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# Multi-omics responses in tree swallow (*Tachycineta bicolor*) nestlings from the Maumee Area of Concern, Maumee River, Ohio

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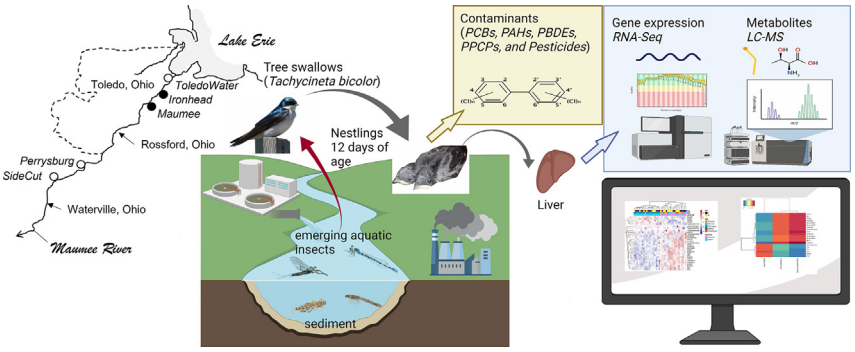
## HIGHLIGHTS

- Tree swallow nestlings were collected in the Maumee River, OH.
- Wastewater treatment plants and industrial land-use sites were included.
- We used a multi-omics approach to identify altered responses to chemical mixtures.
- Upregulated lipogenesis genes and metabolites were found at industrial sites.
- PAHs, oxychlordane, and PBDEs were the most likely driving contaminants.

## GRAPHICAL ABSTRACT

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## ABSTRACT

A multi-omics approach was utilized to identify altered biological responses and functions, and to prioritize contaminants to assess the risks of chemical mixtures in the Maumee Area of Concern (AOC), Maumee River, OH, USA. The Maumee AOC is designated by the United States Environmental Protection Agency as having significant beneficial use impairments, including degradation of fish and wildlife populations, bird or animal deformities or reproduction problems, and loss of fish and wildlife habitat. Tree swallow (*Tachycineta bicolor*) nestlings were collected at five sites along the Maumee River, which included wastewater treatment plants (WWTPs) and industrial land-use sites. Polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo *p* dioxins and furans (PCDD/Fs), and chlorinated pesticide concentrations were elevated in Maumee tree swallows, relative to a remote reference site, Star Lake, WI, USA. Liver tissue was utilized for non-targeted transcriptome and targeted metabolome evaluation. A significantly differentially expressed gene cluster related to a downregulation in cell growth and cell cycle regulation was identified when comparing all Maumee River sites with the reference site. There was an upregulation of lipogenesis genes, such as PPAR signaling (*HMGCS2*, *SLC22A5*), biosynthesis of unsaturated fatty acids (FASN, SCD, ELOVL2, and FADS2), and higher lipogenesis related metabolites, such as docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) at two industrial land-use sites, Ironhead and Maumee, relative to WWTP sites (Perrysburg and SideCut), and the reference site. Toledo Water, in the vicinity of the other two industrial sites and also adjacent to a

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WWTP, showed a mix of signals between industrial land-use and WWTP land-use. PAHs, oxychlordane, and PBDEs were determined to be the most likely causes of the differentiation in biological responses, including *de novo* lipogenesis and biosynthesis of unsaturated fatty acids.

## 1. Introduction

With a goal to achieve a sound long-term management plan and to prevent irreversible damage, the Great Lakes Restoration Initiative was established to assess the effects of human activities on streams, lakes, and drainage basins in the Great Lakes region and to provide a reliable plan for identifying beneficial use impairments, identify their causes, and to develop effective monitoring processes (US Environmental Protection Agency, 2012). The Great Lakes water quality agreement, as an extension of the Clean Water Act, aims to monitor the Great Lake's water quality, specifically focusing on toxic pollutants. Water bodies with elevated contaminants and degradation were assigned by the United States Environmental Protection Agency as Areas of Concern (AOCs) to monitor and restore their beneficial use impairments (BUIs) or designated uses (Hauserman, 2014). The Maumee AOC has been a site of industrial and municipal development with decades of unregulated waste disposal, industrial contamination, sewer overflows, and disposal of dredged materials (US Environmental Protection Agency, 2013). Thus, the Maumee AOC has been designated as having significant BUIs, including degradation of fish and wildlife populations, bird or animal deformities or reproduction problems, and loss of fish and wildlife habitat. The source of contamination is a combination of farming and animal operations in upstream reaches, and wastewater treatment and industrial runoff in the middle and downstream areas of the Maumee River (Cipoletti et al., 2019; Custer et al., 2020).

Legacy and emerging contaminants in the Maumee AOC have raised concerns over the possible causes of tumors and other deformities in wildlife communities, and potential degradation of populations through altered reproduction (Cipoletti et al., 2019; Karr et al., 1985). To monitor and assess the current conditions and effectiveness of the restoration of the Maumee AOC, legacy and emerging contaminants have been measured in sediments and biota, such as mussels (*Eurynia dilatata* and *Lampsilis cardium*) and caged fathead minnows, *Pimephales promelas* (Ankley et al., 2020; Woolnough et al., 2020), and in tree swallows (*Tachycineta bicolor*, Custer et al., 2020). Contaminant distributions were related to land-use, such as agricultural and wastewater treatment plants, and correlated with elevated responses in the aryl hydrocarbon receptor (AhR) pathway (Ankley et al., 2020). The primary environmental contaminants in sediment and biota were identified as legacy contaminants including polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), perfluorinated substances (PFAS), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo *p* dioxins and furans (PCDD/Fs), pesticides, and other emerging environmental contaminants from effluents such as pharmaceuticals and personal care products (PPCPs) (Custer et al., 2020; Woolnough et al., 2020). Halogenated hydrocarbons, such as PCBs, PBDEs, PAHs, and PCDD/Fs were persistent in sediments and further transferred and bioaccumulated into terrestrial organisms, where they can cause deformities and reproductive effects (Bishop et al., 1995, 1999; Custer et al., 2017b; Fry, 1995). Although few emerging contaminants such as PPCPs have been quantified, many are known to cause adverse effects to aquatic and terrestrial organisms, especially with regard to endocrine disruption (Boxall et al., 2012; Ebele et al., 2017).

Tree swallows have long been used as a model to evaluate the movement of sediment contaminants to birds, partially because of the ease of setting up nest boxes and attracting occupants, but also because of their feeding strategies (Custer, 2011). Nestlings are fed aerial stages of benthic aquatic insects with a localized feeding radius of 225 m and have been shown to reflect contaminant profiles in sediments near nest boxes (Custer, 2011; Custer et al., 1998).

Working with contaminant mixtures presents challenges because the complexity of contaminant interactions and effects exceeds our current capacity to predict toxicity. Environmental contaminants can cause additive, synergistic, and antagonistic effects, which have been reported in multiple *in vivo* and *in vitro* studies (Altenburger et al., 2013, 2000; Backhaus et al., 2000). A lack of understanding of the magnitude of chemical interactions still remains. We need an approach to prioritize the chemicals that result in chronic or more complex effects. The biological effects of complex mixtures of pollutants on birds have been shown in multiple recent studies. Species-specific ToxChip polymerase chain reaction assays were shown to be capable of prioritizing environmental contaminants, such as dioxin-like chemicals, organic flame retardants, and environmental mixtures collected from the field or wild avian eggs, and determining perturbed pathways, such as metabolism, immune function, thyroid hormone, oxidative stress, and lipid homeostasis, in chicken and double-crested cormorant hepatocytes (Crump et al., 2019, 2016; Ha et al., 2021; Pagé-Larivière et al., 2018).

Recent technological improvements in omics, including transcriptomics, proteomics, and metabolomics analysis, allow for a better understanding of the alterations in toxicogenomic data in organisms exposed to environmental contaminants, including natural and anthropogenic factors. They also provide a systematic approach to investigate correlations between environmental contaminants and the interaction between different omics responses (Altenburger et al., 2012; Heijne et al., 2005; Porter et al., 2014).

Additionally, these new technological developments, in combination with multivariate analysis, could help determine if subsets of differentially altered genes or metabolites in exposed organisms were statistically significantly enriched for similar functions and pathways (Huang et al., 2017; Yang et al., 2007). Nontargeted biological measurements in metabolomics and transcriptomics have been used in many studies to identify biochemical changes in terrestrial animals related to local contaminant mixture exposures and predicted potential biological effects (Ankley et al., 2020; Tartu et al., 2017).

In the present study, we collected liver tissue from tree swallow nestlings from multiple locations in the Maumee AOC, as well as from a reference area in northern Wisconsin. We evaluated transcriptomic and metabolomic responses and looked for potential correlations between contaminant exposures and biomolecular responses. We also attempted to isolate the dominant contaminants driving major transcriptomic and metabolomic responses. We aimed to improve approaches to create linkages between complex environmental contaminant mixtures and altered biomolecular responses, to serve as a tool in future ecotoxicological research and biomonitoring. Specifically, we investigated whether responses in genetic/metabolomic functions were related to local land-use, information which could be incorporated into an integrated risk assessment plan.

