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Title: Contaminants of Concern and Spatiotemporal Metabolomic Changes in Quagga Mussels (*Dreissena bugensis rostriformis*) from the Milwaukee Estuary (Wisconsin, USA)

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All associated data presented within this manuscript is available within the Supplementary Data file.

ABSTRACT

Environmental metabolomics has emerged as a promising technique in the field of biomonitoring and as an indicator of aquatic ecosystem health. In the Milwaukee Estuary (Wisconsin, USA), previous studies have used a non-targeted metabolomic approach to distinguish between zebra mussels (*Dreissena polymorpha*) collected from sites of varying contamination. To further elucidate the potential effects of contaminants on bivalve health in the Milwaukee Estuary, the present study adopted a caging approach to study the metabolome of quagga mussels (*Dreissena bugensis rostriformis*) deployed in six sites of varying contamination for 2, 5 or 55 days. Caged mussels were co-deployed with two types of passive sampler (POCIS and SPMDs) and data loggers. In conjunction, *in-situ* quagga mussels were collected from the four sites studied previously and analyzed for residues of contaminants and metabolomics using a targeted approach. For the caging study, temporal differences in the metabolomic response were observed with few significant changes observed after 2 and 5 days, but larger differences (up to 97 significantly different metabolites) to the metabolome in all sites after 55 days. A suite of metabolic pathways were altered including biosynthesis and metabolism of amino acids, and up-modulation of phospholipids at all sites, suggesting a potential biological influence such as gametogenesis. In the caging study, average temperatures appeared to have a greater effect on the metabolome than contaminants, despite a large concentration gradient in PAH residues measured in passive samplers and mussel tissue. Conversely, significant differences between the metabolome of mussels collected *in situ* from all three contaminated sites and the offshore reference site were observed. Overall, these findings highlight the importance of contextualizing the effects of environmental conditions and reproductive processes on the metabolome of model organisms to facilitate the wider use of this technique for biomonitoring and environmental health assessments.

Keywords: Metabolome, Quagga Mussel, Great Lakes, Contaminants of Emerging concern, aquatic invertebrates

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INTRODUCTION

The Laurentian Great Lakes comprise approximately one fifth of global surface freshwater and provide a diverse ecosystem for a wide range of aquatic and terrestrial species (Sm et al., 2017). A range of anthropogenic stressors have been identified in the Great Lakes basin, including proliferation of invasive species, global climate change, urban and agricultural development, and chemical pollution (Smith et al., 2019). In 2010, the Great Lakes Restoration Initiative (GLRI) was initiated to coordinate, facilitate, and accelerate efforts to protect and restore the lakes, including remediation of Areas of Concern (AOCs) designated under the Great Lakes Water Quality Agreement. Among the AOCs, the Milwaukee Estuary (WI, USA) was originally designated as an AOC in 1987, with the impacted area expanded in 2008 to include tributaries that act as sources of contaminated sediment to the estuary (USEPA, 2023). The major contaminants within the AOC are legacy contaminants including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and heavy metals (Baldwin et al., 2017; Kimbrough et al., 2014; USEPA, 2023), though recent studies have demonstrated the presence of contaminants of emerging concern (CECs) including pharmaceuticals and personal care products (PPCPs) and alkylphenols (Li et al., 2017; Kimbrough et al., 2018). Furthermore, studies of aquatic organisms such as fish and amphipods either caged in the field or exposed to sediments collected from the Milwaukee Estuary and its tributaries have demonstrated the potential for deleterious effects associated with contaminant exposure (Baldwin et al., 2017; Li et al., 2017).

Among the techniques used for field biomonitoring, metabolomics has shown promise as an emerging tool for elucidating the effects of contaminants on organisms in the natural environment (Hani et al., 2021; Pomfret et al., 2020; Watanabe et al., 2015). Metabolomics is the study of concentration profiles of endogenous metabolites within an organism, with alterations to the metabolome occurring due to stress-driven changes in resource allocation (Pomfret et al., 2020; Watanabe et al., 2015). Typically, studies of the metabolome either adopt a targeted approach, where a specific suite of metabolites are studied based on prior knowledge or hypothesis, or a

non-targeted approach which provides broad, non-selective metabolomic profiling (Viant et al., 2019). Both approaches have been used successfully to determine differences in the metabolome between wild-caught or caged aquatic organisms along contaminant gradients (reviewed in Gil-Sosona et al., 2021). In the Great Lakes, previous studies have documented differences in the metabolome of zebra mussels, *Dreissena polymorpha*, (Watanabe et al., 2015), and fathead minnows, *Pimephales promelas*, in areas of differing contamination (Davis et al., 2016). Watanabe et al., (2015) used ^{1}H-NMR to elucidate differences in the metabolome of *D. polymorpha* collected from either an offshore reference site in Milwaukee Estuary, or three sites within the Milwaukee harbor which are known to be contaminated based on previous monitoring efforts (Kimbrough et al., 2014). The authors found that the metabolome of mussels collected from one of the impacted sites was significantly different to the reference site, driven by differences in levels of 26 individual metabolites (Watanabe et al., 2015), demonstrating the potential use of metabolomics as a tool for assessing bivalve health in the Great Lakes.

In recent years a number of studies have used caged bivalves for metabolomic assessments, which offers the advantage of exposure assessment over defined spatial and temporal scales, as well as the capacity to provide data in areas where bivalves are not typically present (Cappello et al., 2013; Hani et al., 2021). Cages containing bivalves are often co-deployed with data loggers recording environmental conditions such as water temperature and dissolved oxygen (Lehtonen et al., 2019), facilitating a greater understanding of the potential environmental factors that may contribute to observed metabolomic responses. Furthermore, the caging approach allows the ability to study the metabolome over temporal as well as spatial scales, with previous studies suggesting differences in metabolomic responses to pollutants over time. In a study of the grooved carpet shell (*Ruditapes decussatus*) caged at sites receiving urban and agricultural inputs, a significant increase in a suite of metabolites was observed in polluted sites after 7 days of exposure, whereas a significant decrease occurred after 22 days (Campillo et al., 2015). In yellow perch, *Perca flavescens*, exposed *in situ* to municipal effluent, subtle metabolomic effects were

observed after 6 weeks of exposure, compared to minimal metabolomic differences after one or three weeks (Defo et al., 2021). Elucidating the *in-situ* effects of contaminants on the metabolome over temporal scales is important to determine the pollutant response of aquatic biota in the natural environment, and aid in determining the appropriate exposure duration for field environmental biomonitoring.

Taking this into account, the present study adopted a targeted metabolomic approach to determine the impacts of exposure to contaminants of emerging concern (CECs) and legacy compounds on quagga mussels, *Dreissena bugensis rostriformis*, in the Milwaukee Estuary AOC. Two separate studies were conducted, utilizing caged and *in situ* mussels respectively. For the caging study, mussels were collected from a common site and caged at five sites with differing levels of contamination within the AOC, and were sampled after 2, 5 or 55 days of exposure for metabolomic analysis to facilitate a greater understanding of the time-dependent effects of contaminant exposure on the metabolome. Cages were co-deployed with two types of passive sampling devices; polar organic chemical integrative samplers (POCIS) and semi permeable membrane devices (SPMDs) for time-weighted aqueous monitoring of more hydrophilic CECs and PAHs/legacy pesticides, respectively. In addition, caged mussels were analyzed for a suite of compounds after 55 days of exposure to elucidate the exposure profile and differences from source mussels used for caging across the AOC. For the *in-situ* study, tissue chemistry and metabolomic profiles were assessed in mussels collected from four sites of varying contamination as described in Watanabe et al., (2015).

