View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by DigitalCommons@University of Nebraska

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Biochemistry -- Faculty Publications

Biochemistry, Department of

2013

Elucidating Causes of *Diporeia* Decline in the Great Lakes via Metabolomics: Physiological Responses after Exposure to Different Stressors

Suman Maity

Amber Jannasch

Jiri Adamec

James M. Watkins

Thomas Nalepa

See next page for additional authors

Follow this and additional works at: https://digitalcommons.unl.edu/biochemfacpub

Part of the Biochemistry Commons, Biotechnology Commons, and the Other Biochemistry, Biophysics, and Structural Biology Commons

This Article is brought to you for free and open access by the Biochemistry, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Biochemistry -- Faculty Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Suman Maity, Amber Jannasch, Jiri Adamec, James M. Watkins, Thomas Nalepa, Tomas O. Höök, and Maria S. Sepúlveda

Elucidating Causes of *Diporeia* Decline in the Great Lakes via Metabolomics: Physiological Responses after Exposure to Different Stressors

Suman Maity[1,*]
Amber Jannasch[2,†]
Jiri Adamec[3,‡]
James M. Watkins[4,§]
Thomas Nalepa[5,‖]
Tomas O. Höök[1,#]
Maria S. Sepúlveda[1,**]
[1]Department of Forestry and Natural Resources, Purdue University, 195 Marsteller Street, West Lafayette, Indiana 47907; [2]Bindley Bioscience Center at Discovery Park, Purdue University, 1203 West State Street, West Lafayette, Indiana 47907; [3]Department of Biochemistry, Beadle Center N151, University of Nebraska, 1901 Vine Street, Lincoln, Nebraska 68588; [4]Cornell Biological Field Station, 900 Shackelton Point Road, Bridgeport, New York 13030; [5]National Oceanic and Atmospheric Administration, Great Lakes Environmental Research Laboratory, 4840 South State Road, Ann Arbor, Michigan 48108

Accepted 11/14/2012; Electronically Published 1/14/2013

ABSTRACT

The benthic macroinvertebrate *Diporeia* spp. have been extirpated from many areas of the Laurentian Great Lakes, but the mechanisms underlying such declines are not fully understood. *Diporeia* declines coinciding with the invasion of exotic dreissenid mussels (zebra and quagga) have led to the hypothesis that *Diporeia* declines are a result of decreased food availability from increasing competition with dreissenids for diatoms. There is additional evidence that *Diporeia* are negatively affected when in close proximity to dreissenids, probably because of exposure to toxins present in the mussels' pseudofeces. *Diporeia* are also known to be sensitive to anthropogenic contaminants (such as polychlorinated biphenyls [PCBs]) present in Great Lakes sediments. To better understand the physiological responses of *Diporeia* to diverse stressors, we conducted three 28-d experiments evaluating changes in the metabolomes of *Diporeia* (1) fed diatoms (*Cyclotella meneghiniana*) versus starved, (2) exposed (from Lake Michigan and Cayuga Lake) to quagga mussels (*Dreissena bugensis*), and (3) exposed to sediments contaminated with PCBs. The metabolomes of samples were examined using both two-dimensional gas and liquid chromatography coupled with mass spectrometry. Each stressor elicited a unique metabolome response characterized by enhanced citric acid cycle, fatty acid biosynthesis, and protein metabolism in diatom-fed *Diporeia*; impaired glycolysis, protein catabolism, and folate metabolism in *Diporeia* from Lake Michigan irrespective of quagga mussel exposure, suggesting lake-specific adaptation mechanisms; and altered cysteine and phospholipid metabolism during PCB exposure. Subsequent comparisons of these stressor-specific metabolic responses with metabolomes of a feral *Diporeia* population would help identify stressors affecting *Diporeia* populations throughout the Great Lakes.

* Present address: Department of Molecular and Cell Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas 77030; e-mail: maity@bcm.edu.
† E-mail: hopfas@purdue.edu.
‡ E-mail: jadamec2@unl.edu.
§ E-mail: jmw237@cornell.edu.
‖ E-mail: thomas.nalepa@noaa.gov.
E-mail: thook@purdue.edu.
** Corresponding author; e-mail: mssepulv@purdue.edu.

Physiological and Biochemical Zoology 86(2):213–223. 2013. © 2013 by The University of Chicago. All rights reserved. 1522-2152/2013/8602-2080$15.00.
DOI: 10.1086/669132

Introduction

Diporeia spp. were once a major component of the benthic biomass (>70%) in deep-water lakes of North America (Cook and Johnson 1974; Nalepa 1989). Historically, these lipid-rich amphipods constituted a major prey item for various fish species in the Laurentian Great Lakes (Owens and Dittman 2003). Like the European amphipod species, *Diporeia* also feed on diatoms settling from the epilimnetic zones and in turn are consumed by a number of fish species (Johnson 1987; Fitzgerald and Gardner 1993). Diatoms are the main dietary source of polyunsaturated fatty acids (PUFAs), which are essential for reproductive functioning and brood survival in amphipods (Wiklund and Sundelin 2001). So it is likely that diatoms play an important role in *Diporeia* nutrition as well. However, since the 1990s and with the arrival and establishment of nonnative dreissenid mussels (zebra mussel *Dreissena polymorpha*, quagga mussel *Dreissena bugensis*), *Diporeia* populations have experienced severe declines in all of the Great Lakes except Lake Superior (Nalepa et al. 1998, 2005; Dermott 2001; Lozano et al. 2001). Since dreissenids also inhabit the upper sediment layers, it has been hypothesized that they are intercepting settling diatoms before they can reach *Diporeia* (Nalepa et al.