## 2. Materials and methods

### 2.1. Nestling sampling and site descriptions

The Maumee River AOC is located in northwest Ohio. Tree swallow nest boxes (n = 15–20 per site) were placed at five locations along the Maumee River (Fig. 1) and remote reference sites, Star Lake (46°1′28.73″N, 89°28′13.15″W) and Plum Lake (45°59′25.64″N, 89°33′36.00″W). These two reference sites, in the vicinity of northern Wisconsin Lakes, were combined and labelled as Star Lake in this study. The most upstream site was SideCut (41°32′22.14″N, 83°41′41.97″W), followed by Perrysburg (41°33′35.81″N,

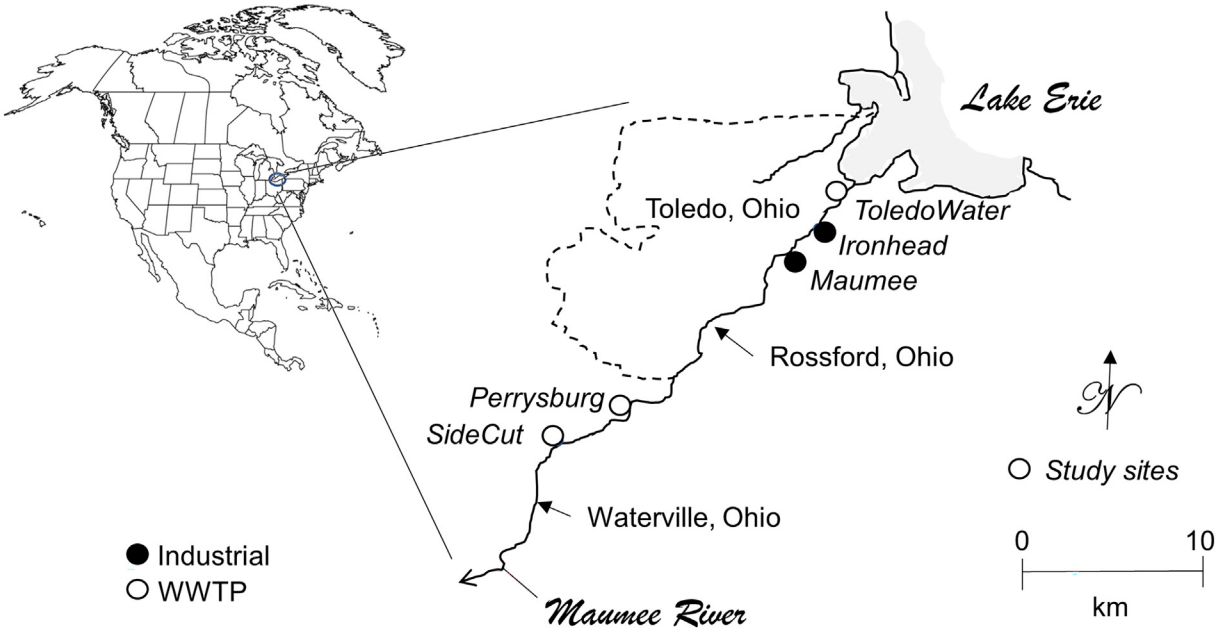


**Fig. 1.** Tree swallow (*Tachycineta bicolor*) study sites in 2016 along the Maumee River, Ohio. Land-use included wastewater treatment plant (WWTP) sites and industrial sites. The most upstream site was SideCut (41°32′22.14″N7, 83°41′41.97″W), followed by Perrysburg (41°33′35.81″N, 83°38′34.61″W), Maumee (41°39′16.05″N, 83°31′10.16″W), Ironhead (41°40′12.07″N, 83°29′38.51″W), and finally Toledo Water (41°41′23.31″N, 83°28′40.21″W).

83°38′34.61″W), Maumee (41°39′16.05″N, 83°31′10.16″W), Ironhead (41°40′12.07″N, 83°29′38.51″W), and finally Toledo Water (41°41′23.31″N, 83°28′40.21″W), which is near the mouth of the river, draining into Lake Erie. There was a gradient from predominantly agricultural chemical inputs upstream to urban and industrial inputs downstream along the Maumee River. Nest boxes at SideCut, Perrysburg, and Toledo Water were located near wastewater treatment plants (WWTPs). Tree swallow nestlings were collected from the nest boxes. Nestling samples were collected for chemical exposure, transcriptomic, and metabolomic analysis. One nestling per nest box was selected for transcriptomic analysis and metabolomic analysis. Individual nestling carcasses were stored at −20 °C for chemical analysis. Emerging contaminants including PBDEs, PFAS, PAHs and non-organochlorine pesticides (N-OCs) were also analyzed. PCBs, organochlorine contaminants, PFAS, PBDEs, and N-OCs were measured in nestling carcasses. PPCPs were measured in nestling livers. Stomach contents collected from the nestlings at the same site were composited and analyzed for PAHs. All chemical analyses were completed by SGS AXYS Analytical (Sidney, British Columbia, CAN) and followed standard USEPA methods (SGS AXYS Analytical Services, 2017). All contaminants data utilized in the current study were previously reported in Custer et al. (2020). Nestlings used in the current paper were collected during the same sampling event as used in Custer et al. (2020). Thus, contaminants and omics data can be directly compared from birds collected in the same year, site, and nest. Tree swallow parents have distinct foraging areas, which leads to similar exposures for nestlings, therefore, nest (rather than nestling) is the appropriate unit of replication for this study.

The total number of nestlings selected for transcriptome and metabolome analyses are provided in Table S1. Liver tissue was collected from 11 to 13 day old (post hatch) nestlings. Nestlings were euthanized by decapitation with minimal effects on biochemical responses. Liver and blood were quickly extracted in the field. Blood was collected *via* funnel into a heparinized tube and an aliquot was snap frozen in liquid nitrogen and stored at −80 °C until analysis. A portion of the liver was excised and frozen in a vapor phase liquid nitrogen dewar while in the field and transferred to a −80 °C freezer for storage prior to metabolomic analysis. A second liver portion from the same nestling was excised and submerged in RNALater for 24 h on ice, and then frozen at −20 °C for RNA extraction. Liver tissues were extracted, processed, and properly preserved within 5 min of euthanasia. All samples were collected under applicable federal (MB123047-0), state (17-33), and local (012716, 012717) permits. Animal procedures were reviewed and approved by the U.S. Geological Survey, Upper Midwest Environmental Sciences Center's Animal Care and Use Committee. Detailed collection methods were described in Custer et al., 2020.

### 2.2. De novo genome assembly from linked reads

Nestling blood samples were used for sequencing and assembly of a reference tree swallow genome. Blood DNA was extracted using the Purispin Fire Monkey Kit (RevoluGen, UK) for high-molecular-weight DNA. Sample quality was evaluated on an Agilent Fragment Analyzer and normalized to ~1.0 ng/μL using a Qubit dsDNA High Sensitivity Assay Kit (ThermoFisher, MA) before library preparation. Libraries were made using the 10× Chromium Genome v2 protocol. Libraries were sequenced on the NovaSeq6000 using 150 bp paired-end reads. Linked reads were assembled with Supernova assembler v2.1.1 and further processed with Arc using XSEDE HPC resources (Towns et al., 2014; Weisenfeld et al., 2017; Yeo et al., 2017), resulting in an N50 of 15.6 Mbp. The assembled genome is available in the National Center for Biotechnology Information Genome under BioProject PRJNA835816. The assembled genome was annotated using the Maker2 pipeline with 89.3 % complete Vertebrata BUSCOs and 85.5 % complete Aves BUSCOs (Holt and Yandell, 2011; Ott et al., 2018; Yeo et al., 2017). Detailed scripts and parameters are described in Supplementary document 1.

### 2.3. RNA isolation and sequencing

Liver tissue was manually homogenized in a sample tube using a disposable pestle and RNA extraction steps were performed using TRI-Reagent and 1-bromo-3-chloropropane (BCP) as described by Molecular Research Center protocol (mrcgene.com; TRI REAGENT-RNA/DNA/PROTEIN ISOLATION REAGENT; Section I: Steps 1–5; May 2014). RNA solubilization was done in nuclease free water and stored at −80 °C until transferred to the analytical facility for sequencing. RNA purity and concentrations were

determined by UV absorbance at 260 nm and 280 nm using a NanoDrop (Thermo, MA). RNA quality was assessed on a 2100 Bioanalyzer (Agilent, CA). For each library preparation, mRNA was purified from 1 μg total RNA using poly-T oligo-attached magnetic beads and was prepared according the TruSeq® Stranded mRNA Sample Preparation Guide (Rev. E) using the Illumina® TruSeq® Stranded mRNA Sample Preparation kits. Quality assessment and quantification was performed using KAPA Library Quantification Kits (Roche Sequencing Solutions, IN, USA) and the libraries were sequenced on an Illumina 2500 to generate paired-end reads of 150-bases. Reads were trimmed using Trimmomatic (Bolger et al., 2014) with a minimum phred of 33. Trimmed reads were aligned to the assembled genome using STAR (Dobin et al., 2013) with an alignment rate of 85 % or higher and final abundance estimation was performed with featureCounts (Liao et al., 2014). Detailed scripts and parameters are described in Supplemental document 1. All RNA sequencing files are available in the National Center for Biotechnology Information Sequence Read Archive under BioProject PRJNA857430.