MATERIALS AND METHODS

Study area & study design

The Milwaukee Estuary AOC includes 13.8 km of rivers, part of the nearshore waters of Lake Michigan, as well as the heavily urbanized inner and outer Milwaukee harbor (Li et al., 2017). The major rivers within the catchment are the Milwaukee, Kinnickinnic, and Menominee Rivers, with the latter two converging with the former in downtown Milwaukee. Both the Milwaukee and

Menominee Rivers are predominantly rural in their upper reaches, before becoming increasingly urbanized further downstream (Choi et al., 2017). The Kinnickinnic River is the shortest of the three, and is heavily urbanized throughout its reaches. As highlighted above, the Milwaukee Estuary was designated as an AOC under the binational Great Lakes Water Quality Agreement in 1987, due to the presence of 11 beneficial use impairments (BUIs) including restrictions on fish and wildlife consumption, eutrophication, and degradation of the benthos (USEPA, 2023). The primary contaminants that led to the listing of the estuary as an AOC were PAHs, PCBs, and metals associated with historical industrial activities, including one of the largest metallurgical coking facilities in the midwestern US (Valentine et al., 2021). Further details on sites for both the caging and *in situ* study are shown in Table 1, including coordinates, environmental conditions (temperature and dissolved oxygen), and a brief site description.

Though zebra mussels were used for the previous metabolomics study conducted in the Milwaukee Estuary (Watanabe et al., 2015), this species has been displaced by quagga mussels in the majority of the Great Lakes basin excluding Lake Superior (Glyshaw et al., 2015). Consequently, quagga mussels were targeted in part due to the large number of individuals required for both caging and *in situ* study designs. Both species have similar life cycles, involving a free-swimming veliger larval stage followed by attachment to hard substrate (Glyshaw et al., 2015). However, the species differ in terms of salinity and thermal tolerance, growth, respiration rates, and the depth at which reproduction typically occurs (Ram et al., 2012). Several studies have used both quagga and zebra mussels interchangeably for contaminant monitoring within the Great Lakes and its tributaries (Evariste et al., 2018; Mills et al., 1993a; Richman and Somers, 2005; 2010), though potential differences in contaminant accumulation and sensitivity have been observed (Evariste et al., 2018; Nowicki and Kashian, 2018). Previous studies of quagga mussels in Lake Michigan have demonstrated maturation of gonads and gamete release in quagga mussels during the time period of the study (Glyshaw et al., 2015).

Caging study. In the present study, quagga mussels were collected from a site in Milwaukee Harbor (LMMB-04), and randomly allocated into cages at one of six sites; LMMB-04, LMMB-05, LMMB-06, LMMB-08, LMMB-11, and LMMB-13 (Figure 1). The LMMB-04 collection site is located in the outer harbor north of the harbor entrance ship channel, adjacent to Juneau Park (Watanabe et al., 2015). The LMMB-05 site was located offshore of Milwaukee Harbor, and has found to be an area of lower contamination based on previous surveys conducted by the NOAA Mussel Watch Program (Figure 1, Kimbrough et al., 2021). Both the aforementioned sites (LMMB-04 and LMMB-05) were included in the previous metabolomic assessment of zebra mussels conducted by Watanabe et al., (2015). LMMB-06 was located on the Northern End of Jones Island, upstream of the Jones Island wastewater treatment plant (WWTP) and close to the USGS water quality station at the mouth of the Milwaukee River. Sites LMMB-08, LMMB-11, and LMMB-13 were located on the Milwaukee, Menominee, and Kinnikinic rivers, respectively.

For the source mussel collection from the LMMB-04 outer harbor site, divers harvested mussels using a metal paint scraper, and mussels were transferred to an aerated 26 L cooler containing site water until deployment. A subsample of mussels was taken to determine baseline levels of contamination and metabolomics at time zero. All samples designated for metabolomics were immediately flash frozen in liquid nitrogen, whereas samples designated for tissue chemistry were rinsed to remove debris, frozen using water ice, and shipped for chemical analysis within two days. Cages were torpedo-shaped metal minnow traps secured approximately 0.5 m above the benthos using steel chain, with each cage containing approximately 300 – 500 mussels per cage. Each deployment contained data loggers for temperature and dissolved oxygen (HOBO U26 logger, Onset Computer Corporation, Bourne, MA, USA) which record data every 5 minutes over the deployment period, as well as POCIS and SPMDs. Cages were retrieved and a subset of mussels was removed following 2, 5 and 55 days of exposure. POCIS and SPMDs were retrieved and analyzed after 55 days of deployment.

In situ study. For the *in situ* mussel study, quagga mussels collected from sites previously sampled by Watanabe et al., (2015) were used. This included the northern harbor site, LMMB-04, highlighted above, where source mussels were collected for caging studies. A second site in the northern harbor, LMMB, adjacent to Lakeshore State Park was also sampled, along with a site adjacent to the Milwaukee Harbor Confined Disposal Facility (Figure 1, LMMB-01), which was located in the southern harbor, ~ 4 km south of LMMB-04. Sample collection was performed by scuba divers who removed mussels from breakwater boulders using a metal paint scraper as described above for source mussels. All metabolomics samples were flash frozen in liquid nitrogen and shipped to SGS AXYS in a dry shipper.

Tissue analyses

PAH samples were analyzed by TDI-Brooks International Inc., College Station, TX, using methods described previously (Kimbrough et al., 2006, 2021). A total of 64 PAHs were analyzed, including alkylated and parent compounds. TDI-Brooks shucked the mussels, homogenized the tissue, and analyzed the homogenate extract for legacy contaminants before shipping an aliquot of the tissue homogenate to SGS AXYS (Sidney, BC, Canada) for CEC analysis. A subset of 16 priority PAHs were used to calculate total parent PAH concentrations following Baldwin et al., (2020). For CECs, the following groups of contaminants were analyzed: pesticides (73 compounds), pharmaceutical and personal care products (PPCPs, 141 compounds), and alkylphenols (4 compounds). The PPCP method was based on EPA method 1694 (U.S.EPA 2007a, b) and analyzed using isotope dilution/surrogate standard quantitation LC/ESI-MS/MS. The pesticide method is based on EPA method 1699 and analyzed by high resolution gas chromatography with high-resolution mass spectrometric detection (HRGC/HRMS). The analysis of the alkylphenols in tissue samples was conducted by two different LC-MS/MS runs, one run in the ESI Negative mode for NP and OP, and the other run in the ESI Positive mode for NP1EO and NP2EO. For all chemical classes, values below three times the method detection limit

(defined as the reporting limit) were considered detected but the concentration was not used for analyses and indicated by 'BRL' (below reporting limit).

POCIS & SPMDs

POCIS deployments were conducted according to established protocols (Alvarez et al., 2010). All POCIS was purchased from EST lab (St. Joseph, Missouri, USA), and consisted of the sorbent Oasis HLB (0.1µm pore size, Waters, Milford, MA). Each deployment contained a protective cannister containing 3 POCIS for a duration of 55 d. POCIS is typically deployed in the field for 30 days (Alvarez et al., 2010); thus, equilibrium was assumed after 55 days of deployment. After retrieval, field-deployed POCIS along with a suite of field blanks were shipped to SGS AXYS on ice for analysis using methods described above.

SPMDs were also purchased from EST lab (St. Joseph, Missouri, USA), and were comprised of additive free lay flat low-density polyethylene with a wall thickness of 70 – 95 µm and a lipid-to-membrane mass ratio of approximately 0.2. For SPMDs, three deuterated performance reference compounds (PRCs) were pre-loaded onto the SPMDs; Anthracene d-10, Fluroanthene d-10, and Dibenzo[a,h]anthracene d-14. The former two PRCs were used to calculate sample specific sampling rates for the PAH compounds, whereas the latter (Dibenzo[a,h]anthracene d-14) was used as a photolysis marker to track potential photodegradation of compounds over the deployment period (Alvarez et al., 2021). Both a field blank and a fabrication blank were used concurrent with SPMDs. Further details on calculation of aqueous concentrations from SPMDs and POCIS are available in the supplementary information, section S1.