2005). Dreissenids can also modify the epilimnetic habitat during filter feeding by increasing water clarity resulting from the higher grazing pressure on diatoms, making diatoms less likely to settle on the lake floor (Scavia and Fahnenstiel 1987; Makarewicz et al. 1999; Vanderploeg et al. 2002). This induced scarcity of food could result in compromised survival and reproductive output by *Diporeia*, as it did in other amphipod species (Sundelin et al. 2008). Previously, impacts of such starvation on the physiology of *Diporeia* have been investigated in laboratory-based mesocosm studies using metabolomics and RNA : DNA ratios, which found disrupted protein and lipid metabolism and declining RNA : DNA ratios under stress (Ryan 2010; Maity et al. 2012*a*).

Dreissenids can also indirectly affect the survival of *Diporeia*. A study by Dermott et al. (2005) reported lower survival rates (almost at a significant level) of *Diporeia* when exposed to pseudofeces from quagga mussels. Therefore, it is possible that these biodeposits can exert a negative impact on *Diporeia*, but clear evidence supporting this hypothesis is still lacking. However, *Diporeia* populations are still abundant in Cayuga Lake, a lake also densely populated by quagga mussels. Thus, it is possible that *Diporeia* from this lake have “adapted” to the presence of these mussels, compared to *Diporeia* from other lakes that are declining in the presence of dreissenids.

While there are multiple lines of evidence that dreissenids negatively affect *Diporeia*, the interactions between these invertebrates are not straightforward. For example, in Cayuga Lake, a stable *Diporeia* population is known to coexist with dreissenids (Nalepa et al. 2006; Watkins et al. 2007, 2012). In addition, several sites within the Great Lakes experienced large declines in *Diporeia* numbers well before the invasion of dreissenids (Watkins et al. 2007; Nalepa et al. 2009). Therefore, studies that evaluate the physiological response of *Diporeia* to the presence of dreissenids are warranted as a way to better understand the mechanistic relationship between these taxons.

Exposure of *Diporeia* to environmental pollutants has also been hypothesized as a potential cause of their decline. Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) are present at high concentrations (>200 μg/g) in some areas within the Great Lakes (Cieniawski and Collier 2003; Hornbuckle et al. 2006). PCBs are known to elicit a myriad of toxic effects ranging from immunosuppression to cancer (Silkworth and Antrim 1985; Aznar et al. 2005). Lipid-rich *Diporeia* would be an ideal target for lipophilic-PCB mediated toxicity, which could potentially increase the susceptibility of *Diporeia* to secondary stressors (e.g., pathogens; Landrum et al. 1998, 2001). Furthermore, *Diporeia* are not only likely to be exposed to lipophilic contaminants but also highly sensitive to their effects (Gossiaux et al. 1993; Landrum and Nalepa 1998; Ralston-Hooper et al. 2008). Indeed, the extirpation of *Diporeia* from the Niagara River has been linked to PCB contamination (Nalepa 1991). Dreissenids can potentially increase the bioavailability of a number of organic pollutants including PCBs in the benthic sediment via their feces or pseudofeces accumulation (Bruner et al. 1994; Gossiaux et al. 1998; Ma et al. 1999). Thus, the changing chemical characteristics of benthic sediment might also affect the survival of *Diporeia*.

Here, we report on the results of three separate experiments conducted to gain a better understanding of the potential indirect (food competition and increased PCB exposure) and direct (exposure to pseudofeces) impacts of dreissenids on the physiology of *Diporeia*. In the first experiment, *Diporeia* were starved and their metabolomes were compared to those fed with diatoms (to test the “food competition” hypothesis). In the second experiment, *Diporeia* were exposed to quagga mussels and their metabolomes were compared to those with no dreissenids (to test the “direct dreissenid effect” hypothesis). In the third and final experiment, *Diporeia* were chronically exposed to sediments spiked with a mixture of PCBs (to test the “pollution” hypothesis). We hypothesized that each stressor would result in specific metabolite changes. To the best of our knowledge, this study constitutes the first analyses of physiological responses measured as changes in metabolite concentrations in *Diporeia* exposed to these stressors. The long-term goal of these studies is to better understand the physiological responses of *Diporeia* to different environmental stressors and thereby facilitate elucidation of mechanisms underlying their drastic decline in the Great Lakes.

Material and Methods

Diatom Feeding Experiment

A commercial strain of *Cyclotella meneghiniana* (one of the most abundant diatom species in Lake Michigan) was purchased from Bigelow Laboratory for Ocean Sciences (West Boothbay Harbor, ME) for culture. Following the vendor’s instructions, we reared diatoms in L1+Si media (11 practical salinity units) at 20°C and a 14L : 10D cycle for 30 d. Diatom cultures were then centrifuged at 3,400 rpm for 60 min at 4°C, and the supernatant was collected. Next, 5 mL molecular-grade water was added to the pellet and vortexed for 2 min. The number of cells per milliliter from each aliquot was quantified under a microscope using a hemocytometer before preserving at −80°C for future use.

