ecotechnology* **2021**, *5*, 100081.
(33) Ekman, D. R.; Ankley, G. T.; Blazer, V. S.; Collette, T. W.; Garcia-Reyero, N.; Iwanowicz, L. R.; Jorgenson, Z. G.; Lee, K. E.; Mazik, P. M.; Miller, D. H.; Perkins, E. J.; Smith, E. T.; Tietge, J. E.; Villeneuve, D. L. Biological Effects−Based Tools for Monitoring Impacted Surface Waters in the Great Lakes: A Multiagency Program in Support of the Great Lakes Restoration Initiative. *Environ. Pract.* **2013**, *15*, 409−426.
(34) Cipoletti, N.; Jorgenson, Z. G.; Banda, J. A.; Kohno, S.; Hummel, S. L.; Schoenfuss, H. L. Biological Consequences of Agricultural and Urban Land-Use along the Maumee River, a Major Tributary to the Laurentian Great Lakes Watershed. *J. Great Lakes Res.* **2020**, *46*, 1001−1014.
(35) Curtis-Quick, J. A.; Ulanov, A. V.; Li, Z.; Bieber, J. F.; Tucker-Retter, E. K.; Suski, C. D. Why the Stall? Using Metabolomics to Define the Lack of Upstream Movement of Invasive Bigheaded Carp in the Illinois River. *PLoS One* **2021**, *16* (10), e0258150.
(36) Skelton, D. M.; Ekman, D. R.; Martinović-Weigelt, D.; Ankley, G. T.; Villeneuve, D. L.; Teng, Q.; Collette, T. W. Metabolomics for in Situ Environmental Monitoring of Surface Waters Impacted by Contaminants from Both Point and Nonpoint Sources. *Environ. Sci. Technol.* **2014**, *48*, 2395−2403.
(37) Izral, N. M.; Brua, R. B.; Culp, J. M.; Yates, A. G. Crayfish Tissue Metabolomes Effectively Distinguish Impacts of Wastewater and Agriculture in Aquatic Ecosystems. *Sci. Total Environ.* **2021**, *760*, 143322.
(38) Kimbrough, K. L.; Jacob, A.; Regan, S.; Davenport, E.; Edwards, M.; Leight, A. K.; Freitag, A.; Rider, M.; Johnson, W. E. Characterization of Polycyclic Aromatic Hydrocarbons in the Great Lakes Basin Using Dreissenid Mussels. *Environ. Monit. Assess.* **2021**, *193* (12), 116 DOI: 10.1007/s10661-021-09401-7.
(39) Richards, R. P.; Baker, D. B. Pesticide Concentration Patterns in Agricultural Drainage Networks in the Lake Erie Basin. *Environ. Toxicol. Chem.* **1993**, *12* (1), 13−26.
(40) Peter Richards, R.; Baker, D. B.; Kramer, J. W.; Ellen Ewing, D. Annual Loads of Herbicides in Lake Erie Tributaries of Michigan and Ohio. *J. Great Lakes Res.* **1996**, *22* (2), 414−428.
(41) Alvarez, D. A.; Corsi, S. R.; De Cicco, L. A.; Villeneuve, D. L.; Baldwin, A. K. Identifying Chemicals and Mixtures of Potential Biological Concern Detected in Passive Samplers from Great Lakes Tributaries Using High-Throughput Data and Biological Pathways. *Environ. Toxicol. Chem.* **2021**, *40* (8), 2165−2182.
(42) Ankley, G. T.; Berninger, J. P.; Blackwell, B. R.; Cavallin, J. E.; Collette, T. W.; Ekman, D. R.; Fay, K. A.; Feifarek, D. J.; Jensen, K. M.; Kahl, M. D.; Mosley, J. D.; Poole, S. T.; Randolph, E. C.; Rearick, D.; Schroeder, A. L.; Swintek, J.; Villeneuve, D. L. Pathway-Based Approaches for Assessing Biological Hazards of Complex Mixtures of Contaminants: A Case Study in the Maumee River. *Environ. Toxicol. Chem.* **2021**, *40* (4), 1098−1122.
(43) EPA. National Recommended Water Quality Criteria - Aquatic Life Criteria Table.