Metabolomics & quality control

All metabolomics samples were extracted and analyzed at SGS AXYS using LC-MS/MS (or flow-injection MS/MS for lipids) following the standard operating procedure MLM-001 as described in Benskin et al., (2013) and Legrand et al., (2021). Briefly, mussels were removed from their shells, exterior water removed and the whole body homogenized. 100-150mg of the sample were extracted using chloroform and methanol (2X) sequentially with a bead blender, and

four aliquots of the extract were used to analyze the five lists of metabolites: amino acids and biogenic amines (43 metabolites), fatty acids (18 metabolites) and hexose (1 metabolite), phospholipids and acylcarnitines (144 metabolites) and metabolites associated with energy pathways (17 metabolites). Individual samples were processed, plated and analyzed in batches of between 76 and 81 samples. Each of the extracts were spiked with isotopically labeled standard or non-isotopically labeled standards (for lipids analysis only) to provide for internal standard-relative quantitation. Amino acid and biogenic amines were analyzed as their phenylthiocarbamyl derivatives after derivatization with Phenylisothiocyanate (PITC, also known as Edman's reagent). Each batch contained an additional 8 quality control samples including 3 lab blanks to evaluate the potential for contamination introduced by the analytical procedure, and 5 replicates of an Internal Reference Material (IRM) sample (IRM SC7176). The IRM sample consisted of a homogenate of dreissenid mussels collected from the Great Lakes and analyzed previously in 2016.

Amino acids, biogenic amines, fatty acids, hexose, and metabolites associated with energy pathways were analyzed by LC-MS/MS, using an Agilent 1100 HPLC coupled to an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, ON, Canada). Phospholipid and acylcarnitine analytes were analyzed using flow-injection tandem mass spectrometry (FI-MS/MS). Mass spectrometry performance across the run was monitored by replicate injections of a mid-point calibration sample approximately once every 20 samples.

Details on the batching of samples is shown in the Table S1 (Supplementary Data). Due to the potential for interbatch variation (Han and Li, 2020), statistical comparisons were conducted within individual batches. For example, source mussels from LMMB-04 (t0) and all three timepoints for each individual site (*e.g.*, LMMB-06 t2, t5 and t55) were analyzed together within a single batch, thus comparisons were focused on comparing temporal differences within sites, and between timepoints and source mussels. The IRM samples were used to perform quality assurance/quality control (QAQC) on the dataset. First, metabolites that exhibited variability ≥

30% relative standard deviation (RSD) between batches were removed from the dataset (Legrand et al., 2022). Compounds that were not detected in ≥ 50% of IRM samples across all batches were also removed (Trushina et al., 2013). Prior to statistical analysis, all samples were normalized by sum and Pareto scaled (Defo et al., 2021; LeGrand et al., 2022).

Statistical analyses

Metabolomic data was analyzed using MetaboAnalyst v5.0 (Pang et al., 2022). Site and timepoint differences between metabolomes were first assessed using principal component analysis (PCA). Differences in PC scores between sites and source mussels were analyzed at each time point using a non-parametric Kruskal-Wallis and posthoc Dunn's test. The number of significantly different metabolites between timepoints and sites was assessed using analysis of variance (ANOVA) with a posthoc Tukey's honestly significant difference (HSD). Pathway analysis was conducted on sites showing significant PCA score differences from source mussels at each individual timepoint using *Danio rerio* as a model organism (Xia and Wishart, 2011). PCA was used to assess the potential associations between metabolite changes, levels of contaminants and measured environmental conditions. For the LMMB-04 site where data loggers failed, an arbitrary value of 14 °C was used based on the proximity of this site to LMMB-06. Analyses were conducted with and without addition of temperature for LMMB-04 to determine the potential influence on findings. For this analysis, only metabolites that showed significant differences from source mussels after 55 days were used, since tissue residues were measured at this timepoint only. Correlation analysis (Spearman's rank-order correlation) was conducted to determine the relationship between individual contaminant groups, temperature, and metabolites showing significant differences identified using PCA. Correlation analyses were conducted using R Studio (v 4.3.1).

RESULTS

Mussel caging temporal study

POCIS & SPMD chemistry. Results of the time-weighted average concentrations for POCIS and SPMDs are shown in Tables 2 and 3, respectively. Raw chemistry data for POCIS and SPMDs is available in the Supplementary Data, Tables S2 and S3, respectively. The total number of compounds detected ranged from 17 to 35 (Table 2), with the fewest detected at the offshore LMMB-05 site (17 compounds), and the greatest detected at LMMB-05 and the Kinnickinnic River site, LMMB-13 (35 compounds at both sites). Among the pesticides, the number of compounds detected ranged from 10 (LMMB-05) to 16 (LMMB-13), with the PPCPs ranging from 5 compounds detected (LMMB-05) to 19 (LMMB-06). Finally, the number of hormones detected ranged from 1 (LMMB-04, LMMB-06, and LMMB-11) to 3 (LMMB-13). Of the CEC classes (defined here as alkylphenols, pharmaceuticals, and pesticides) analyzed in POCIS, pesticides were detected at the highest concentrations across all sites excluding LMMB-06, with pesticide concentrations ranging from 51 – 239 ng/L across all sites. The current-use herbicide atrazine was recorded at the highest concentrations of the pesticides in all samples excluding LMMB-06, with concentrations ranging from 23.1 – 111 ng/L. For the PPCPs, highest concentrations were recorded at the LMMB-13 site, with the plasticizer, bisphenol A, recorded at a maximum concentration of 149 ng/L. Total concentrations of PPCPs ranged from 3.91 ng/L (LMMB-05) to 249 ng/L (LMMB-13) across all sites. Hormones were recorded at low concentrations across all sites, ranging from 1.36 – 2.28 ng/L, with the steroid hormone, Androstenedione, the only compound recorded above the reporting limit.

For SPMDs, the highest concentrations of parent and alkylated PAHs were recorded in sites LMMB-11 and LMMB-13, located in the Menominee and Kinnickinnic Rivers, respectively (Table 3). Fluoranthene was detected at highest concentrations of the parent PAHs across all sites, ranging from 2.68 – 176 ng/L. Total parent PAH concentrations were lowest at the offshore site, LMMB-05, with concentrations of 6.64 ng/L, compared to maximum concentrations of 503 ng/L at the Kinnickinnic River site, LMMB-13. The same pattern was observed for the alkylated PAHs, with lowest concentrations of 7 ng/L observed at LMMB-05, and highest concentrations

of 440 ng/L observed at LMMB-13. Across all sites, C1-Fluoranthenes/Pyrenes were recorded at the highest concentrations of the alkylated PAHs. Concentrations of individual legacy pesticides were typically < 1 ng/L at all sites excluding LMMB-13, which had total concentrations of 1.05 ng/L.

Tissue chemistry. Body residues of contaminants as well as fold change from source quagga mussels is shown in Table 4. Raw tissue chemistry data for CECs and PAHs are available in the Supplementary Data, Tables S4 and S5, respectively. Across all compound classes, lowest levels of contamination were found at the offshore site, LMMB-05, with over an order of magnitude lower PAH levels compared to the site of highest contamination, LMMB-13 (Figure 2A and 2B). Total pesticide residues were highest at the LMMB-11 site, with a 2.3-fold increase from source mussels. The legacy pesticide degradate, 4,4'-DDE, was the highest pesticide recorded across all sites. Alkylphenol resides were highest at the LMMB-13 site, with total alkylphenol residues of 478 ng/g wet weight, representing a 9.5-fold increase from the source mussels. For the parent PAH compounds, chrysene was recorded at the highest concentration across all sites, with total parent PAH residues ranging from 533 – 27744 ng/g dry weight at LMMB-05 and LMMB-13, respectively. Similarly, levels of alkylated PAHs ranged from 431 – 24329 ng/g dry weight at LMMB-05 and LMMB-13, respectively.

Metabolome changes over time relative to source mussels. All raw, non-normalized metabolomic data organized by batch is available in the Supplementary Data, Tables S6-S10. PCA plots of individuals and mean PC score plots of quagga mussel metabolomes for all timepoints are shown in Figure 3 and S1, respectively. After 2 days, few differences from source mussels were evident (Figure 3A), with only a single site, LMMB-08, showing significant differences in PC scores from t0 mussels along PC2 (Dunn's test, FDR correction, $p < 0.05$). Overall, 51 metabolites were significantly different in LMMB-08 after 2 days relative to source mussels (Table 5), which was driven mostly by a decrease in levels of 22 amino acids and biological amines, and an increase in 14 phospholipids and acylcarnitines (ANOVA, Posthoc

Tukey's HSD, $p < 0.05$). The fewest number of significantly different metabolites were recorded in mussels caged at the source mussel collection site, LMMB-04 (3 metabolites significantly different, Table 5).