In April 2009, live *Diporeia* were collected from southern Lake Michigan on board the Environmental Protection Agency’s R/V *Lake Guardian*. Using a ponar grab (0.23-m^2 sampling area with 500-μm screen), we collected *Diporeia* from the sediment-water interface (temperature, ~4°C), and live animals were sorted out after sieving the sediment through a 0.5-mm mesh screen and placed in coolers filled with prechilled (4°C) lake water and sediment. Live organisms were transported to the laboratory and acclimated for 48 h in a dark walk-in chamber set at 4°C in order to simulate deep-lake water conditions. These environmental conditions were replicated in all experiments. Next, one live animal was randomly assigned to each of 50 Erlenmeyer flasks (125 mL) filled with ashed sand (70 g) and reconstituted hard water (80 mL; Ralston-Hooper et al. 2008). Half of the water in each flask was replaced every week throughout the experiment. The treatment (starved) group was not fed during the trial, and the control group was

fed diatoms twice a week to allow for a daily feeding rate of 0.03 mg diatoms per individual *Diporeia* (estimated feeding rate from Quigley and Vanderploeg 1991; Dermott et al. 2005). At the completion of the experiment, live organisms were collected, and randomly selected animals per treatment ($n = 5$) were assigned for metabolomic analysis as described below.

Quagga Mussel Exposure Experiment

In the summer of 2008, live *Diporeia* and quagga mussels were collected from Cayuga Lake (Myer's Point, 43°37′N, 79°35′W, 45 m) and Lake Michigan (site C-6, 42°79′N, 87°45′W, 93 m) and brought back to the laboratory inside coolers with ice. In the laboratory, 10 individual *Diporeia* from each site were exposed to quaggas (collected near Muskegon in Lake Michigan and Myer's Point in Cayuga Lake; density, 2,000 individuals/m^2) in 250-mL glass jars prefilled with combusted sand substratum and Cayuga Lake water. Animals were not fed during the experiment because we could not control how much, if any, of the food provided would be eaten by the mussels compared to *Diporeia.* Animals were held at 4°C in complete darkness for 28 d. At the end of the experiment, a selected number of live organisms ($n = 7$ per condition) were set aside for metabolomic analysis.

PCB Exposure

Animals for this experiment were collected concomitantly with the animals used for the diatom feeding experiment (described above). After arrival to the laboratory, organisms were allowed to acclimate for 48 h at 4°C before the start of the experiment. On the basis of reports describing the total body burdens of PCBs in *Diporeia* (Wong et al. 2004) and a biota sediment accumulation factor of 1 (Landrum et al. 1998, 2001; Kukkonen et al. 2004), we exposed *Diporeia* to a mixture of eight PCB congeners (2-chlorobiphenyl, 2.3-dichlorobiphenyl, 2.4.5-trichlorobiphenyl, 2.2′.4.4′-tetrachlorobipheyl, 2.2′.3′.4.6.-pentachlorobiphenyl, 2.2′.4.4′.5.6′-hexachlorobiphenyl, 2.2′.3.3′.4.4′.6-heptachlorobiphenyl, and 2.2′.3.3′.4.5′.6.6′-octachlorobiphenyl) purchased from ChemService (West Chester, PA). The sediment for this experiment was collected from Lake Michigan and sterilized by autoclaving it. The control sediment was then spiked with analytical-grade acetone (0.5 mL/100 g). Sediment was placed inside a stainless steel container and stirred for 45 min with a handheld blender for homogenous mixing (Kukkonen et al. 2004). Next, the sediment was resuspended in reconstituted modified hard water (RMHW; 1 : 1 w/v), and the supernatant was decanted four times. Each Erlenmeyer flask (125 mL) was filled with either control sediment or treated sediment (40 g) and RMHW (80 mL). Each treatment was replicated 25 times (one *Diporeia* per flask). The experiment was kept in complete darkness at 4°C for 28 d. At the completion of the study, live *Diporeia* were collected and preserved at −80°C, with $n = 5$ per treatment randomly selected for metabolomics analyses.

Extraction and Analysis of PCBs from Spiked Sediments. Sediment samples were prepared by extracting with equal volumes of acetone and hexane 25 mL of acetone and 25 mL of hexane per 10 g of sediment (wet mass) using published protocols (Brannon and Karn 1990). The solvents were added into glass vials containing the sediment and allowed to shake overnight at 37°C. The following morning the samples were centrifuged at 1,200 rpm for 10 min, and the supernatant was transferred to a fresh glass vial. After the samples were dried with nitrogen gas, each sample was reconstituted in 100 μL of acetone and vortexed for 10 min before being transferred to an autosampler vial. Samples were analyzed with an Agilent 6890N gas chromatograph (GC) coupled to a Leco Pegasus III time-of-flight mass spectrometer (TOF-MS). An Agilent HP-5MS column (30 m × 0.250 mm, 0.25-μm film) was used for GC analysis. The initial temperature was 100°C, held for 2 min. A ramp of 15°C/min was used until reaching 160°C, followed by 5°C/min up to 270°C. This temperature was held for 1 min and then returned to 100°C before the next injection. The carrier gas was helium with a flow rate of 1 mL/min, and an inlet split ratio of 10 was used. Each sample had an injection volume of 3 μL. The GC inlet temperature was set to 225°C. The transfer line from GC to MS was set to 250°C. MS data were collected from 60–800 mass units (u) with an acquisition rate of 20 spectra/s in one-dimensional mode only. The detector voltage was 1,700 V. Electron impact was used with electron energy set to −70 eV. All samples were processed with Leco ChromaTOF (ver. 3.32) software. The detected PCB level in the spiked sediment was 4.5 μg/g, which is below previously detected PCB levels (200 μg/g) in contaminated sediments from the Great Lakes region (Cieniawski and Collier 2003).