### 2.4. Metabolomics extraction and analysis

Approximately 50 to 100 mg of liver samples were homogenized and sequentially extracted with methanol at SGS AXYS Analytical Services LTD (Sidney, BC, Canada), on three 96-well plate batches and analyzed by LC-MS/MS, following SGS AXYS Standard Operation Procedure MLM-001, Determination of Metabolites in Biological Samples (Targeted Metabolomics) (Benskin et al., 2014). Targeted metabolomics included MABA: Amino acids and biogenic amines (43 metabolites), MBIA: Bile acids (13 metabolites), MFAA: Fatty acids (18 metabolites), HEX: and Hexose (1 metabolite), MLIP: Phospholipids and acylcarnitines (144 metabolites), MNRG: Metabolites associated with energy pathways (17 metabolites). Internal reference matrix (IRM) with known characterized range of the calibrated range, selected blank samples, and study samples were included in the analysis. Background levels of metabolites, as indicated by blank sample values, were used to adjust the metabolite reporting limits. Reporting limits were calculated as the average blank value plus three times the blank standard deviation. Metabolites were detected in each sample and only metabolite concentrations greater than this limit were included in the analyses. Analysis for this study focused on metabolites with reliable detections; any metabolites not detected in at least 80 % of samples were removed.

### 2.5. Transcriptome and metabolome data processing

Nestlings from each site were selected to compare omic responses between sites (Table S1). For transcriptome data, a count matrix was generated using featureCounts and was independently filtered by only including genes with at least one count from all individuals at the same site. Differentially expressed genes (DEGs) were determined following count normalization and quasi-likelihood F-test using EdgeR (Robinson et al., 2010). Top DEGs with a false discovery rate (FDR) of <0.05 were determined by 1) comparison between all the Maumee River sites and Star Lake reference site, 2) pairwise comparison between individual site and Star Lake reference site, and 3) comparison between Maumee sites with an ANOVA-like design.

Biological process (BP) gene ontology (GO) terms and KEGG pathways were determined using G-profiler (Reimand et al., 2007).

Differentially altered metabolites were determined by linear regression fitting using linear mixed-effects models (Bates et al., 2015) followed by Benjamini-Hochberg Procedure (BH) for correcting multiple comparisons.

### 2.6. Multivariate analysis and data visualization

R package Vegan was used in ordination analysis including multidimensional scaling ordination (MDS) in Euclidean distance matrices and non-metric multidimensional scaling (NMDS) in Bray-Curtis dissimilarity for multivariate analysis of contaminants and top transcriptomic responses (Oksanen et al., 2020). Separation between land-use was analyzed using vegan adonis multivariate test using 999 permutations. Top correlated contaminants were selected using vegan envfit using 999 permutations and plotted on the MDS ordination plot. The top 200 most differentially expressed genes (DEGs) were plotted on NMDS ordination and consensus clustered using R package DEGreport (Pantano, 2019). Differentially expressed genes (DEGs) between different Maumee River sites and Star Lake were examined using R package UpSetR (Gehlenborg, 2019). BP GO terms, KEGG terms, and differentially altered metabolites (DAM) were identified at each site and visualized using R package ggplot2 (Wickham, 2016). Top DEGs and DAMs among Maumee River sites were visualized by heatmap using R package Pheatmap (Kolde, 2019).

Partial least-squares discriminant analysis (PLS-DA) was used for both DEGs and DAMs between Maumee sites to illustrate site separation. Sparse partial least-squares (sPLS) analysis was used to correlate top variates from

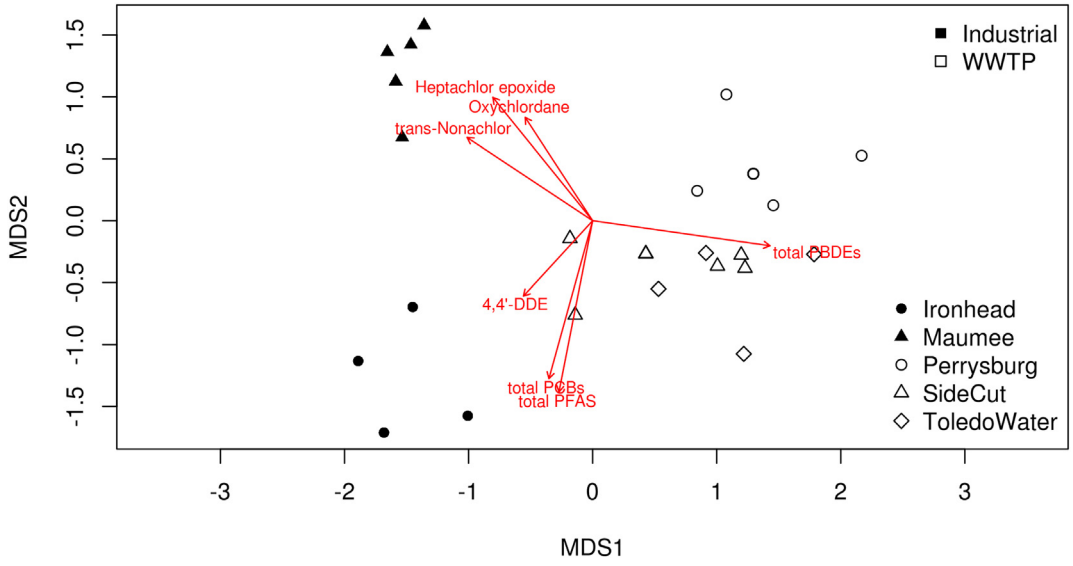


**Fig. 2.** Major variation in contaminant profiles on a multidimensional scaling ordination plot in Euclidean distance at tree swallow study sites along the Maumee River, Ohio. Sites are color-coded by land-use. Environmental contaminants including polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), perfluorinated substances (PFAS), and chlorinated pesticides, significantly correlated with the separation between sites ($p < 0.001$, permutation test) and are shown as vectors. The separation between land-use is significant ($p < 0.001$) based on 999 permutations.

DEGs and DAMs together to present the potential interaction between DEGs and DAMs, or with 13 major contaminant classes (Custer et al., 2020). MixOmics, R was used in the PLS and sPLS analysis described above (Rohart et al., 2017).

## 3. Results

### 3.1. Contaminant profiles

Contaminant concentrations in nestling carcasses and stomach contents were highly correlated with local land-use. The separation between sites based on major legacy and emerging contaminant concentrations showed a significant differentiation between the three WWTP sites (Perrysburg, SideCut, and Toledo Water), and the two industrial sites (Ironhead and Maumee), in a multidimensional scaling configuration using Euclidean distance ($p < 0.001$, permutation test) (Fig. 2). This current analysis was adapted from Custer et al., 2020. Top contaminants correlated with the separation of individual sites included PCBs, PBDEs, and per- and polyfluoroalkyl substances (PFAS) ($p < 0.001$, permutation test), followed by multiple chlorinated pesticides, such as oxychlordane.

Detailed contaminant concentrations in nestling carcasses and stomach contents were reported in Custer et al., 2020. Total PCBs, PBDEs, and legacy pesticides were elevated at all study sites along the Maumee River, relative to Star Lake, while concentrations of perfluorooctane sulfonate (PFOS) and total PFAS were similar between all sites along the Maumee River and only slightly elevated relative to Star Lake. Few personal care products in livers and non-organochlorine agricultural chemicals in carcasses were detected. Higher levels of PBDEs were measured in nestling carcasses at WWTP sites than from industrial sites (Fig. 2). Total and parent PAHs were elevated in pooled nestling stomach contents at Toledo Water, relative to the two upstream WWTP sites, Perrysburg and SideCut (Fig. S1). There was a general trend of higher concentrations of parent PAHs and parent to total PAH ratios in pooled stomach contents (by site) at downstream locations than at upstream locations. However, there was a unique contaminant signature of both elevated concentrations of PAHs and PBDEs at Toledo Water, which is a downstream WWTP site and located closer to the other two industrial sites. Detailed contaminant profiles from tree swallows at study sites along the Maumee River, OH were previously reported in Custer et al. (2020).