After 5 days, greater overall separation of sites from source mussels was observed in PCA plots (Figures 3B and S1). Two sites, LMMB-08 and LMMB-11 were significantly different than source mussels along PC2 (Dunn's Test, FDR correction, $p < 0.05$), which accounted for 14.9% of variance. Consistent with these findings, the greatest number of significantly different metabolites relative to the source were observed in LMMB-08 (38 metabolites) and LMMB-11 (36 metabolites), with the fewest observed at LMMB-06 (12 metabolites).

Following 55 days of caging, greater separation of all sites from source mussels was evident using PCA (Figures 3C and S1). PC1 scores of two sites, LMMB-05 and LMMB-08, were significantly different from t0 mussels (Dunn's Test, FDR correction, $p < 0.05$), with LMMB-13 approaching significance ($p = 0.06$). At each site, the greatest number of significantly different metabolites relative to source mussels was observed after 55 days compared to the 2 and 5-day timepoints, with sites LMMB-08 and LMMB-11 again showing the most differences, with 97 and 92 significantly different metabolites respectively. After 55 days, the metabolites showing the greatest fold-change between LMMB-08 and t0 were methionine sulfoxide (12.5-fold reduction) and PC aa C34:4 (4-fold reduction), with methione sulfoxide (7.7-fold reduction) and myristic acid (4.1-fold reduction) showing the greatest fold-change between LMMB-11 and t0. The greatest fold change between LMMB-05 and t0 was observed for tyrosine (3.3-fold reduction) and phenylalanine (3.2-fold reduction), whereas two phospholipids, PC aa C36:1 and PC aa C32:0, showed the greatest fold change between LMMB-04 after 55 days compared to t0 (1.8 and 2.8-fold increase).

In contrast to the day 2 and day 5 timepoints, some changes in metabolites were consistent across all sites. Mussels caged at every site, including the collection site LMMB-04, showed an increase in glycerophospholipids (Table 5) relative to the source mussels with a suite

of 10 metabolites common to all sites. Levels of the phospholipid PC aa C36:1 across all sites after 55 days relative to t0 is shown in Figure S2. Across all sites, concentrations of PC aa C36:1 were 2-fold higher after 55 days compared to t0.

The PCA plot of significantly different metabolites after 55 days in relation to measured contaminants in POCIS, SPMDs and tissue is shown in Figure S3. To avoid overplotting and due to the changes in phospholipid levels being observed at all sites, phospholipids and acylcarnitines were removed from the analysis. As can be seen from the PCA biplot, levels of many of the amino acids that were responsible for driving metabolome differences between sites, such as methionine sulfoxide (Met-SO), did not appear related to any of the measured environmental conditions or contaminants (Figure S3). However, a suite of amino acids including asparagine (asn), proline (pro), serine (ser), and isoleucine (ile) appeared negatively related to average temperature. Correlation analysis found significant negative relationships between a suite of six amino acids and average temperature (Spearman's rho, $p < 0.05$, Figure S7). In terms of contaminants, concentrations of phenethylamine (PEA) appeared related to levels of contaminants including aqueous and tissue residues of PAHs, as well as tissue residues of alkylphenols (Figure S3), whilst γ-Aminobutyric acid (GABA) appeared related to aqueous concentrations of pesticides measured using POCIS. A significant positive relationship between concentrations of aqueous PAHs and PEA was found (Spearman's rho, $p < 0.05$, Figure S4).

Within-site temporal changes to the metabolome. PCA plots of the within-site metabolome changes are shown in Figure S5, with the number of significantly different metabolites between timepoints shown in Table 6. For all sites, the greatest separation was evident after 55 days relative to other timepoints, which was largely driven by an increase in phospholipids at this timepoint as highlighted above. For all sites excluding LMMB-08, greater separation between the metabolome at days 2 and 55 was observed compared with differences between day 0 (source) and day 55 (Figure S5). This was reinforced by a greater number of significantly different metabolites between days 55 and day 2 compared with days 55 and day 0

for all sites excluding LMMB-08 (Table 6). This suggests that changes to the metabolome after 2 days were transient and different to those recorded after 55 days, with the exception of the site with the most significantly different metabolites, LMMB-08, where changes after days 2 and 5 were more similar to day 55 metabolomic profiles (Figure S5).

Pathway analysis. Pathway analysis was conducted to identify potential biological pathways impacted in sites after 2, 5 and 55 days. Only sites exhibiting significantly different PC scores from source mussels were used for pathway analysis within each timepoint. Significantly altered pathways for each timepoint are displayed in Figure 4. After 2 days, a total of 9 pathways were significantly altered between LMMB-08 and t0 mussels, with pathways relating to amino acid biosynthesis and metabolism most effected. Fewer pathways were significantly altered after 5 days, with the most effected pathways consistent with those altered after 2 days; phenylalanine, tryptophan and tyrosine biosynthesis (pathway impact = 1.0), alanine, aspartate, and glutamate metabolism (pathway impact = 0.450), and phenylalanine metabolism (pathway impact = 0.595). Of the 5 altered pathways, 3 pathways were significantly effected in LMMB-08, with all 5 significantly altered in LMMB-11 relative to source mussels (Figure 4). The greatest number of pathways were significantly altered after 55 days, with a total of 11 pathways significantly altered (Figure 4).

In situ study

In situ mussel tissue chemistry. Total residues of CECs and PAHs (parent and alkylated) for *in situ* mussels are shown in Figure 2. Of the four sites selected for the *in situ* study, LMMB-04 and LMMB-01 had the highest levels of both CECs and PAHs, with the lowest levels recorded in the offshore LMMB-05 site. Maximum levels of PAHs in the *in situ* mussels were over five-fold lower than those in mussels caged in the tributary of highest contamination, the Kinnikinic River (LMMB-13).

In situ mussel metabolomics. PCA analysis of *in situ* mussel metabolomes collected from four sites in the Milwaukee Estuary AOC is shown in Figure 5. All sites showed significant separation

from the offshore site of lower contamination, LMMB-05. Along PC1, which was responsible for 50.7% of the variance, PC scores for both LMMB-04 and LMMB were significantly different from LMMB-05 (Dunn's test, FDR correction, $p < 0.05$). Along PC2 (15.3% of the variance), PC scores of all sites were significantly different from LMMB-05 (Dunn's test, $p < 0.05$). No significant differences among PC scores of LMMB, LMMB-01 and LMMB-04 were recorded (Dunn's Test, FDR Correction, $p > 0.05$). Overall, the greatest number of significantly different metabolites were observed between LMMB-04 and LMMB-05, with a total of 82 significantly different metabolites driven largely by greater phospholipids and acylcarnitines in LMMB-05 (50 metabolites, Supplementary Information, Table S1), and lower levels of 21 amino acids in LMMB-05 relative to LMMB-04 (ANOVA, Posthoc Tukey's HSD, $p < 0.05$). The fewest number of significantly different metabolites was observed between LMMB and LMMB-01, with 4 significantly different metabolites (ANOVA, Posthoc Tukey's HSD, $p < 0.05$, Table S1). In terms of fold change between the reference (LMMB-05) and sites showing significant differences (LMMB-04, LMMB-01, and LMMB), the largest fold change was observed for glycine, which was four-fold lower on average in LMMB-05 compared to the altered sites (Figure S6A). The phospholipid, phosphatidylcholine acyl-alkyl C42:3 (PC ae C42:3), was also a major driver of the differences between LMMB-05 and the other sites, being 1.8-fold higher in LMMB-05 compared to all other sites (Figure S6B).

To determine the potential pathways altered between the sites exhibiting significant differences, pathway analysis was conducted. Only metabolites that were significantly different in all three sites comparisons relative to LMMB-05, the site of lowest contamination, were used for this analysis. Overall, a total of 7 pathways were significantly altered (Figure 6). The three pathways showing the greatest effectswere linoleic acid metabolism, D-glutamine and D-glutamate metabolism, and alanine, aspartate and glutamate metabolism.