Metabolomic Analyses

Metabolomics data from whole organisms were collected using a combination of mass spectrometry coupled with two-dimensional gas chromatography (GCXGC) and liquid chromatography (LC) following protocols described by Maity et al. (2012*a*, 2012*b*) and Ralston-Hooper et al. (2008) with some modifications. Briefly, each sample (composed of a single *Diporeia*, 0.0021–0.0098 g) was homogenized, methanol (300 μL) and chloroform (450 μL) were added, and the sample was centrifuged in order to separate polar (methanol : water) and nonpolar (chloroform) phases. After the solvents were allowed to evaporate at 45°C using a Savant SPD 131DDA SpeedVac concentrator (Thermo Electron Corporation, Milford, MA), dried sample pellets were collected for metabolomics analysis. The dried nonpolar (chloroform)-phase extract was resuspended in an LC-MS mobile phase solution (50% water, 25% methanol, 25% acetonitrile, and 0.1% piperidine) and analyzed using LC/TOF-MS. The polar (methanol : water) phase was analyzed using two-dimensional GCXGC/TOF-MS. The polar phase was derivatized in two steps. First, a 30-μL solution of methoxyamine hydrochloride (20 mg) dissolved in 1 mL anhydrous pyridine was added to the dried pellet and placed on a shaker for 30 min at 60°C. Next, 45 μL of N-methyl-N-(trimethylsilyl)

trifluoroacetamide was added to this mixture and the solution was heated for 1 h at 60°C before transferring it for GCXGC/TOF-MS analysis.

Instrumental Conditions. Nonpolar metabolites were analyzed using LC based on an Agilent 1100 (Agilent Technologies, Santa Clara, CA) platform with a Zorbax-C8 column (2.1 mm × 150 mm, 5 μm; Agilent Technologies). The instrumental parameters were as follows: initial injection volume, 8 μL; flow rate, 300 μL/min; gradient of two separate mobile phases A (water + 0.1% piperidine) and B (50 : 50 v/v acetonitrile : methanol + 0.1% piperidine), A : B at 50 : 50 for 5 min followed by a linear increase to 100% B over 20 min, a return to A : B at 50 : 50 for 5 min, and an additional 10 min of A : B at 50 : 50 to reequilibrate the column. After the chromatographic separation, nonpolar metabolites were analyzed in negative electrospray ionization mode using LC/TOF-MS (Agilent G6200 series, LC/MSD TOF). Instrumental conditions were as follows: capillary voltage, 3,500 V; flow rate of desolvation gas, 9.0 L/h; desolvation temperature, 350°C; nebulizer pressure, 40 psi; fragmentor voltage, 175 V; skimmer voltage, 65 V; octapole resonant frequency, 250 V. With a detectable mass range of 100–1,800 da and scan rate of 1 spectrum/s, Agilent Masshunter (ver. 01.03) was used to process mass spectra.

Derivatized polar samples were analyzed in a Pegasus III GCXGC/TOF-MS (Leco, St. Joseph, MI). Samples (2 μL) were injected into the GC column under the following instrumental settings: split-mode ratio, 20; flow rate carrier gas (helium), 1.5 mL/min; inlet port temperature, 280°C. The dimensions of the first and second columns were as follows: Restek Rtx-225 matrix, 0.25 mm × 30 m, 0.25 μm, and Agilent HP-5 matrix, 0.32 mm × 2 m, 0.25 μm, respectively. The temperature of the first column was gradually increased from 50° to 240°C (7°C/min) and held at 270°C for 5 min. The temperature of the second column was raised following a similar pattern with an offset of +50°C. The MS spectra was acquired under the following instrumental conditions: ionization mode, electron impact; data acquisition using TOF measured for ion fragments; temperature, 200°C; detectable mass range, 30–800 da; detector voltage, 1,700 V; electron energy, −70 V; scan rate, 100 spectra/s. An acquisition delay of 150 s was applied at the start of the run.

Data Processing and Statistical Analysis. Processing of LC-MS spectra involved identification, alignment, and merging of common peaks across multiple samples. Two software packages (XMASS and XAlign) designed by Bindley Biosciences Center at Purdue University were used for this purpose (Zhang et al. 2005). Peaks were normalized using a constant mean under the assumption of equal intensity of total ion current of all spectra compared. Next, the value of mass over charge (m/z) of each peak was accepted as proxy for their potential identity, and the tentative list of potential metabolites was prepared after searching the Human Metabolome Database (http://www.hmdb.ca; Wishart et al. 2007).

Spectra from GCXGC-MS were processed and analyzed using two different software packages, ChromatTOF (ver. 3.32, Leco) and MSort, an in-house software (Oh et al. 2008). Multiple entries of single peak were merged and aligned across samples by calculating the retention time (RT) and similarity value (SV) indices. The first and second RT windows were set at 1% and 5%, respectively, with a correlational value of 0.95 as minimum threshold. Each spectrum was searched against the National Institute of Standards and Technology (NIST) database (http://www.nist.gov/srd/nist1.cfm) and compared for an overall SV with available spectrum in the library. On the basis of the SV the potential identity of the metabolite was determined. For example, an SV of 1,000 signifies a complete match, while an SV between 600 and 800 indicates a fair possibility (Cristoni et al. 2009). We set up the minimum threshold of SV at 600 for peak identification.