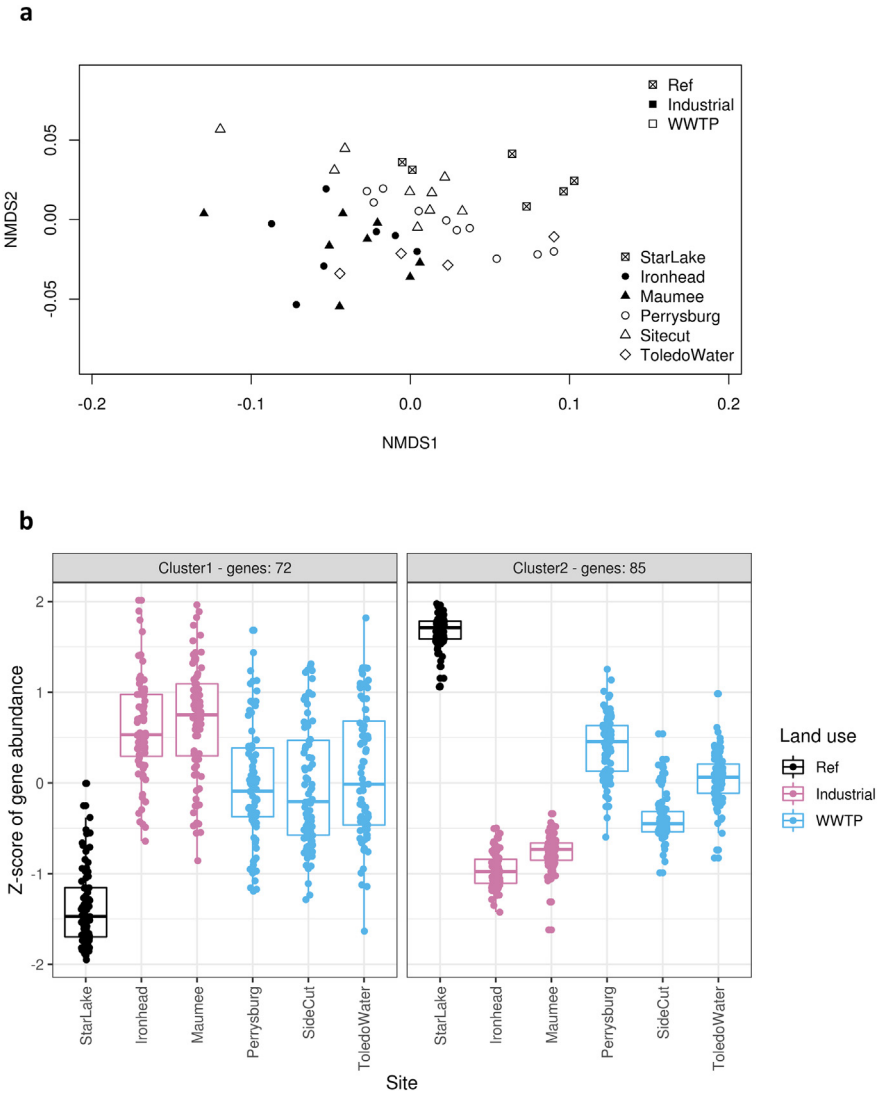


**Fig. 3.** (a) Non-metric multidimensional analysis of the top 200 most significant differentially expressed genes between the Maumee River sites and the reference site, Star Lake, Wisconsin. Individual samples are shape- and land-use coded. (b) Two major co-expressed consensus clusters in the top 200 most significant differentially expressed genes (DEGs) between the Maumee River sites and the reference site, Star Lake. Sites are color-coded by land-use. Z-scores were calculated for each gene and plotted at individual sites.

### 3.2. General tree swallow transcriptomic responses at sites along the Maumee River

Between the Maumee River sites and Star Lake reference site, we found a downregulation in cell cycle regulating genes, such as cyclin-dependent kinases (*CDK2*, *CDK6*), cell division cycle 25A (*CDC25A*), serine/threonine-protein kinase (*PLK1*), E2F transcription factors (*E2Fs*) and an upregulation in lipogenesis genes involved in cholesterol and fatty acids biosynthesis, such as mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 1 (*HMGCS1*), sterol regulatory-element binding protein (*SREBF2*), HMG-CoA reductase (*HMGCR*), mevalonate kinase (*MVK*), squalene epoxidase (*SQLE*), lanosterol synthase (*LSS*), lanosterol 14α-demethylase (*CYP51A1*), (*CHCR7*) fatty acid synthase (*FASN*), fatty acid elongases (*ELOVL6*) (Table S2a). Transcriptomic responses based on the top 200 most significantly DEGs (*FDR* < 0.05) between the Maumee River sites and the reference site, Star Lake, showed a separation between the reference and all Maumee River sites on an NMDS ordination plot (Fig. 3a). There was also a separation between two downstream industrial sites Maumee and Ironhead, and upstream WWTP sites SideCut and Perrysburg. Transcriptomic responses at Toledo Water were intermediate between the other industrial and WWTP sites. Transcriptomic responses at the upstream WWTP sites were closer to the responses at Star Lake. There were two co-expressed gene clusters in the top 200 DEGs (FDR < 0.05, Fig. 3b). Compared to WWTP sites and Star Lake, an upregulation in cluster 1 genes was measured at industrial sites, including Ironhead and Maumee. A downregulation in cluster 2 genes relative to Star Lake was observed at all sites. When examining the gene functions and pathways in co-expressed cluster 1 and cluster 2, lipid and sterol biosynthetic processes related genes dominated the co-expressed gene cluster 1, and mitotic cell cycle phase transition and meiotic nuclear division related genes dominated the co-expressed gene cluster 2 (Fig. S2a, b).

The downregulation of genes related to cell proliferation in cluster 2 was significant at the Maumee River sites relative to Star Lake (FDR < 0.05). However, the differentiation in cluster 2 gene expression among Maumee River sites was not significant, suggesting that these sites shared similar profiles in cell cycle regulation.

### 3.3. Transcriptomic responses between individual Maumee sites and Star Lake

When comparing individual Maumee River sites with Star Lake, the highest number of DEGs (Table S2b) were identified at Ironhead (n = 1478), followed by Maumee (n = 1228), and SideCut (n = 775) with an intermediate number of DEGs, and finally Perrysburg (n = 195) and Toledo Water (n = 0) with the fewest (*FDR* < 0.05) (Fig. 4a, b). The upregulated DEGs were most similar between Maumee and Ironhead, followed by SideCut and Perrysburg; however, most altered DEGs had similar functions (Fig. 5a, b). The pattern was somewhat similar for the downregulated genes, a greater number of DEGs were shared among Ironhead, Maumee, and SideCut, compared to the number shared between Maumee and Ironhead. Perrysburg shared fewer downregulated DEGs with Ironhead, Maumee, and SideCut. Toledo Water still had no DEGs, whereas Perrysburg had 58 unique DEGs. There was greater within group variation at Toledo Water (Fig. 3a), which likely contributed to the lack of DEGs.

Relative to Star Lake, Ironhead and Maumee exhibited similar BP GO functions but fewer terms were over-represented at Perrysburg and Toledo Water (Fig. 5a). Lipid and amino acid metabolic and steroid biosynthetic process related DEGs dominated the upregulated DEGs at Ironhead and Maumee but not at Perrysburg or SideCut. Downregulated DEGs related to cell cycle regulation and DNA replication were measured at Ironhead, Maumee, and SideCut. A higher number of KEGG pathways were enriched relative to Star Lake at Ironhead and Maumee than at Perrysburg and SideCut (Fig. 5b). Upregulated DEGs were related to amino acid, energy, and fatty acid metabolism related KEGG pathways. Downregulated DEGs were associated with cell cycle, DNA replication, and cellular senescence related KEGG pathways.

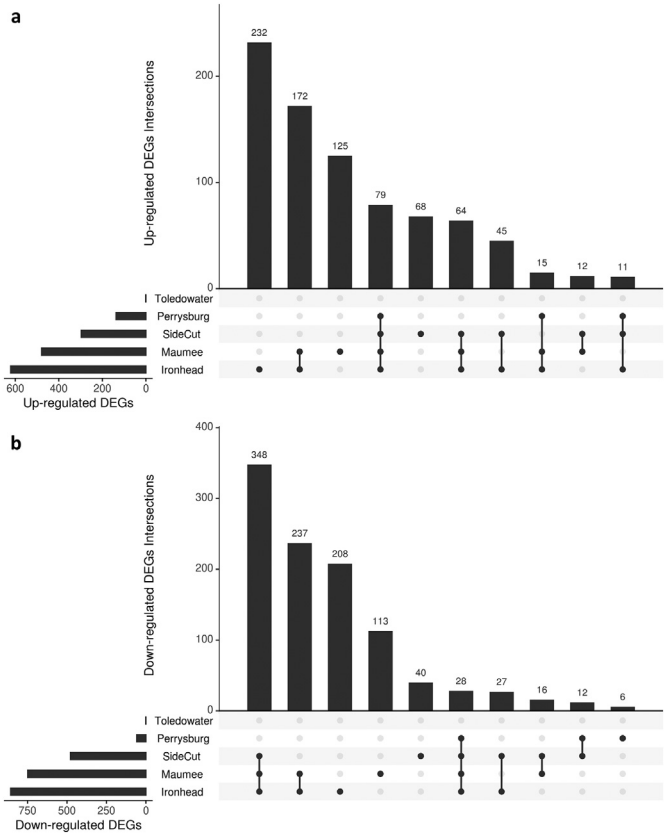


**Fig. 4.** (a) Upregulated and (b) downregulated differentially expressed genes (DEGs) at study sites along the Maumee River, OH relative to Star Lake, WI. No significant DEGs were identified at Toledo Water. The total number of DEGs at each site relative to the reference site, Star Lake, is indicated by the horizontal bars on the left. The unique DEGs at each site are labelled on the vertical bars shown above the single dot for that site. The intersected DEGs between sites are labelled on the vertical bars shown above the vertically-linked dots.