(44) Richard, A. M.; Judson, R. S.; Houck, K. A.; Grulke, C. M.; Volarath, P.; Thillainadarajah, I.; Yang, C.; Rathman, J.; Martin, M. T.; Wambaugh, J. F.; Knudsen, T. B.; Kancherla, J.; Mansouri, K.; Patlewicz, G.; Williams, A. J.; Little, S. B.; Crofton, K. M.; Thomas, R. S. ToxCast Chemical Landscape: Paving the Road to 21st Century Toxicology. *Chem. Res. Toxicol.* **2016**, *29* (8), 1225−1251.
(45) Juhel, G.; Bayen, S.; Goh, C.; Lee, W. K.; Kelly, B. C. Use of a Suite of Biomarkers to Assess the Effects of Carbamazepine, Bisphenol A, Atrazine, and Their Mixtures on Green Mussels, Perna Viridis. *Environ. Toxicol. Chem.* **2017**, *36* (2), 429−441.

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(46) Kim, H.; Homan, M. Evaluation of Pharmaceuticals and Personal Care Products (PPCPs) in Drinking Water Originating from Lake Erie. *J. Great Lakes Res.* **2020**, *46*, 1321−1330.
(47) US Environmental Protection Agency (US EPA). *Maumee River Great Lakes Area of Concern Information*. US Environmental Protection Agency: Chicago, IL. Available from: https://www.epa.gov/great-lakes-aocs/maumee-aoc (accessed 20 November 2023).
(48) Cheung, C. C. C.; Zheng, G. J.; Li, A. M. Y.; Richardson, B. J.; Lam, P. K. S. Relationships between Tissue Concentrations of Polycyclic Aromatic Hydrocarbons and Antioxidative Responses of Marine Mussels, *Perna Viridis*. *Aquat. Toxicol.* **2001**, *52* (3−4), 189−203.
(49) André, C.; Pilote, M.; Gagnon, C.; Gagné, F. Ecotoxicological Impacts of Oil Sand Mining Activity to Endemic Caged Mussels *Pyganodon Grandis*. *Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol.* **2022**, *251*, 109193 DOI: 10.1016/j.cbpc.2021.109193.
(50) Kimbrough, K. L.; Johnson, W. E.; Lauenstein, G. G.; Christensen, J. D.; Apeti, D. A. An Assessment of Two Decades of Contaminant Monitoring in the Nation's Coastal Zone. Silver Spring, MD. NOAA Technical Memorandum NOS NCCOS 74 2008; Vol. 105 2008.
(51) de Solla, S. R.; Gilroy, A. M.; Klinck, J. S.; King, L. E.; McInnis, R.; Struger, J.; Backus, S. M.; Gillis, P. L. Bioaccumulation of Pharmaceuticals and Personal Care Products in the Unionid Mussel *Lasmigona Costata* in a River Receiving Wastewater Effluent. *Chemosphere* **2016**, *146*, 486−496.
(52) Sehonova, P.; Svobodova, Z.; Dolezelova, P.; Vosmerova, P.; Faggio, C. Effects of Waterborne Antidepressants on Non-Target Animals Living in the Aquatic Environment: A Review. *Sci. Total Environ.* **2018**, *631*−*632*, 789−794.
(53) Henry, T. B.; Kwon, J. W.; Armbrust, K. L.; Black, M. C. Acute and Chronic Toxicity of Five Selective Serotonin Reuptake Inhibitors in Ceriodaphnia Dubia. *Environ. Toxicol. Chem.* **2004**, *23* (9), 2229−2233.
(54) Gilroy, È. A. M.; Gillis, P. L.; King, L. E.; Bendo, N. A.; Salerno, J.; Giacomin, M.; de Solla, S. R. The Effects of Pharmaceuticals on a Unionid Mussel (*Lampsilis Siliquoidea*): An Examination of Acute and Chronic Endpoints of Toxicity across Life Stages. *Environ. Toxicol. Chem.* **2017**, *36* (6), 1572−1583.
(55) James, C. A.; Lanksbury, J.; Khangaonkar, T.; West, J. Evaluating Exposures of Bay Mussels (Mytilus Trossulus) to Contaminants of Emerging Concern through Environmental Sampling and Hydrodynamic Modeling. *Sci. Total Environ.* **2020**, *709*, 136098.
(56) Riva, C.; Porte, C.; Binelli, A.; Provini, A. Evaluation of 4-Nonylphenol in Vivo Exposure in Dreissena Polymorpha: Bioaccumulation, Steroid Levels and Oxidative Stress. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **2010**, *152* (2), 175−181.