Both LC-MS and GCXGC/TOF-MS data were analyzed using multivariate statistical methods implemented in R open-source statistical software (ver. 2.9.2, R Foundation for Statistical Computing, Vienna). Missing values pose a common problem for "omics" data (Chich et al. 2007; Karpievitch et al. 2010). Because of a limited sample size, coupled with the prohibitive cost of repeated measures, and to minimize loss of information, we deemed missing value imputation essential for any "omics" data (Unnebrink and Windeler 2001; Colinge et al. 2005). In this study, missing values were imputed with the group mean if missing values were present in half (or less) of the total samples per group; otherwise, missing values were replaced by 1 (Colinge et al. 2005). Data were analyzed using heuristic methods of dimension reduction (principal component analysis [PCA]). Two-sample t-tests adjusted for false discovery rate (Benjamini and Hochberg 1995) were used to compare significant ($\alpha = 0.05$) metabolite concentrations between control and experimental samples.

Results

Diporeia responded with changes in a unique set of metabolites when exposed to one of the following three environmental stressors: lack of food, coexposure to quagga mussels, or exposure to a mixture of PCBs. For example, PCAs demonstrated a clear separation between diatom-fed versus starved *Diporeia* (fig. 1*a*; combined contribution of the first two PCs explained 57% of the total variation) and PCB-exposed versus control *Diporeia* (fig. 1*c*; combined contribution of the first two PCs explained 67% of the total variation). While the quagga mussel coexposure experiment revealed a strong lake effect (fig. 1*b*; combined contribution of the first two PCs explained 52% of the total variation), *Diporeia* from Cayuga Lake tended to cluster into two separate groups based on the presence/absence of quagga mussels (although organisms from Lake Michigan did not). In contrast to metabolite responses, survival rates did not differ among treatments (>80% across all experiments; data not presented).

The metabolites that were up- or downregulated after stressor exposure varied for the three different stressors (for altered

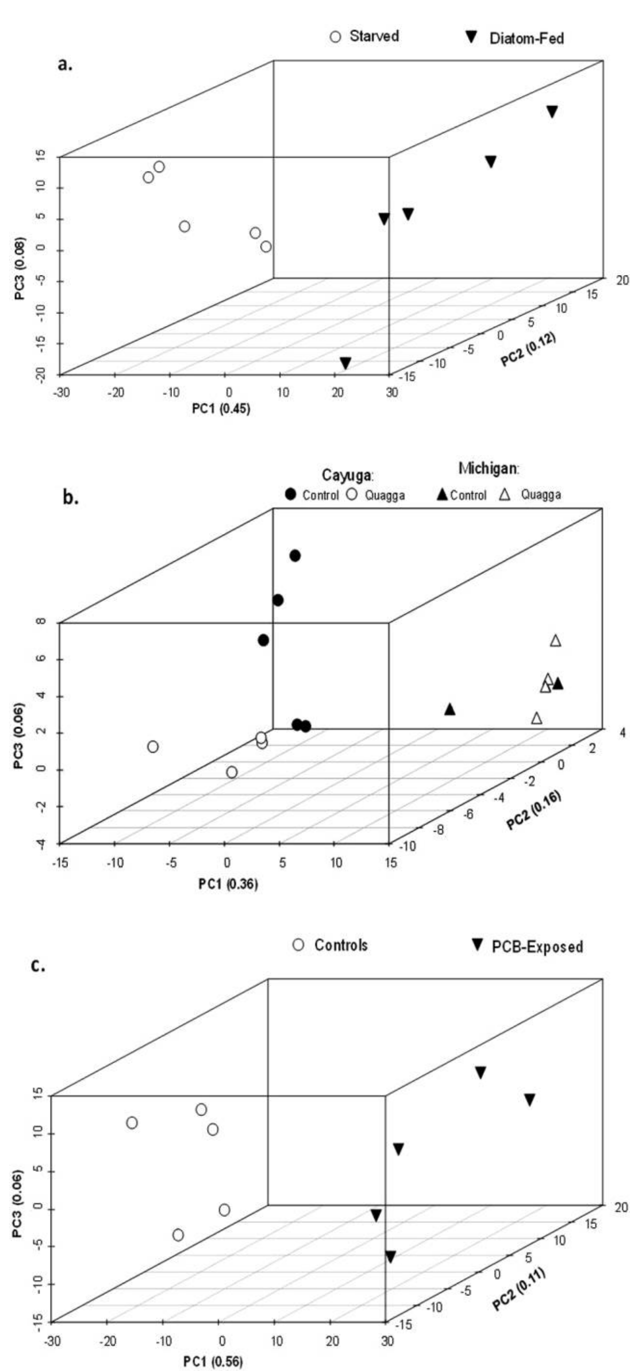

Figure 1. Principal component analysis plot of *Diporeia* samples showing differential metabolic profiles between control and treatment groups. *a*, Diatom feeding; *b*, quagga exposure; *c*, polychlorinated biphenyl (PCB) exposure experiments. Each symbol represents the metabolome of a single *Diporeia* along three principal components (PCs). Proportion of variation explained is shown in parentheses.

polar and nonpolar phase–extracted metabolites, see tables 1, 2, respectively). A total of nine metabolites (four polar phase and five nonpolar phase), seven metabolites (three polar phase and four nonpolar phase), and four nonpolar metabolites were significantly altered in the diatom feeding, quagga mussel coexposure, and PCB exposure experiments, respectively. These metabolites fell into different classes, including fatty acids, phospholipids, amino acids, by-products of amino acid metabolism, and pheromones. Below we discuss the potential mechanisms and biological implications of these metabolite changes.