### 3.4. Transcriptomic responses in nestling livers between Maumee sites

All the DEGs (n = 30), identified between Maumee sites, were phospholipase A2 group IB (*PLA2G1B*), fatty acids metabolism/synthesis related genes such as malic enzyme 1 (*ME1*), solute carrier family 22 member 5 (*SLC22A5*, carnitine transporter), hcy-binding domain-containing protein (*BHMT2*, methyltransferase), arachidonate 5-lipoxygenase (*ALOX5*), fatty acid elongase 2 (*ELOVL2*), fatty acid desaturase 2 (*FADS2*), and stearoyl-CoA desaturase (*SCD*), and sterol biosynthesis related genes, such as sterol regulatory element-binding protein (*SCAP*) and lanosterol synthase (*LSS*) (Fig. S3). These DEGs were over-represented in lipid metabolic process, monocarboxylic acid biosynthetic process, and small molecule biosynthetic process BP GO terms, and corresponded to biosynthesis of unsaturated fatty acids, PPAR signaling pathway, alpha-linolenic acid metabolism KEGG terms. There was good separation between the industrial sites (Ironhead and Maumee) and two of the WWTP sites (Perrysburg and SideCut). Toledo Water was labelled as WWTP land-use, but its transcriptomic responses in these top DEGs were closer to industrial sites which were in close proximity (Fig. 6a).

### 3.5. Metabolomic responses in nestling livers between Maumee sites

When comparing among Maumee River sites, we found the highest number of DAMs between WWTP sites (Perrysburg and SideCut) and industrial sites (*i.e.*, Maumee and Ironhead). The highest number of DAMs was associated with phospholipids, followed by amino acids, and fatty

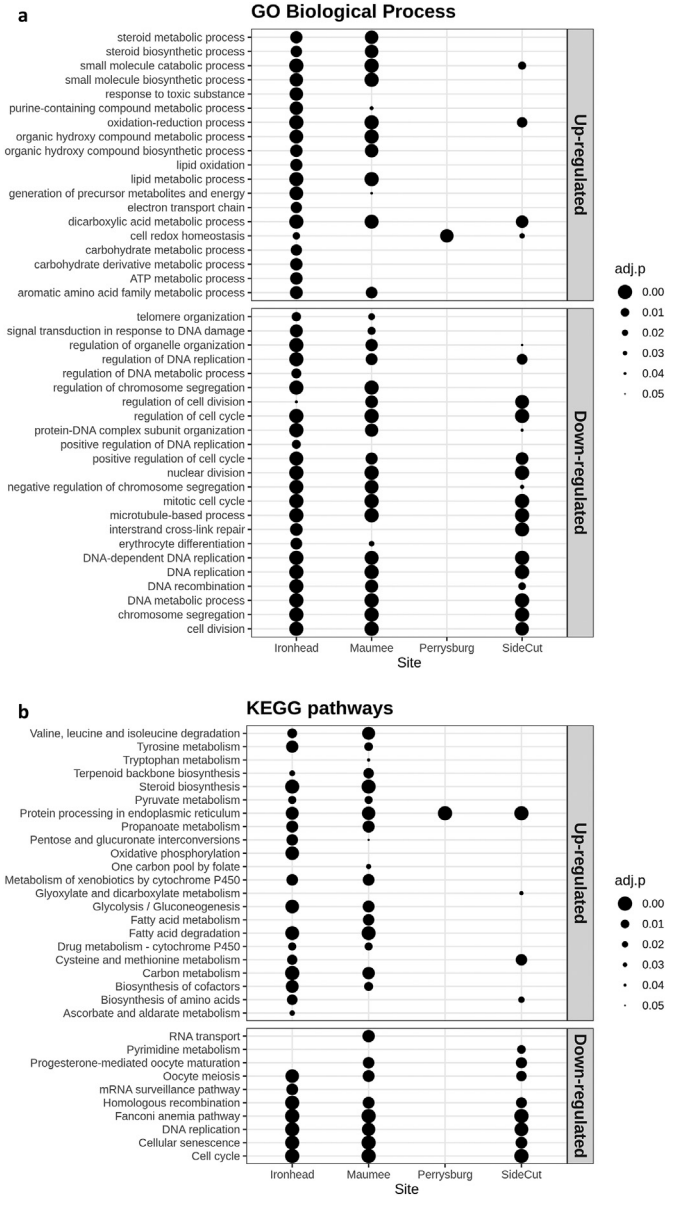


**Fig. 5.** Enriched (a) Gene Ontology (GO) Biological Process (BP) functional terms (b) KEGG pathways in upregulated and downregulated differentially expressed genes (DEGs) at study sites along the Maumee River, OH relative to Star Lake, WI. Enriched terms were determined using over-representation analysis (ORA) with adjusted *p*-value (*adj.p*) < 0.05. Larger dots indicate more differences (either Up- or Downregulated) relative to Star Lake.

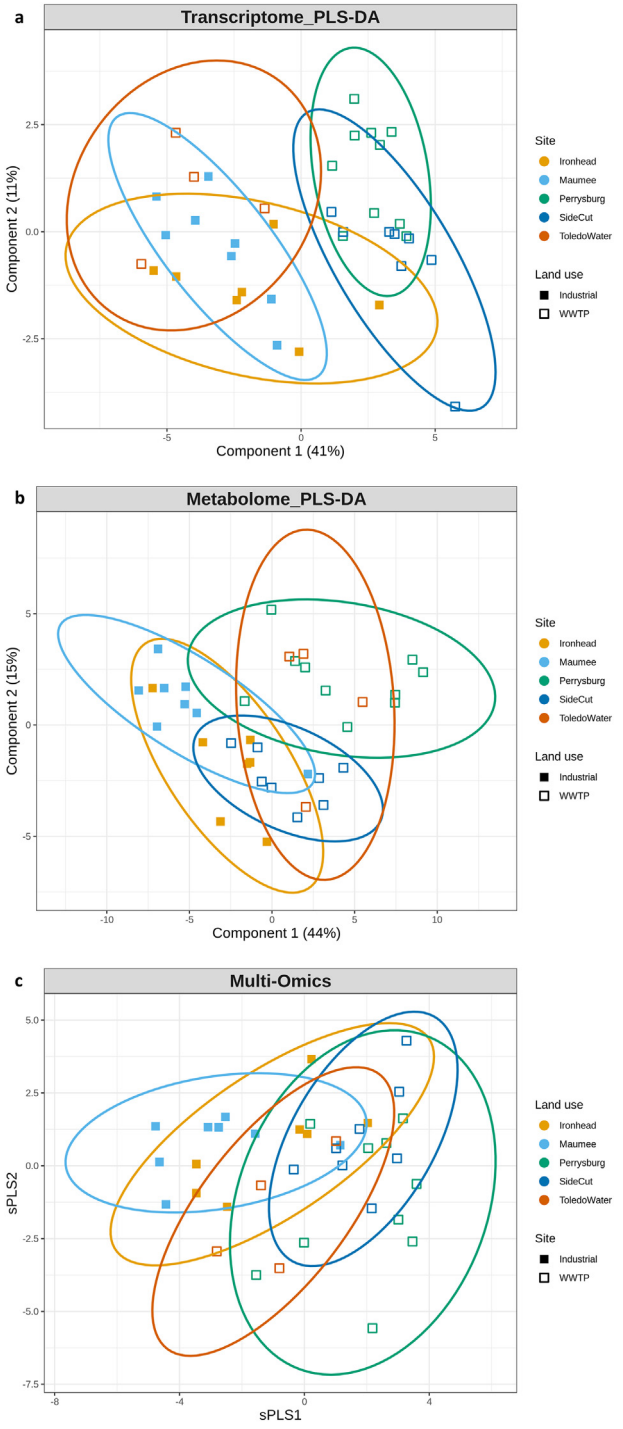


**Fig. 6.** (a) Thirty significantly differentially expressed genes (DEGs) were identified at all study sites along the Maumee River, OH relative to each other and used in a partial least-squares discriminant analysis (PLS-DA). (b) Partial least-squares discriminant analysis (PLS-DA) of metabolomic responses in significantly differentially altered metabolites (DAMs) at tree swallow study sites along the Maumee Rive, OH. (c) Combining transcriptomic with metabolomic responses at tree swallow study sites along the Maumee River, OH, in 2016. Differentially expressed genes (DEGs) and differentially altered metabolites (DAMs) between Maumee River sites were combined in a sparse-partial least-squares analysis (sPLS). Individual samples are color- and land-use coded with the 95 % confidence interval ellipses.

acids. There were few energy metabolites identified in the DAMs (Fig. S4). PLS-DA analysis showed that Perrysburg separated well from Maumee and Ironhead, the two industrial sites (Fig. 6b), although the separation of SideCut from the two industrial sites was not observed. A general trend of higher phospholipids, unsaturated fatty acids, and glutathione (GSH) was observed at industrial sites (Maumee and Ironhead), relative to WWTP sites (Perrysburg, SideCut, and Toledo Water). However, amino acids were elevated at WWTP sites relative to industrial sites (Fig. 7). Glutathione (GSH) and docosapentaenoic acid (DPA) were the two most altered metabolites. The amount of GSH was significantly higher at Ironhead followed by Maumee, Perrysburg, and SideCut (Fig. S5a). The amount of DPA at Maumee was significantly higher than at Perrysburg and SideCut (Fig. S5b). Responses in GSH and DPA were in the same direction except at Toledo Water. These significant DAMs were most associated with alpha linolenic acid and linoleic acid metabolism, urea cycle, and aspartate metabolism KEGG pathways (Fig. S6).