DISCUSSION

Time-Specific metabolomic changes

After 2 days, significant differences in the metabolome of mussels caged at a single site, LMMB-08, were evident compared to source mussels. The pathways showing the greatest impacts at LMMB-08 were related to biosynthesis and metabolism of phenylalanine, alanine, and tyrosine. Phenylalanine metabolism has been shown to be impacted in bivalves in response to a suite of stressors including effluent from wastewater treatment plants (Dumas et al., 2020), polystyrene microplastics (Huang et al., 2021), and pathogens (Nguyen et al., 2018). Pathways relating to metabolism and biosynthesis of tryptophan, tyrosine, and phenylalanine are known to be inter-related and trigger the synthesis of neurotransmitters including catecholamines and serotonin receptors (Blanco and Blanco, 2017). A significant reduction in arginine was also observed in LMMB-08 mussels after two days of caging. Arginine is known to play a role in some energy metabolism pathways, with concentrations thought to be related to adenosine tri-and di-phosphate (ATP/ADP) (Fasulo et al., 2012). Arginine is the final product of the reaction between phosphoarginine and ADP, with phosphoarginine being the primary phosphagen for ATP regeneration (Fasulo et al., 2012; Fan et al., 1991). Thus, the reduction in arginine suggests perturbation to energy metabolism and regeneration in LMMB-08 mussels.

After 5 days of caging, two sites showed significant separation from source mussels; LMMB-08 and LMMB-11. Metabolomes after 55 days of caging showed the most differences from source mussels across all sites, with all sites excluding LMMB-06 and LMMB-13 showing significant separation using PCA. However, the metabolome of mussels collected and caged at the same site (LMMB-04) exhibited significant changes after 55 days, emphasizing the importance of determining and contextualizing natural changes to metabolic profiles. Furthermore, up-modulation of phospholipids was observed across all sites after 55 days, suggesting that these changes were likely due to natural temporal changes in lipid profiles over the deployment period. The present study was conducted from early-June to late-July, during which time previous studies have documented maturation of gonads and gamete release in quagga mussels inhabiting Lake Michigan (Glyshaw et al., 2015). Phospholipids are known to increase

during gonad maturation in bivalves (Martinez-Pita et al., 2012), thus the up-modulation of these metabolites at all sites after 55 days may be attributed to gonadal maturation in mussels over the deployment period.

Analysis of PCA plots and the number of significantly different metabolites between timepoints suggested that for the majority of sites excluding LMMB-08, initial changes to the metabolome after 2 days were transient and not reflective of metabolite profiles after 55 days. For example, all sites excluding LMMB-08 showed up-modulation of amino acids and biological amines after 2 days, compared with a decrease across all sites after 55 days. Similar findings were recorded in caged clams, *Ruditapes decussatus*, exposed to urban and agricultural inputs for 7 or 22 days, with an initial increase in levels of amino acids after 7 days of caging at polluted sites relative to the control, followed by a decline after 22 days (Campillo et al., 2015). In the present study, metabolomic profiles after 5 days were more similar to day 55 profiles, suggesting that a 5-day exposure duration may be more useful in determining final metabolite profiles compared with 2 days. In general, metabolomics is thought to provide a rapid reflection of an individual's stress response, and can potentially serve as an early warning of deleterious effects (Pomfret et al., 2020). However, metabolomic responses in bivalves are known to be sensitive to a suite of abiotic and biotic factors, including diet, sex, age, and water quality parameters (Jones et al., 2008; Rosenblum et al., 2006). Thus, it is possible that the transient changes observed in the metabolome of most sites after 2 days may be reflective of stress associated with handling and deployment in cages, with subsequent changes more representative of potential contamination and environmental effects. Previous studies have demonstrated that transport and handling of gastropods can induce shifts to the metabolome including alterations to amino acid metabolism (Alfaro et al., 2021; Nguyen et al., 2020).

Potential drivers of metabolome changes

Overall, the LMMB-08 site located on the Milwaukee River consistently showed the largest differences from source mussels, even after 2 days of caging. Similarly, the LMMB-11

site located in the Menominee River showed separation from source mussels after 5 and 55 days. Generally, levels of measured contaminants were comparable in mussel tissue between the LMMB-08 and source mussels (Figures 1 and 2), with marginally higher levels of PAHs and alkylphenols, but lower concentrations of pesticides and PPCP in LMMB-08. Differences in aqueous concentrations of CECs measured using POCIS were observed between LMMB-08 and LMMB-04, with total time-weighted averages of 292 and 71.6 ng/L, respectively. Concentrations of contaminants in mussels caged at LMMB-11 and in passive samplers were higher than the collection site across all classes excluding PPCPs, with parent and alkylated PAH levels 2.8 and 4.2-fold higher relative to source mussels. However, the most highly contaminated site, LMMB-13, showed no significant differences from source mussels at any timepoints, though differences approached significance after 55 days. Furthermore, the offshore site of lowest contamination, LMMB-05, showed significant metabolomic alterations after 55 days, with a greater number of significantly different metabolites (74) relative to the site of highest contamination, LMMB-13 (68).

To further understanding the relationship between contaminants and metabolomic changes, PCA was conducted using metabolites showing significant differences from source mussels after 55 days. Of these metabolites, only 2 of the 27 amino acids showing differences appeared related to levels of contaminants, indicating that the majority of observed changes were indicative of a general stress or biological response rather than directly attributed to contaminants. Phenethylamine (PEA) was significantly associated with tissue concentrations of several contaminants, including alkylphenols and PAHs. PEA is a biogenic trace amine which is thought to play a role as a neurotransmitter in invertebrates (Borowsky et al., 2001). Previous studies have demonstrated that pesticides and alkylphenol exposure may influence levels of neurotransmitters in bivalves (Cooper & Bidwell, 2006; Dias et al., 2021; Vidal-Liñán et al., 2015), though a decrease is typically observed. The inhibitory neurotransmitter GABA, which plays a role in reducing neuronal excitability in vertebrates and invertebrates (Tano et al., 2009), was positively

related to aqueous pesticide levels measured using POCIS. Studies using murine models have demonstrated that atrazine exposure, which was recorded at the highest concentrations in POCIS at most of the sites (Table 2), led to an increase in levels of GABA (Chávez-Pichardo et al., 2020). Thus, it is possible that elevated aqueous atrazine exposure may have led to an increase in GABA due to its neurotoxic effects (Stradtman and Freeman, 2021), though this possibility has not been studied in bivalves to the authors knowledge. However, GABA was also positively related to temperature, which has previously been shown in bivalves (Dunphy et al., 2018). Due to the challenges in disentangling the effects of environmental conditions and contaminants in field-based metabolomic studies, future research should focus on identifying putative markers of xenobiotic exposure in controlled laboratory exposures that can be applied to studies in the natural environment.

To further understand potential drivers of metabolomic differences between sites, temperature and dissolved oxygen was monitored continuously over the deployment period at all sites excluding LMMB-04 wherein equipment failure precluded data collection. Both of the sites showing greater metabolomic changes, LMMB-08 and LMMB-11, exhibited the highest temperatures over the deployment periods, with temperatures ranging from 18.3 – 27.7 and 17.3 – 25.2 at LMMB-08 and LMMB-11, respectively (Table 1). Previous studies have suggested that temperatures of 30 °C induce lethality in quagga mussels, with temperatures ≥ 25 °C likely to induce thermal stress (Mills et al., 1993b, 1996). As highlighted above, maximum temperatures at both LMMB-08 and LMMB-11 exceeded the 25 °C threshold over the 55-day deployment period, thus metabolic changes may be partly attributed to thermal stress. Previous metabolomic studies of male blue mussels, *Mytilus edulis*, exposed to elevated temperatures found reductions in several amino acids including asparagine, isoleucine, and tyrosine that were also reduced in sites showing significant differences in the present study (Ellis et al., 2014). Furthermore, the PCA of significantly different metabolites after 55 days indicated negative relationships between a number of amino acids, including asparagine, isoleucine, valine, and threonine with average

temperature over the 55-day period (Figure S3). The relationship between six amino acids and average temperature over the 55-day deployment period is shown in Figure S7, with all showing significant negative relationships (Spearman's rho, $p < 0.05$), whether the estimated temperature data for LMMB-04 was included or not. These findings suggest that temperature-induced stress rather than levels of contaminants may have been responsible for the consistent differences in the metabolomes of mussels caged at sites LMMB-08 and LMMB-11. Future studies should focus on determining changes to the metabolome in quagga mussels collected seasonally to elucidate the influence of environmental factors and reproductive cycles on levels of endogenous metabolites.