Discussion

Diatom Feeding Experiment

The main objective of this experiment was to identify metabolite changes in *Diporeia* fed a diatom diet in relation to starved animals. This is important because a major hypothesis for their decline is a decrease of phytoplankton availability due to increased competition with dreissenids. Thus, a better understanding of the physiological response of this amphipod to starvation is needed. In order to determine whether *Diporeia* were ingesting the diatoms being offered, we examined a representative number of organisms using standard histological techniques and observed plant material in the gastrointestinal tract (data not shown). This finding, coupled with the metabolite profile observed in the diatom-fed group (overall increased glycolysis, lipidogenesis, and protein synthesis), is indicative of organisms having preyed on diatoms during the trial. However, it is important to note that the feeding regime utilized in this experiment is probably different from real environmental conditions; *Diporeia* are likely to feed intermittently (during spring, more than twice per week) on a variety of phytoplankton and bacterial species. Thus, extrapolation of these data to Great Lakes conditions should be done with caution.

Increased concentrations of palmitoleic, octadecenoic (stearic), and malonic acids in the diatom-fed group (tables 1, 2) are indicative of increased lipidogenesis and decreased lipid peroxidation. Palmitoleic and octadecenoic acids are important for fatty acid and triacylglycerol synthesis. Crustaceans are known to store high levels of palmitoleic acid, which is utilized during growth and molting (Sheen and Wu 1999). In addition, the ratio between stearic (i.e., octadecenoic) acid and oleic acid is crucial for maintaining plasma membrane fluidity, intercellular signaling, and immune function. A higher abundance of octadecenoic acid suggests a healthy organism. Elevated malonic acid levels are indicative of not only enhanced acetyl-CoA carboxylase activity and thus lipidogenesis but also reduced lipid oxidation (Cake et al. 1995). Malonic acid is also an important component of cuticular lipids in invertebrates (Buckner et al. 1996). If a similar function exists in *Diporeia*, a decrease of malonic acid could result in alterations in molting cycle and cuticle formation.

Higher concentrations of urea (table 1) and lower concentrations of L-pipecolic acid (table 2) suggest increased protein synthesis, and therefore enhanced growth, in the diatom-fed group. Urea is a by-product of excess proteins, and in crustaceans the cellular concentrations of a number of compounds including glutamine, L-aspartate, creatine, and arginine are closely linked with urea production (Wright 1995; Weihrauch et al. 2004). L-pipecolic acid is a by-product of lysine degra-

Table 1: List of significant metabolites (mean ± SE; $N = 5$ individual *Diporeia* per condition) extracted from polar fraction that were altered during the diatom feeding and quagga mussel coexposure experiments

Metabolite name	Identification criteria based on SV[a]	Mean expression level: Fed (control)	Mean expression level: Starved	*P* value[b]	Direction of change[c]
Diatom feeding:					
Urea	785	18.24 ± .11	ND	<.0001	↓
1-I2MU	871	10.02 ± .60	ND	<.001	↓
Octadecanoic acid	908	10.96 ± .11	4.54 ± 2.43	.04	↓
Glycoside[d]	794	11.16 ± .23	ND	<.0001	↓
		Cayuga Lake	Lake Michigan		
Quagga coexposure:					
Heptacosane	772	6.23 ± 2.55	14.79 ± .28	.03	↑
L-proline	798	15.51 ± .05	14.59 ± .20	<.001	↓
G3P	773	5.69 ± 2.29	14.09 ± .10	.02	↑

Note. For the quagga mussel coexposure experiment, data shown are the comparison of *Diporeia* between lakes, since presence of quagga mussels caused little to no effect on the metabolomes. No significant polar metabolites were detected in the polychlorinated biphenyl exposure experiment. ND = not detected; 1-I2MU = 1-iodo-2-methylundecane; G3P = glyceraldehyde-3-phosphate.

[a]Identification based on mass spectra similarity value (SV) match against National Institute of Standards and Technology library.

[b]$P < 0.05$ (Benjamini Hochberg corrected).

[c]Calculated in relation to the control group.

[d]Undetermined type.

dation, and lysine is an essential amino acid stored to meet energy needs during periods of food deprivation (Cowey and Forster 1971; Lasser and Allen 1976; Miyajima et al. 1976). Lysine is also a precursor of carnitine, which is involved in lipid biosynthesis (Dall and Smith 1987). During starvation, amino acid reserves would be progressively metabolized to sustain basic physiological processes leading to their increase in cells. Therefore, an absence of L-pipecolic acid in the diatom-fed group suggests little to no protein catabolism.

Other interesting metabolites that showed significant differences between the diatom-fed and starved groups included 7C-aglycone, glycoside, and 1-iodo-2-methylundecane (1-I2MU; tables 1, 2). 7C-aglycone is a breakdown product of vitamin K metabolism, and it is produced via beta oxidation of phylloquinone (Wishart et al. 2007). Since the primary dietary source of phylloquinone is plant based, the lack of diatoms likely caused a rapid depletion of vitamin K in the starved group.

Glycosidic groups are important components of plant flavonoids and sterol biosynthetic pathways. A number of different sterol biosynthetic pathways have been elucidated in crustaceans over the years (Kanazawa 2001). Glycosidic sterols are derived from plants and are involved in the production of eicosanoids, crucial for the initiation of molting in crustaceans. They are also important for normal reproduction and development (Martin-Creuzburg and Elert 2004) and are needed for the synthesis of PUFAs (Tornabene et al. 1974). Thus, starvation can quickly lead to potential reproductive and developmental side effects.