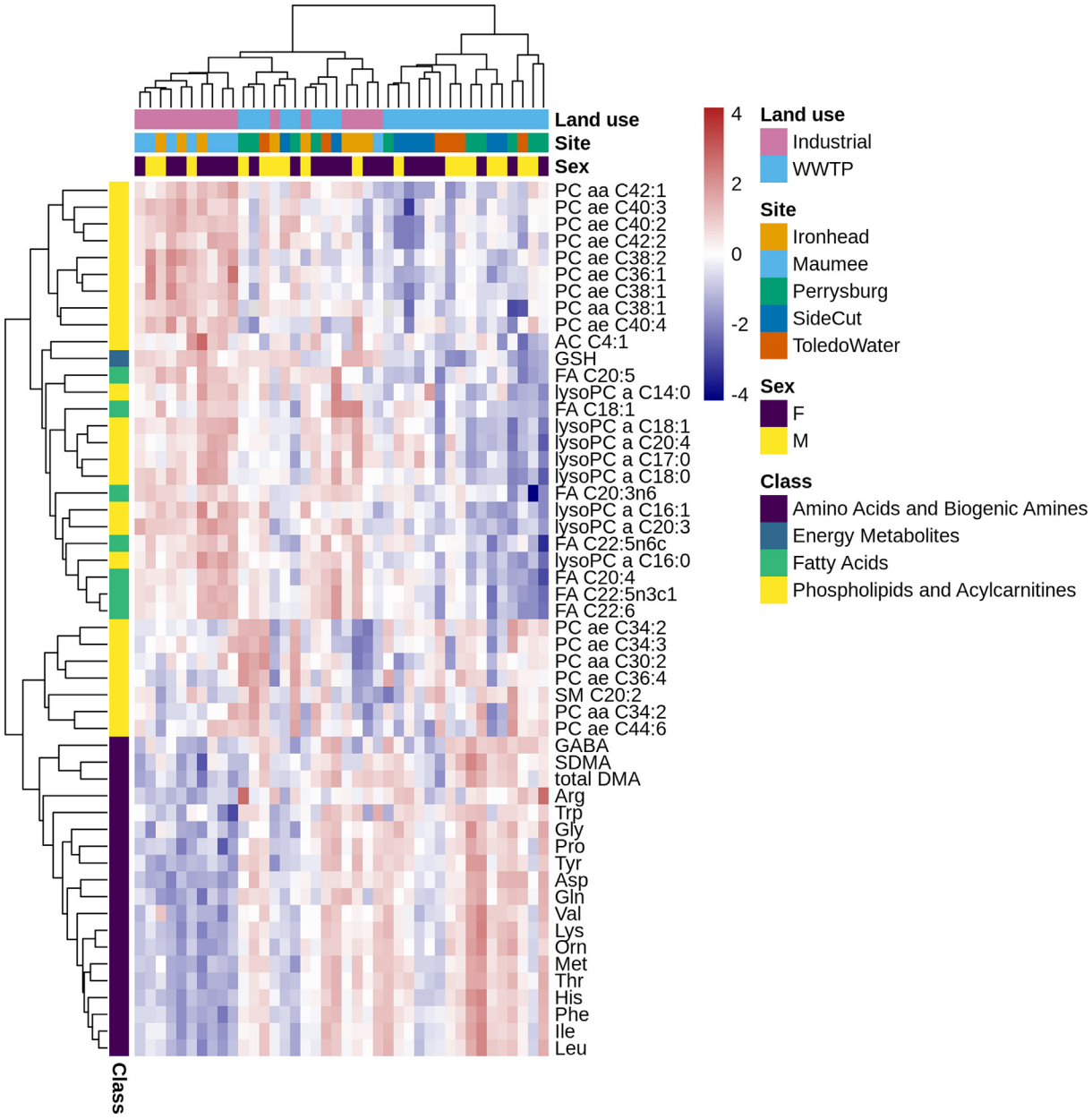


**Fig. 7.** Heatmap of differentially altered metabolites (DAMs) at tree swallow study sites along the Maumee River, OH. Individual responses were normalized as Z-score, clustered, and plotted at each column with annotation of sex, site, and land-use. The metabolite class is annotated on the left side of the heatmap.

### *3.6. Combing transcriptomic responses with metabolomic responses*

Combining transcriptomic and metabolomic responses in a sPLS analysis identified a clear separation between industrial and WWTP sites (Fig. 6c). The combined responses at Maumee and Ironhead separated the most from Perrysburg and SideCut, with Toledo Water in between. The upregulated combined responses that differentiated between industrial sites and WWTP sites corresponded to biosynthesis of unsaturated fatty acids, linoleic acid metabolism, and PPAR signaling pathways (FDR < 0.05). Downregulated combined responses that differentiated between the two land uses included arginine biosynthesis and aminoacyl-tRNA biosynthesis pathways (FDR < 0.05). The top differentiating genes and metabolites between sites indicated an induced *de novo* lipogenesis from glucose to triglyceride but also the synthesis of cholesterol, fatty acids, and phospholipids, and a potential inflammation pathway at industrial sites relative to WWTP sites (Fig. 8).

### *3.7. Correlation between transcriptomic responses, metabolomic responses, and contaminant profiles*

There was a clear positive correlation between lipogenesis genes such as SCD, FADS2, ELOVL2, ME1, PLA2G1B and phospholipids, such as acyl-alkyl phosphatidylcholine (PC ae), and a negative correlation between lipogenesis genes and amino acids (Fig. 9a). The top contaminants in nestling carcasses and stomach contents that covaried with both transcriptomic and metabolomic responses were total PBDEs, oxychlordane, and total parent PAHs. Total parent PAHs in stomach contents and oxychlordane in nestling carcasses correlated with an upregulation in lipogenesis genes and phospholipid metabolites of PC ae compounds, and a downregulation in amino acids. PBDEs in nestling carcasses also correlated with these responses but in the opposite direction (Fig. 9b).

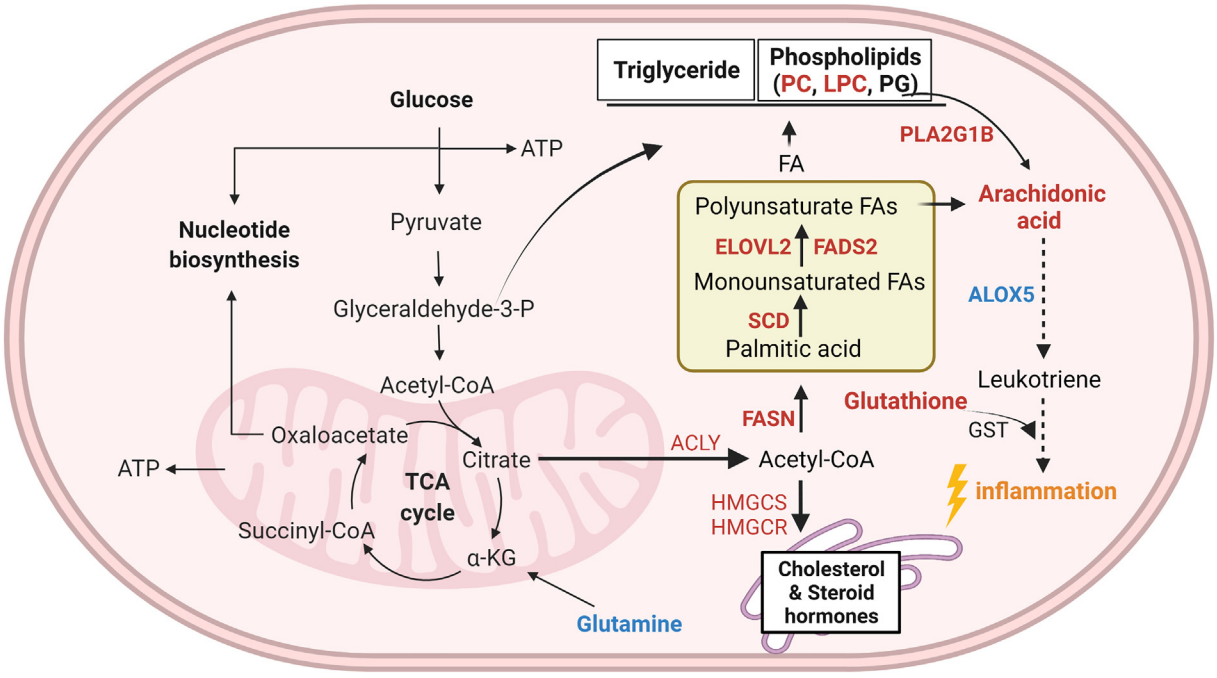


**Fig. 8.** This diagram describes lipogenesis genes and metabolites regulation at tree swallow study sites along the Maumee River, OH, which included *de novo* lipogenesis from glucose to triglyceride but also the synthesis of cholesterol, fatty acids, and phospholipids, and a potential inflammation pathway. Metabolites or genes are colored in red for upregulation and blue for downregulation.