In situ mussel study

For the *in situ* study, quagga mussels were collected from four sites in the Milwaukee Estuary AOC which were the focus of a previous non-targeted metabolomics study using zebra mussels (*Dreissena polymorpha*, Watanabe et al., 2015). The Watanabe et al., (2015) study found that mussels collected from a contaminant impacted site in the south harbor, LMMB-1, exhibited a significantly different metabolomic profile than those collected from an offshore reference site, LMMB-05, though no significant differences were observed between the reference and other impacted sites. The observed metabolic differences between LMMB-1 and LMMB-05 were largely driven by alterations to levels of amino acids, with a number of nucleotides and osmolytes also impacted (Watanabe et al., 2015). Comparatively, the present study found significant differences in the metabolome of all three impacted sites relative to the reference site (Figure 5), with the greatest number of significant differences observed between LMMB-04 and LMMB-05.

Several of the altered metabolites driving differences between the impacted and reference sites in the present study are similar to those observed by Watanabe et al., (2015). For example, levels of the free amino acid, glycine, were on average four-fold higher in mussels collected from the impacted sites relative to the reference site in the present study, with Watanabe et al., (2015) also finding significant up-regulation of this compound in LMMB-01 relative to LMMB-05. In a recent study of zebra mussels caged downstream of WWTPs, glycine was identified as one of

four metabolites driving significant differences in the metabolome between reference and impacted sites (Hani et al., 2021), suggesting that this metabolite could potentially be used as an indicator of contaminant impacts. Glycine is known to be catalyzed anaerobically to produce energy, and also plays an important role in the metabolism of glutathione, which is an important cellular thiol involved in protection of cells from oxidative stress (Hani et al., 2021; Lee and Lee, 2011). Thus, the higher levels of glycine observed in all three impacted sites may reflect an increase in oxidative stress at sites of higher contamination. This is further supported by significantly higher levels of methionine sulfoxide at all three impacted sites relative to the reference site, with methionine sulfoxide formed by the oxidation of methionine and a known biomarker of oxidative stress in bivalves (Noor et al., 2021).

Using pathway analysis, linoleic acid metabolism was found to be the most altered pathway in impacted sites relative to the offshore reference site (Figure 6), driven by higher levels of linoleic acid in LMMB-05 relative to all other sites. Previous studies have demonstrated alterations to fatty acid metabolism and composition in bivalves exposed to contaminants in the laboratory (Falfushynska et al., 2019; Mesquita et al., 2018) and field (Filiminova et al., 2016; Perrat et al., 2013). For example, exposure to the pharmaceutical atorvastatin was found to suppress mRNA expression of fatty acid metabolism genes in *Mytilus edulis* (Falfushynska et al., 2019), whilst exposure to PAHs and trace metals in *M. galloprovincialis* caged in the wild was shown to alter compositions of polyunsaturated fatty acids (PUFAs, Signa et al., 2015). Other altered pathways were similar to those observed in the caging study, including biosynthesis and metabolism of amino acids including leucine, arginine, and glutamate, with potential drivers discussed above. Several recent studies have suggested that zebra and quagga mussels may have different responses to stressors (Evariste et al., 2018; Nowicki and Kashian, 2018), therefore it is interesting to note that all three impacted sites showed differences from the reference in the present study, compared with only a single site in the previous study with *D. polymorpha*.

Though differences in the specific metabolic analysis and timing of sampling preclude a direct comparison, this could suggest a greater sensitivity of quagga mussels to metabolic stress.

CONCLUSIONS

Overall, the present study found that the duration of caging had a significant impact on metabolic responses of quagga mussels at six sites within the Milwaukee Estuary, with 55 days of caging shown to produce the greatest number of significantly different metabolites. Subsequent pathway analysis suggested that biosynthesis and metabolism of amino acids were most impacted. Furthermore, many of the initial changes after 2 days were transient and not reflective of metabolic changes after longer deployment durations. After 55 days of caging at all sites regardless of contamination, an up-modulation of phospholipids and acylcarnities was observed, which was likely due to gametogenesis and alterations to lipid metabolism prior to spawning. This finding highlights the importance of considering natural seasonal alterations to model organism metabolomes before use in environmental monitoring studies. Furthermore, the sites showing the greatest differences from source mussels appeared to be those characterized by the greatest temperature extremes rather than those exhibiting the highest levels of contaminants, with levels of several amino acids negatively related to average temperatures over the deployment period. This suggests that temperature may have had a greater influence on metabolic responses than levels of contaminants, despite an order of magnitude gradient in PAH residues. In a separate *in-situ* study, metabolic profiles of quagga mussels collected from an offshore site of lower contamination were significantly different than three harbor sites with greater levels of contamination. Whilst environmental metabolomics remains a useful tool for distinguishing among populations in biomonitoring, further research is needed to elucidate the drivers of change to metabolomes.

Table 1. Site details, coordinates, and environmental conditions (average temperature and dissolved oxygen) at eight sites within the Milwaukee Estuary AOC. Temperature and dissolved oxygen are provided over the 2, 5- and 55-day deployment periods, with the range in parentheses.

nd = no data due to an equipment failure in data loggers deployed at LMMB-04. Temperature and dissolved oxygen were not measured for the *in situ* study.

Table 2. Summary of CECs detected in POCIS deployed for 55 days at six sites in the Milwaukee Estuary (WI, USA) area of concern. CEC = Contaminants of Emerging Concern. TWA = Time Weighted Average. PPCPs = Pharmaceuticals and Personal Care Products.

Table 3. Time weighted average (TWA) concentrations of parent PAHs, alkylated PAHs, and legacy pesticides in SPMDs deployed at 6 sites in the Milwaukee Estuary AOC in 2017. SPMD = Semipermeable Membrane Device. PAH = Polycyclic Aromatic Hydrocarbon.

Table 4. Contaminants of emerging concern and PAHs in source quagga mussel tissue and after 55 days of caging in six sites of varying contamination in the Milwaukee Estuary, Wisconsin, USA. NA = not applicable.

Table 5. The number of significantly different metabolites (ANOVA, Posthoc Tukey's HSD) relative to source mussels after caging in six sites in the Milwaukee Estuary (WI, USA) Area of Concern after 2, 5 and 55 days of caging. Arrows represent increases or decreases relative to source mussels.

Table 6. Within-site timepoint comparisons of the number of significantly different metabolites in six sites in the Milwaukee Estuary AOC. Metabolite differences are shown as totals and also grouped by type.

Figure 1. Location of quagga mussels caged (circles), caged and collected *in situ* (squares) or collected *in situ* only (triangles) in the Milwaukee Estuary in 2017.

Figure 2. Body residues of A) contaminants of emerging concern and B) alkylated and parent PAHs in caged and *in situ* quagga mussels collected from the Milwaukee Estuary AOC in 2017.

Figure 3. Principal component analysis of quagga mussel metabolomes after 2 (A), 5 (B) or 55 (C) days of caging at six sites in the Milwaukee Estuary AOC in 2017. Differences are shown relative to the source mussel metabolome (indicated as t0).

Figure 4. Pathway analysis of significantly different metabolites relative to source (t0) mussels after 2, 5 and 55 days. Only metabolites from sites showing significant differences from source mussels were used for pathway analysis. Letters represent sites where significant pathways were identified: a= LMMB-08, b = LMMB-11, c = LMMB-05.