We previously reported the presence of 1-I2MU in *Diporeia* that were starved for 60 d (Maity et al. 2012*b*). 1-I2MU levels ranged widely across individuals and decreased during our starvation trials. We are unsure of its function in *Diporeia*, but it resembles 1-iodoundecane, a pheromone produced after estrogen stimulus and present in cow urine during estrus (Achiraman et al. 2010). An elevated level of this metabolite in diatom-fed *Diporeia* might be indicative of "normal" reproductive function compared to the starved group.

Quagga Exposure Study

We conducted this experiment to test how *Diporeia* respond to the presence of quagga mussels. There is some indication that *Diporeia* might be negatively affected by the presence of these mussels as a result of the toxic effects of their pseudofeces (Dermott et al. 2005). However, *Diporeia* populations are still abundant in Cayuga Lake, a lake also densely populated by quagga mussels. To test the hypothesis that *Diporeia* from this lake are less sensitive to the presence of these mussels, we exposed *Diporeia* from both lakes to quagga mussels and examined their metabolomes. We found a strong lake effect on metabolite profiles with quaggas not being a determining factor (fig. 1*b*). In other words, irrespective of the presence of quagga mussels, *Diporeia* from Cayuga Lake and Lake Michigan responded differently. On the basis of the comparison between *Diporeia* from Lake Michigan and Cayuga Lake, a common set of metabolites was identified that were differentially expressed between two lakes. The biological roles of these metabolites are discussed below.

Glyceraldehyde 3-phosphate (G3P) was upregulated in *Diporeia* from Lake Michigan (table 1). G3P is an important

Table 2: List of significant metabolites (mean ± SE; $N = 5$ individual *Diporeia* per condition) extracted from nonpolar fraction that were altered during the diatom feeding, quagga mussel coexposure, and polychlorinated biphenyl exposure experiments

Metabolite name	HMDB no. (m/z value)[a]	Mean expression level: Fed (control)	Mean expression level: Starved	*P* value[b]	Direction of change[c]
Diatom feeding:					
Malonic acid	HMDB00691 (102.974)	4.17 ± .02	ND	<.0001	↓
L-pipecolic acid	HMDB00716 (128.071)	ND	7.00 ± .09	<.001	↓
DHAP	HMDB01473 (168.991)	ND	5.81 ± .004	<.001	↑
Palmitoleic acid	HMDB03229 (253.219)	5.90 ± .04	ND	<.001	↓
7C-aglycone	HMDB04808 (297.142)	ND	5.54 ± .06	<.001	↑
		Cayuga Lake	Lake Michigan		
Quagga coexposure:					
Glycyl-L-leucine	HMDB00759 (187.117)	2.98 ± .97	6.54 ± .02	.02	↑
13-cis-retinal	HMDB06220 (283.221)	6.72 ± .22	2.87 ± 1.19	.01	↓
LPA	HMDB07850 (437.228)	4.37 ± 1.65	10.04 ± .05	.03	↑
2,5,-DAPNTP	HMDB06821 (511.912)	4.74 ± 1.83	11.07 ± .14	.03	↑
		Controls	Exposed		
PCB exposure:					
3-mercaptopyruvic acid	HMDB01368 (118.994)	ND	5.21 ± .09	<.001	↑
12-KETE	HMDB13633 (317.219)	5.77 ± .09	ND	<.0001	↓
LysoPE	HMDB11502 (438.284)	ND	4.86 ± .04	<.0001	↑
PG	HMDB10585 (719.481)	8.70 ± .31	7.84 ± .20	.04	↓

Note. For the quagga mussel experiment, data shown are the comparison of *Diporeia* between lakes, since presence of quagga mussels caused little to no effect on the metabolomes. ND = not detected; DHAP = dihydroxyacetone phosphate; LPA = lysophosphatidic acid; 2,5,-DAPNTP = 2,5-diaminopyrimidine nucleoside triphosphate; 12-KETE = 12-keto-eicosatetraenoic acid; LysoPE = lysophosphatidylethanolamine; PG = phosphatidylglycerol.

[a]Human Metabolome Database (HMDB) accession number and (mass over charge ratio).

[b]$P < 0.05$ (Benjamini Hochberg corrected).

[c]Calculated in relation to the control group.

component of both the glycolytic and gluconeogenic pathways (Wishart et al. 2007). Since G3P is linked with sterol biosynthetic pathways, changes in G3P concentrations will impact lipid production. G3P concentration can also be used as an indirect estimate of GAPDH activity, the target of S-glutathione that helps minimize oxidative stress. This step renders GAPDH inactive, thus raising the concentration of G3P in the cell (Klatt and Lamas 2000). GAPDH is also thought to be relevant in controlling apoptosis, making it an important indicator for overall physiological status (Chuang et al. 2005; Lauritano et al. 2011). Thus, an accumulation of G3P in exposed animals indicates less availability of GAPDH. We speculate that this could be due to an elevated stress response and possibly result in hindered lipid production in affected animals.