## 4. Discussion

Environmental pollutants, including PCBs, PFAS, PBDEs, PAHs, and chlorinated pesticides, were found at higher levels in tree swallow nestlings in the Maumee AOC, relative to non-AOCs around the Great Lakes and also compared to Star Lake (Custer et al., 2017a). Within the Maumee AOC, there was local variation in contaminant profiles, which was dependent on site proximity to wastewater treatment plants or industrial land-use. However, it is extremely difficult to predict biochemical responses based on contaminant profiles of complex mixtures without considering the complexity of potential additive, antagonist, and synergistic effects between contaminants (van Broekhuizen et al., 2017). The inclusion of cumulative and synergistic effects in a regulatory framework has been proposed, although the methods to assess such effects are not currently available (Kortenkamp and Faust, 2018). Therefore, addressing the complexity of contaminant mixtures from source-based assessments, such as the effluent of wastewater treatment plants, and assessing their effects using a modern multi-omics approach can unveil the potential perturbations in functions and pathways (Crump et al., 2019; Li et al., 2017).

Between the Maumee River sites and Star Lake, we found a downregulation in cell cycle regulation related genes and an upregulation in lipogenesis genes. Among the Maumee River sites, significant variation was only related to lipogenesis genes, especially the biosynthesis of unsaturated fatty acids such as fatty acid and stearoyl-CoA desaturase (*FADS2*, *SCD*) and fatty acid elongase (*ELOVL2*). Within these DEGs, *ALOX5*, *SCD*, *SLC22A5* were indicated in hepatic steatosis IPA terms (Lai et al., 2020).

The downregulation in cell cycle related genes could be related to the elevated PAHs, PCBs, PFAS, and chlorinated pesticides in the Maumee AOC. The suppression of cell cycle related gene expression by elevated concentrations of halogenated hydrocarbons has been reported, and can cause disturbances in normal cell growth, or even apoptosis (Hardesty et al., 2017; Mandal, 2005; Porter et al., 2014). However, the expression of cell cycle related genes did not vary significantly between individual sites along the Maumee River. Cell cycle regulation and cell proliferation perturbation are downstream responses of aryl hydrocarbon receptor (AhR) agonists such as PAHs, dioxin-like PCBs, and other persistent organic contaminants (Doskey et al., 2020). Gene expression of cytochrome P450 1A (CYP1A) and its related enzymes often respond to minor perturbations by a variety of factors (Doskey et al., 2020; Hu et al., 2007). This pathway was generally induced at all Maumee sites, and thus was not informative in discriminating among Maumee sites. However, there was a significant increase in EROD activity in tree swallow liver cells at the downstream industrial sites compared to both Star Lake reference and sites farther upstream on the Maumee River (Custer et al., 2020). Cell cycle regulated genes, such as aryl hydrocarbon receptor (AhR) downstream targets, could share the same properties as CYP1A and may not be able to differentiate minor changes in environmental contaminants.

The upregulation in lipogenesis related genes indicated a potential for steatosis in the liver induced by endocrine disruptors (EDCs), including PCDDs, PAHs, PCBs, organochlorines, and perfluoroalkyls, which could bind or interact with nuclear receptors (NRs), such as AhR, constitutive androstane receptor (CAR), estrogen receptor alpha (ERα), peroxisome proliferator-activated receptors (PPARs), and pregnane X receptor (PXR) (Foulds et al., 2017; Küblbeck et al., 2020). These EDC-NR interactions could then alter key energy metabolism processes, such as hepatic glucose production and lipid metabolism. Differentially altered metabolites between the Maumee River sites were concentrated in phospholipids, amino acids, and fatty acids rather than energy metabolites, which indicated metabolomic responses were still highly related to fatty acid, phospholipid synthesis, and *de novo* lipogenesis. While both amino acid and carbohydrate metabolites could be transformed into lipids (DeBerardinis et al., 2008), we found a clear negative correlation between the level of amino acids and lipids/phospholipids in metabolites. When combining transcriptomic and metabolomic responses, there was a clear positive correlation between the number of phospholipids, fatty acids and lipogenesis genes, and a negative correlation between amino acids and lipogenesis genes. This pattern indicates a preference in modulating energy metabolism by prioritizing lipogenesis over amino acids to synthesize unsaturated fatty acids. Higher unsaturated fatty acids, such as docosapentaenoic acid (DPA), were found at Ironhead, Maumee, and Toledo Water than Perrysburg and SideCut, which was the same pattern as the relative level of lipogenesis gene expression among the Maumee River sites. We found a lower level of glutathione at WWTP sites than industrial sites, which was consistent with recent studies that livers of glutathione-deficient rats had lower expression of

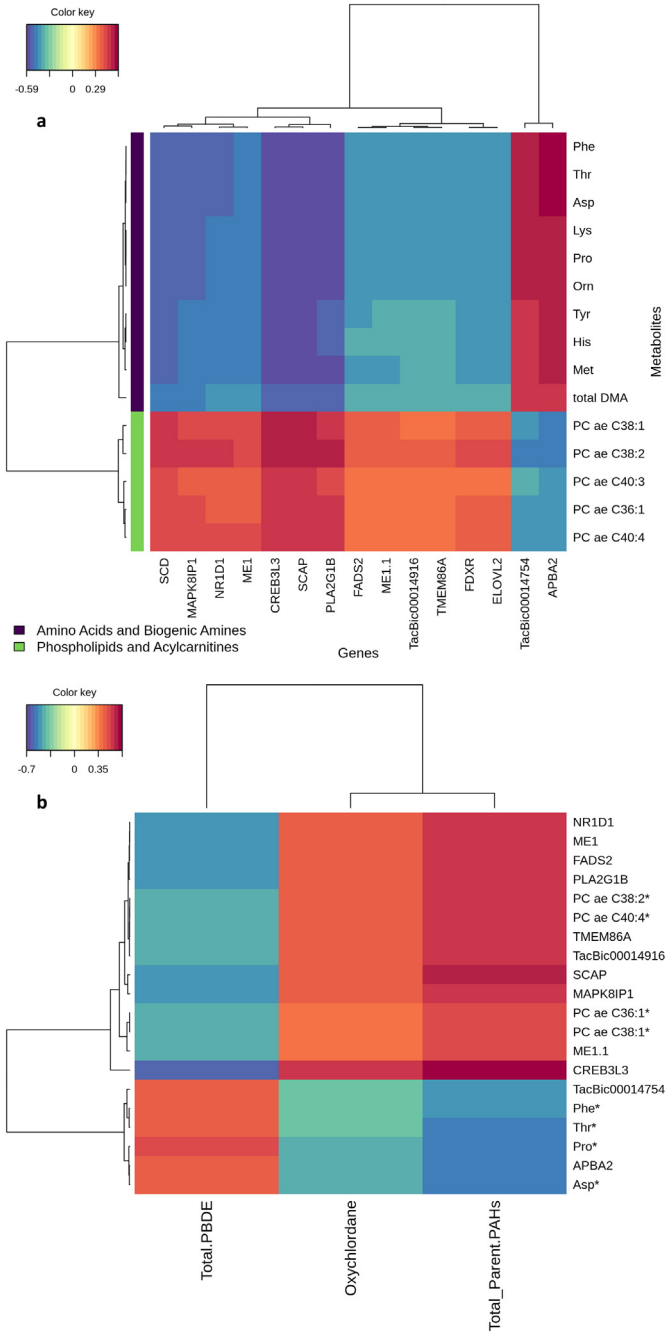


**Fig. 9.** (a) Heatmap of correlation scores between top gens and metabolites at tree swallow study sites along the Maumee River, OH, in 2016. Differentially expressed genes (DEGs) and differentially altered metabolites (DAMs) were selected by their correlation score in a sparse-partial least-squares analysis (sPLS). Metabolites are displayed on the vertical axis and genes are displayed on the horizontal axis. The metabolite class is annotated on the left. (b) Heatmap of correlation scores between contaminants and both transcriptomic and metabolomic responses. Top contaminants that covaried with both the top differentially genes (DEGs) and differentially altered metabolites (DAMs) at tree swallow study sites along the Maumee River, OH, in 2016 were selected and displayed as columns. DEGs and DAMs are displayed on the vertical axis. DAMs are annotated with an asterisk.

lipogenesis genes such as sterol regulatory element-binding protein (SREBP) and acetyl-CoA carboxylase (ACC) genes, and fatty acid synthase (FAS) (Brandsch et al., 2010).