(57) Faria, M.; López, M. A.; Fernández-Sanjuan, M.; Lacorte, S.; Barata, C. Comparative Toxicity of Single and Combined Mixtures of Selected Pollutants among Larval Stages of the Native Freshwater Mussels (Unio Elongatulus) and the Invasive Zebra Mussel (Dreissena Polymorpha). *Sci. Total Environ.* **2010**, *408* (12), 2452−2458.
(58) Chen, H.; Diao, X.; Wang, H.; Zhou, H. An Integrated Metabolomic and Proteomic Study of Toxic Effects of Benzo[a]Pyrene on Gills of the Pearl Oyster *Pinctada Martensii*. *Ecotoxicol. Environ. Saf.* **2018**, *156*, 330−336.
(59) Chen, H.; Song, Q.; Diao, X.; Zhou, H. Proteomic and Metabolomic Analysis on the Toxicological Effects of Benzo[a]Pyrene in Pearl Oyster *Pinctada Martensii*. *Aquat. Toxicol.* **2016**, *175*, 81−89.
(60) Brew, D. W.; Black, M. C.; Santos, M.; Rodgers, J.; Henderson, W. M. Metabolomic Investigations of the Temporal Effects of Exposure to Pharmaceuticals and Personal Care Products and Their Mixture in the Eastern Oyster (*Crassostrea Virginica*). *Environ. Toxicol. Chem.* **2020**, *39* (2), 419−436.
(61) Müller, M.; Mentel, M.; van Hellemond, J. J.; Henze, K.; Woehle, C.; Gould, S. B.; Yu, R.-Y.; van der Giezen, M.; Tielens, A. G. M.; Martin, W. F. Biochemistry and Evolution of Anaerobic Energy Metabolism in Eukaryotes. *Microbiol. Mol. Biol. Rev.* **2012**, *76* (2), 444−495.
(62) Scavia, D.; David Allan, J.; Arend, K. K.; Bartell, S.; Beletsky, D.; Bosch, N. S.; Brandt, S. B.; Briland, R. D.; Daloğlu, I.; DePinto, J. V.; Dolan, D. M.; Evans, M. A.; Farmer, T. M.; Goto, D.; Han, H.; Höök, T. O.; Knight, R.; Ludsin, S. A.; Mason, D.; Michalak, A. M.; Peter Richards, R.; Roberts, J. J.; Rucinski, D. K.; Rutherford, E.; Schwab, D. J.; Sesterhenn, T. M.; Zhang, H.; Zhou, Y. Assessing and Addressing the Re-Eutrophication of Lake Erie: Central Basin Hypoxia. *J. Great Lakes Res.* **2014**, *40* (2), 226−246.
(63) Belivermiş, M.; Swarzenski, P. W.; Oberhänsli, F.; Melvin, S. D.; Metian, M. Effects of Variable Deoxygenation on Trace Element Bioaccumulation and Resulting Metabolome Profiles in the Blue Mussel (*Mytilus Edulis*). *Chemosphere* 2020, *250*. DOI: 10.1016/j.chemosphere.2020.126314.
(64) Tuffnail, W.; Mills, G. A.; Cary, P.; Greenwood, R. An Environmental 1H NMR Metabolomic Study of the Exposure of the Marine Mussel *Mytilus Edulis* to Atrazine, Lindane, Hypoxia and Starvation. *Metabolomics* **2009**, *5* (1), 33−43.
(65) Alexander Jr, J. E.; McMahon, R. F. Respiratory Response to Temperature and Hypoxia in the Zebra Mussel *Dreissena Polymorpha*. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **2004**, *137* (2), 425−434.
(66) Cipoletti, N.; Jorgenson, Z. G.; Banda, J. A.; Kohno, S.; Hummel, S. L.; Schoenfuss, H. L. Biological Consequences of Agricultural and Urban Land-Use along the Maumee River, a Major Tributary to the Laurentian Great Lakes Watershed. *J. Great Lakes Res.* **2020**, *46* (4), 1001−1014.
(67) Roznere, I.; Watters, G. T.; Wolfe, B. A.; Daly, M. Nontargeted Metabolomics Reveals Biochemical Pathways Altered in Response to Captivity and Food Limitation in the Freshwater Mussel *Amblema Plicata*. *Comp. Biochem. Physiol., - Part D: Genomics Proteomics* **2014**, *12*, 53−60.

https://doi.org/10.1021/acs.est.3c00812
Environ. Sci. Technol. XXXX, XXX, XXX−XXX