Figure 5. Principal component analysis of the metabolome of quagga mussels collected *in situ* from four sites in the Milwaukee Estuary AOC. The graph on the right displays the mean PC scores with the error bars representing the standard error of the mean.

Figure 6. Pathway analysis of all common significant metabolites in quagga mussels between the offshore site of lower contamination (LMMB-05) and three contaminated sites in the Milwaukee Estuary AOC. The y-axis describes the log transformed p-values from the pathway enrichment analysis, with only significant pathways (FDR p-value < 0.05) labelled. The x-axis represents the pathway impact values from the pathway topology analysis. The circle radius is based on this impact value, which ranges 0.0 to 1.0, and the larger radius indicates a greater impact value.

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Table 1. Site details, coordinates, and environmental conditions (average temperature and dissolved oxygen) at eight sites within the Milwaukee Estuary AOC. Temperature and dissolved oxygen are provided over the 2, 5- and 55-day deployment periods, with the range in parentheses. nd = no data due to an equipment failure in data loggers deployed at LMMB-04. Temperature and dissolved oxygen were not measured for the *in situ* study.

Site Code	Site Description	Coordinates	Average Temperature (°C)	Average Dissolved Oxygen (mg/L)
		Caging Study		
LMMB-04	Northern Outer Harbor	43.043, -87.888	nd	nd
LMMB-05	Lake Michigan Offshore	43.05943, -87.86472	T2: 11.6 (11.1 - 13.2) T5: 11.2 (9.00 - 16.0) T55: 12.6 (6.24 - 22.1)	T2: 13.0 (10.6 - 15.1) T5: 12.7 (10.3 – 14.4) T55: 11.8 (7.45 – 16.5)
LMMB-06	North Jones Island	43.0247, -87.8976	T2: 12.9 (11.6 – 15.0) T5: 13.1 (11.6 – 16.1) T55: 14.4 (8.46 – 23.3)	T2: 10.7 (9.10 – 11.9) T5: 10.9 (9.10 – 12.3) T55: 9.51 (4.01– 12.6)
LMMB-08	Upper Milwaukee River	43.05679, -87.8983	T2: 22.2 (20.7 – 23.1) T5: 23.0 (20.7 – 25.6) T55:23.7 (18.3 – 27.7)	T2: 8.33 (6.25 – 11.5) T5: 7.83 (4.00 – 11.8) T55: 7.00 (1.92 – 11.8)

LMMB-11	Upper Menominee River	43.03296, -87.9396	T2: 20.8 (19.0 – 22.5) T5: 21.7 (19.0 – 24.0) T55: 22.0 (17.3 – 25.2)	T2: 6.16 (4.02 – 8.46) T5: 6.07 (4 – 8.46) T55: 5.66 (4 – 8.46)
LMMB-13	Upper Kinnikinic River	43.0046, -87.913	T2: 19.2 (16.9 – 23.7) T5: 19.1 (16.7 – 23.7) T55: 20.9 (16.7 – 24.7)	T2: 9.23 (8.73 – 9.60) T5: 5.10 (4.02 – 9.60) T55: 5.49 (4.02 – 9.60)
		In Situ Study		
LMMB	Outer Harbor, Lakeshore Park	43.0329, -87.894	nd	nd
LMMB-01	Milwaukee Harbor Confined Disposal Facility (CDF)	43.0079, -87.888	nd	nd

Table 2. Summary of CECs detected in POCIS deployed for 55 days at six sites in the Milwaukee Estuary (WI, USA) area of concern. CEC = Contaminants of Emerging Concern. TWA = Time Weighted Average. PPCPs = Pharmaceuticals and Personal Care Products.

Site	Number of Compounds Detected	Maximum TWA Concentration (Compound Name, ng/L)	Total TWA Concentration (ng/L)
		Pesticides	
LMMB-4	11	Atrazine, 23.1	52.6
LMMB-5	10	Atrazine, 34.9	51
LMMB-6	15	Atrazine, 31.8	68.7
LMMB-8	12	Atrazine, 111	239
LMMB-11	15	Metolachlor, 77.7	238
LMMB-13	16	Atrazine, 63.2	146
		PPCPs	
LMMB-4	12	DEET, 10.4	17.5
LMMB-5	5	DEET, 3.50	3.91
LMMB-6	19	Hydrochlorothiazide, 68.6	137
LMMB-8	14	Hydrochlorothiazide, 28.7	52.1

LMMB-11	13	DEET, 55.9	60.5
LMMB-13	16	Bisphenol A, 149	249
		Hormones	
LMMB-4	1	Androstenedione, 1.47	1.47
LMMB-5	2	Androstenedione, 2.28	2.28
LMMB-6	1	Androstenedione, 2.19	2.19
LMMB-8	2	Androstenedione, 1.36	1.36
LMMB-11	1	Androstenedione, 2.18	2.18
LMMB-13	3	Androstenedione, 1.96	1.96
		Total	
LMMB-4	24	Atrazine, 23.1	71.6
LMMB-5	17	Atrazine, 34.9	57.2
LMMB-6	35	Hydrochlorothiazide, 68.6	208
LMMB-8	28	Atrazine, 111	292
LMMB-11	29	Metolachlor, 77.7	301
LMMB-13	35	Bisphenol A, 149	397

Table 3. Time weighted average (TWA) concentrations of parent PAHs, alkylated PAHs, and legacy pesticides in SPMDs deployed at 6 sites in the Milwaukee Estuary AOC in 2017. SPMD = Semipermeable Membrane Device. PAH = Polycyclic Aromatic Hydrocarbon.

Site	Number of Compounds Detected	Maximum TWA Concentration (Compound Name, ng/L)	Total TWA Concentration (ng/L)
		Parent PAHs	
LMMB-04	15	Fluoranthene, 7.44	23
LMMB-05	15	Fluoranthene, 2.68	6.64
LMMB-06	15	Fluoranthene, 6.74	20.7
LMMB-08	15	Fluoranthene, 10.5	30.3
LMMB-11	15	Fluoranthene, 97.8	294
LMMB-13	15	Fluoranthene, 176	503
		Alkylated PAHs	
LMMB-04	19	C1-Fluoranthenes, 4.68	20.5

LMMB-05	18	C1-Naphthalenes, 1.22	7
LMMB-06	20	C1-Fluoranthenes, 5.76	17.3
LMMB-08	19	C1-Fluoranthenes, 6.09	19.4
LMMB-11	20	C1-Fluoranthenes, 50.3	213
LMMB-13	20	C1-Fluoranthenes, 86.1	440
		Legacy Pesticides	
LMMB-04	19	Dieldrin, 0.043	0.196
LMMB-05	18	Dieldrin, 0.089	0.223
LMMB-06	20	p,p'-DDD, 0.096	0.525
LMMB-08	17	Dieldrin, 0.100	0.329
LMMB-11	20	p,p'-DDE, 0.204	0.953
LMMB-13	21	Dieldrin, 0.314	1.05

Table 4. Contaminants of emerging concern and PAHs in source quagga mussel tissue and after 55 days of caging in six sites of varying contamination in the Milwaukee Estuary, Wisconsin, USA. NA = not applicable. BRL = Below Reporting Limit.