Omega-3 unsaturated fatty acids, such as α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), and DPA, could protect rat hepatocytes from TCDD-induced hepatoxicity and hepatic fat accumulation (Parker et al., 2012; Turkez et al., 2016, 2012). Arachidonic acid (AA), released by phospholipase A2 from membrane phospholipids, could be transformed by lipoxygenase (*ALOX5*) into proinflammatory leukotrienes after exposure to TCDD in female Sprague-Dawley rats (Doskey et al., 2020). Comparing combined transcriptomic and metabolomic responses at Maumee and Ironhead with responses in TCDD-dosed rats, they both had upregulation in phospholipase A2 (PLA2G1B) and higher AA; however, ω−3 fatty acids, such DPA and DHA, were higher at Maumee and Ironhead relative to Perrysburg and SideCut but attenuated in TCDD-exposed rats. Moreover, ALOX5, as a pro-inflammatory biomarker, was not induced at Maumee and Ironhead despite higher levels of AA. The co-occurrence of higher glutathione and ω−3 unsaturated fatty acids at Maumee and Ironhead suggest that nestlings could be in chronic stress but still able to resist potential inflammation and oxidative stress resulting from environmental pollutant exposures (Fig. 8).

*De novo* lipogenesis related genes were reported to be induced, primarily in the liver of early life stage birds, when either fed a high carbohydrate diet (Ding et al., 2012; Smith and McWilliams, 2009) or in response to stress (Cai et al., 2009). Stress corticosterones have been reported to induce lipogenesis genes and other metabolism and systemic responses in chickens (Zaytsoff et al., 2019). Complex mixtures of pollutants have also been reported to cause stress responses. Great tits (*Parus major*) living in an urban environment showed an induced gene expression in immune and inflammatory responses, detoxification, protection against oxidative stress, and lipid metabolism (Watson et al., 2017). Multiple arctic seabirds monitored for persistent organic pollutants showed a positive correlation between the stress hormone corticosterone and plasma total PCB levels (Dietz et al., 2019). ToxChip *in vitro* assays showed that lipogenesis is one of the top responses to environmental contaminants. Organohalogen contaminants extracted from double-crested cormorant egg and organic flame retardants extracted from passive air samplers could both cause alteration in lipogenesis related responses in *in vitro* assays (Crump et al., 2019, 2016). Artic birds (murre *Uria lomvia*, and guillemot *Cepphus grille*) exposed to polycyclic aromatic compounds also showed perturbation in lipogenesis related responses (Zahaby et al., 2021).

However, the long-term effects of early life stress in wild birds are difficult to estimate and often misleading when birds are capable of developing resilience and plasticity (Drummond and Ancona, 2015). The current multi-omics approach only aims to provide a snapshot of the current molecular and physiological states of nestlings to estimate site conditions. We acknowledge the limitation of extrapolating between molecular responses and an organism's health and performance.

Combined results from both transcriptomic and metabolomic responses indicated total parent PAHs, oxychlordane, and PBDEs were the most likely drivers for the perturbation in lipogenesis and amino acid metabolism. Polycyclic aromatic hydrocarbons, which originate from industrial combustion activities, and in particular benzo[*a*]pyrene (BaP), are more potent inducers of AhR or ER activity than 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or pesticides. Benzo[*a*]pyrene and pesticide mixtures are known to have additive effects on ER activity. Thus, PAHs activate AhR downstream pathways, potentially resulting in additive effects with oxychlordane on the ER and lead to an upregulation in lipogenesis-related gene expression (Foulds et al., 2017; Tq et al., 2020). Polybrominated diphenyl ethers function as agonists or antagonists for the ER (Kojima et al., 2013). Polybrominated diphenyl ether concentrations were negatively correlated with lipogenesis related gene expression among Maumee River sites, although a causal relationship is not clear. Polybrominated diphenyl ether concentrations were elevated at Perrysburg and SideCut, whereas both PAHs and PBDEs were elevated at Toledo Water. Because Toledo Water clustered with Ironhead and Maumee in terms of lipogenesis related gene expression, the positive correlation of PAHs appears to outweigh the potential negative correlation of PBDEs with lipogenesis genes. Similar correlations between aromatic hydrocarbon and lipogenesis related expression have been found in polar bears (*Ursus maritimus*) and double-crested cormorants (*Phalacrocorax auratus*), which

exhibit positive correlations with PCBs and oxychlordane, but negative correlations with PBDEs (Tartu et al., 2017; Xia et al., 2020).

Compared to other bioindicators of cytochrome *p*450 activity (EROD), oxidative stress, DNA damage, or thyroid functions, the current multi-omics approach is more sensitive to alterations in land-use and contaminant profiles. Although EROD activity also reflected variation in land-use (Custer et al., 2020), the multi-omics approach could also identify disturbed functions and downstream biochemical effects to provide a broad insight in non-targeted analysis. Measuring selective molecular bioindicator endpoints can provide insight into the specific biological mechanisms and processes and biochemical responses; however, selecting bioindicators to address specific environmental contamination requires a comprehensive characterization of contamination and a good understanding of its related mode of action (Handy et al., 2003). Contrary to pre-selected bioindicators, multi-omics provides a broad and integrated picture of the way an organism exposed to pollutant mixtures responds. Non-targeted transcriptome and metabolome analyses allow for the determination of altered biological processes and the discovery of targeted bioindicators, and further overcome the limitation in interpretation of transcriptome data in the context of biological functions when transcriptome data alone does not always correlate with phenotypic variation (Misra et al., 2019; Vineis et al., 2013).

Other organisms, including mussels (*Eurynia dilatata* and *Lampsilis cardium*) and caged fathead minnows (*Pimephales promelas*), were collected by others (Cipoletti et al., 2019; Woolnough et al., 2020), or staged in similar locations along the Maumee River. However, the contaminants measured in the mussel and fish tissues and the water column differed somewhat from those measured in tree swallow nestlings. Pesticides, pharmaceuticals, and wastewater indicators such as plasticizers, PAHs, sterols, pain relievers, and personal care products, were the dominant contaminants in the water column, fathead minnow tissue, and mussel tissue (Ankley et al., 2020; Cipoletti et al., 2019; Woolnough et al., 2020). However, in tree swallow nestlings, legacy contaminants including PCBs, PBDEs, and organochlorine pesticides were the primary pollutants, with limited personal care products being detected occasionally. PPCPs in general are not persistent and can be easily metabolized and excreted from swallow nestlings. However, mussel and fathead minnow are constantly exposed to these PPCPs through the continuous release to the environment. Therefore, only those high level PPCPs measured in mussel, such as N,N-diethyl-meta-toluamide (DEET) and iopamidol, could be also measured in swallow nestlings (Kimbrough et al., 2018; Woolnough et al., 2020). This differentiation in contaminant profiles further highlights the importance of including terrestrial birds in studies of AOCs, including potential toxicological effects.

There is a need to prioritize contaminants for long-term, targeted contaminants monitoring and to determine their toxicological effects in the environment for environmental risk assessments, planning management activities, and setting up quantitative management goals. In the current study, integrating transcriptomic and metabolomic signals provide a path for combining multiple lines of evidence to extract comprehensive source-based functional perturbations. Even with substantial environmental and chemical complexity, we were able to identify patterns and see clear separation between sites based on adjacent land-use. Additionally, lipogenesis genes identified in the current study could be used for long-term monitoring for potential oxidative stress inducers and general stressors in the environment.

## 5. Conclusions

We demonstrated the value of multivariate analysis approaches in identifying and prioritizing contaminants correlated with perturbed functions and pathways in field-collected wild bird populations. Although parent PAHs, oxychlordane, and total PBDEs were the most potent drivers of multi-omics responses, induced lipogenesis genes in unsaturated fatty acids synthesis, elevated $\omega-3$ unsaturated fatty acids, such as DPA and DHA, arachidonic acid and its lipoxygenase (ALOX5) might be useful as sensitive bioindicators of persistent organic pollutant mixtures to provide insights on the biological condition of an AOC and help determine the progress of management goals.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159130.

## CRediT authorship contribution statement

**Chi Yen Tseng:** Conceptualization, Methodology, Data curation, Software, Formal analysis, Validation, Writing – original draft, Visualization. **Christine M. Custer:** Project administration, Investigation, Supervision, Funding acquisition, Resources, Writing – review & editing. **Thomas W. Custer:** Project administration, Supervision, Funding acquisition, Resources. **Paul M. Dummer:** Data curation, Investigation, Writing – review & editing. **Natalie Karouna-Renier:** Resources, Investigation, Writing – review & editing. **Cole W. Matson:** Supervision, Writing – review & editing.

## Data availability

Raw sequences pertaining to the present study are deposited in National Center for Biotechnology Information Sequence Read Archive under PRJNA835816. Metadata associated with the present study are available as a US Geological Survey data release doi:https://doi.org/10.5066/P9PJSC9S. Data, associated metadata, and calculation tools are also available from the corresponding author (Cole Matson: cole_matson@baylor.edu).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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