Site	Number of compounds detected	Maximum Concentration (Compound, ng/g wet weight)	Total Concentration, Fold Change (ng/g wet weight)
		PPCP	
LMMB-04 (Source)	16	Sertraline, 34.8	62.6 (NA)
LMMB-04	10	Etoposide, 5.77	15.7 (0.25)
LMMB-05	8	Sertraline, 14.6	15.3 (0.24)
LMMB-06	18	Triclocarban, 11.5	28.1 (0.45)
LMMB-08	16	Sertraline, 11.9	21 (0.34)
LMMB-11	6	BRL	BRL (NA)
LMMB-13	6	Triclocarban, 3.67	5.7 (0.09)
		Pesticide	
LMMB-04 (Source)	12	4,4'-DDE, 3.18	6.70 (NA)
LMMB-04	17	4,4'-DDE, 6.26	13 (1.94)
LMMB-05	10	4,4'-DDE, 1.08	1.74 (0.26)
LMMB-06	16	4,4'-DDE, 8.10	15.9 (2.37)
LMMB-08	15	4,4'-DDE, 4.25	8.72 (1.30)
LMMB-11	17	4,4'-DDE, 16.3	30.4 (4.54)
LMMB-13	17	4,4'-DDE, 6.77	22.9 (3.42)
		Alkylphenols	
LMMB-04 (Source)	3	NP1EO, 34.1	50.6 (NA)
LMMB-04	3	4-Nonylphenol, 33.9	66.5 (1.31)
LMMB-05	3	NP1EO, 10.7	14.7 (0.29)
LMMB-06	3	4-Nonylphenol, 122	214 (4.23)
LMMB-08	3	NP1EO, 42.4	77.6 (1.53)
LMMB-11	4	4-Nonylphenol, 77	159 (3.14)
LMMB-13	4	4-Nonylphenol, 401	478 (9.45)
		Parent PAHs	
LMMB-04 (Source)	15	Chrysene, 874	4177 (NA)
LMMB-04	15	Chrysene, 861	4111 (0.98)
LMMB-05	15	Chrysene, 120	554 (0.13)

LMMB-06	15	Chrysene, 1892	8102 (1.94)
LMMB-08	15	Chrysene, 1193	6179 (1.48)
LMMB-11	15	Chrysene, 2440	13577 (3.25)
LMMB-13	15	Chrysene, 4904	27796 (6.65)
		Alkylated PAHs	
LMMB-04 (Source)	21	C1-Fluoranthenes, 347	2335 (NA)
LMMB-04	24	C1-Fluoranthenes, 426	3316 (1.42)
LMMB-05	12	C3-Phenanthrenes, 60.7	431 (0.18)
LMMB-06	18	C1-Phenanthrenes, 830	5379 (2.30)
LMMB-08	19	C1-Phenanthrenes, 555	4000 (1.71)
LMMB-11	25	C1-Phenanthrenes, 1551	9711 (4.16)
LMMB-13	27	C1-Phenanthrenes, 4235	24329 (10.4)

Table 5. The number of significantly different metabolites (ANOVA, Posthoc Tukey's HSD) relative to source mussels after caging in six sites in the Milwaukee Estuary (WI, USA) Area of Concern after 2, 5 and 55 days of caging. Arrows represent increases or decreases relative to source mussels.

Site	Time (d)	Total Number of Differences	All	Phospholipids and Acylcarnitines	Fatty acids	Amino acids and Biogenic amines	Energy Metabolites
Metabolic Differences After 2 Days Relative to Source Mussels							
LMMB-04	2	3	1↑, 2↓	0↑,0↓	0↑,2↓	1↑,0↓	0↑,0↓
LMMB-05	2	20	11↑, 9 ↓	0↑, 7↓	1↑,1↓	10↑,1↓	0↑,0↓
LMMB-06	2	35	15↑,11↓	0↑,8↓	0↑,3↓	15↑,0↓	0↑,0↓
LMMB-08	2	51	19↑,32↓	14↑, 6↓	3↑,4↓	2↑,22↓	0↑,0↓
LMMB-11	2	31	14↑,17↓	5↑, 8↓	1↑,4↓	8↑,4↓	0↑,1↓
LMMB-13	2	22	15↑,7↓	0↑, 6↓	0↑,0↓	15↑,1↓	0↑,0↓
Metabolic Differences After 5 Days Relative to Source Mussels							

LMMB-04	5	13	10↑,3↓	0↑,1↓	0↑,1↓	11↑,0↓	0↑,0↓
LMMB-05	5	22	16↑, 6 ↓	16↑, 1↓	0↑,3↓	0↑,2↓	0↑,0↓
LMMB-06	5	11	7↑,4↓	1↑,4↓	0↑,0↓	6↑,0↓	0↑,0↓
LMMB-08	5	38	18↑,20↓	16↑, 5↓	0↑,2↓	2↑,13↓	0↑,0↓
LMMB-11	5	36	22↑,14↓	16↑, 2↓	1↑,2↓	4↑,10↓	1↑,0↓
LMMB-13	5	12	12↑,0↓	10↑, 0↓	0↑,0↓	2↑,0↓	0↑,0↓
Metabolic Differences After 55 Days Relative to Source Mussels							
LMMB-04	55	27	25↑,2↓	25↑,2↓	0↑,0↓	0↑,0↓	0↑,0↓
LMMB-05	55	74	56↑, 18↓	56↑,0↓	0↑,4↓	0↑,14↓	0↑,0↓
LMMB-06	55	50	32↑,18↓	31↑,3↓	1↑,3↓	0↑,12↓	0↑,0↓
LMMB-08	55	97	64↑,33↓	62↑,5↓	0↑,5↓	2↑,23↓	0↑,0↓
LMMB-11	55	92	56↑,26↓	53↑,5↓	1↑,3↓	2↑,18↓	0↑,0↓
LMMB-13	55	68	49↑,19↓	46↑,2↓	0↑,4↓	2↑,13↓	1↑,0↓

Table 6. Within-site timepoint comparisons of the number of significantly different metabolites in six sites in the Milwaukee Estuary AOC. Metabolite differences are shown as totals and also grouped by type.

Site	Time (d)	Total Number of Differences	All	Phospholipids and Acylcarnitines	Fatty acids	Amino acids and Biogenic amines	Energy Metabolites
Day 5 vs Day 2 Comparisons							
LMMB-04	D5 vs D2	5	2↑, 3↓	0↑,2↓	0↑,0↓	2↑,1↓	0↑,0↓
LMMB-05	D5 vs D2	84	68↑,16↓	68↑,1↓	0↑,0↓	0↑,15↓	0↑,0↓
LMMB-06	D5 vs D2	13	5↑,8↓	3↑,2↓	0↑,1↓	2↑,5↓	0↑,0↓
LMMB-08	D5 vs D2	17	11↑,6↓	11↑, 3↓	0↑,2↓	0↑,1↓	0↑,0↓
LMMB-11	D5 vs D2	39	20↑19↓	20↑,0↓	0↑,0↓	0↑,19↓	0↑,0↓
LMMB-13	D5 vs D2	34	20↑,14↓	19↑,0↓	0↑,0↓	1↑,14↓	0↑,0↓
Day 55 vs Day 2 Comparisons							
LMMB-04	D55 vs D2	31	22↑, 9 ↓	21↑,3↓	0↑,0↓	1↑,6↓	0↑,0↓
LMMB-05	D55 vs D2	97	73↑,24↓	73↑, 0↓	0↑,2↓	0↑,22↓	0↑,0↓
LMMB-06	D55 vs D2	67	42↑,25 ↓	40↑, 3↓	2↑,1↓	0↑,21↓	0↑,0↓
LMMB-08	D55 vs D2	72	48↑,24↓	46↑, 0↓	1↑,3↓	1↑,21↓	0↑,0↓
LMMB-11	D55 vs D2	99	76↑, 23↓	70↑,0↓	0↑,1↓	0↑,22↓	0↑,0↓
LMMB-13	D55 vs D2	84	58↑,26↓	55↑,1↓	1↑,3↓	2↑,23↓	0↑,1↓
Day 55 vs Day 5 Comparisons							
LMMB-04	D55 vs D5	45	35↑,10↓	34↑,1↓	1↑,0↓	0↑,9↓	0↑,0↓
LMMB-05	D55 vs D5	39	20↑,19↓	19↑, 0↓	1↑,0↓	0↑,19↓	0↑,0↓

LMMB-06	D55 vs D5	71	46↑,25↓	43↑,1↓	2↑,3↓	1↑,21↓	0↑,0↓
LMMB-08	D55 vs D5	78	54↑,24↓	51↑, 0↓	2↑,3↓	1↑,21↓	0↑,0↓
LMMB-11	D55 vs D5	57	37↑,20↓	33↑,0↓	2↑,0↓	2↑,20↓	0↑,0↓
LMMB-13	D55 vs D5	52	23↑,29↓	20↑,3↓	1↑,4↓	1↑22↓	1↑,0↓