Changes in the concentration of several metabolites point toward an increased oxidative damage and potential stress response in *Diporeia* from Lake Michigan compared to animals from Cayuga Lake. The nonpolar compound 13-cis-retinal was consistently downregulated in *Diporeia* from Lake Michigan (table 2). This metabolite is a type of retinoid acid belonging to the polyisoprenoid lipid family (Chen et al. 2010). These lipids perform an array of biological functions in animals. For instance, they act as the substrate binding site for lipocalin-type prostaglandin D synthase (Zsila et al. 2004), leading to anti-inflammatory responses (Wishart et al. 2007). They are also related to sterol biosynthesis and possess antioxidant properties (Chen et al. 2010). A decrease in the concentration of this metabolite might affect sterol production and increase oxidative damage in *Diporeia* from Lake Michigan.

The level of another nonpolar metabolite, 2,5-diaminopyrimidine nucleoside triphosphate (table 2), was also increased in *Diporeia* from Lake Michigan. This metabolite is a by-product of folate metabolism (Wishart et al. 2007). Activation of folate metabolism is indicative of enhanced antioxidant glutathione production to counteract radical oxygen species-mediated cellular damage (Quinlivan et al. 2006). Thus, in general, *Diporeia* from Lake Michigan were experiencing elevated oxidative stress.

Dipeptides such as glycyl-L-leucin are readily utilized in healthy animals as precursors for different chemical reactions (Wishart et al. 2007). Leucine is an important amino acid that performs diverse cellular functions including synthesis of pro-

teoglycans (Iozzo 1999). Under stress, the reduced activity of dipeptidase enzymes results in an elevated level of dipeptides such as glycyl-L-leucine (Egorova et al. 2008). Thus, an increase in the concentration of glycyl-L-leucine in *Diporeia* from Lake Michigan might be indicative of decreased amino acid utilization due to increased stress.

PCB Exposure Study

Another potential environmental stressor that could help explain the disappearance of *Diporeia* is exposure to POPs such as PCBs. Because of their high lipid content, *Diporeia* are likely to bioaccumulate POPs with high octanol-water partition coefficients (Helm et al. 2008; Kuo et al. 2010). Overall, exposure to a mixture of PCBs resulted in disrupted lipid metabolism, increased oxidative stress, and induction of aryl-hydrocarbon receptor (AhR)–mediated pathways as discussed below.

The phospholipid lyso-phosphatidyl ethanolamine increased in PCB-exposed *Diporeia* (table 2). Although not significant, the phospholipid precursor's octa- and hexa-decanoic acids were more abundant in the PCB-exposed group. Their accumulation indicates reduced production of essential lipids in PCB-exposed *Diporeia*. Another phospholipid, phosphoglyceride also declined in exposed animals. In crustaceans, phospholipids are crucial for the production of cholesterol, which, in turn, is important for effective control of molting cycle, larval development, and oocyte formation (Lee and Puppione 1978; Coutteau et al. 1997; Gonzalez-Felix et al. 2002; Sánchez-Paz et al. 2006). Thus, a decline in phospholipids could have a broad range of effects.

The other nonpolar metabolite, 3-mercaptopyruvic acid (table 2), is a by-product of amino acid cysteine (Wishart et al. 2007). It contains a thiol group that acts as an effective mediator to control protein oxidation. Because of its role as an antioxidant, cysteine also has affinity for heavy-metal ions and thus acts as an important indicator of metal toxicity. Reversible oxidation of proteins often contributes to the activation and deactivation of the transcription factors responsible for cell signaling or protein kinase activity within the cytoplasm (Cumming et al. 2004). The disulfide bonds produced by two cysteine molecules are also critical in maintaining the functionality of proteins (Wishart et al. 2007). A disrupted production of cysteine could affect detoxification and transcription mechanisms in affected *Diporeia*.

12-KETE, a by-product of arachidonic acid metabolism, was decreased in PCB-exposed organisms (table 2). Arachidonic acid (a type of PUFA) is essential for a range of biological functions including enhancing growth and survival in prawn larvae, increasing fecundity, and maintaining homeostasis (Kanazawa et al. 1977; D'Abramo and Sheen 1993; Rees et al. 1994). In crustaceans, PUFAs are especially important as a source of energy during prolonged starvation (Bychek et al. 2005). They also aid in eicosanoid production, hormone synthesis, and the production of prostaglandins and leukotrienes important in immune function (Smith and Borgeat 1985; Blomquist et al. 1991). Declining arachidonic acid might have significant impact on these physiological processes. Also, 12-KETE is an important conversion component of 12-HETE, a ligand for the activation of AhR-mediated pathways (Spokas et al. 1999). PCBs are known AhR agonists (Cooke et al. 2001; Pocar et al. 2006), and thus an increased activity of the AhR pathway is an expected outcome.

Conclusions and Future Research Needs

Overall, our results suggest that a different set of metabolites is expressed in response to each stressor: (*a*) a diatom-rich diet enhanced fatty acid biosynthesis, whereas starvation resulted in altered glycolysis and sterol biosynthetic pathways; (*b*) lake type has more impact than quagga exposure on *Diporeia* metabolomes, and animals from Lake Michigan responded with an induction of oxidative stress and altered lipid metabolism when compared to samples from Cayuga Lake; and (*c*) exposure of *Diporeia* to a mixture of PCBs resulted in disrupted phospholipid and eicosanoid production, decreased PUFA levels, and increased AhR-mediated activity.

Our results should not be viewed as the final answer but rather as a first step for identifying relevant metabolites and biochemical pathways associated with stress and potential lower fitness in *Diporeia*. Furthermore, our studies have laid the groundwork for future hypothesis-driven and more targeted approaches that should be conducted next. For example, the stressor-specific metabolite profiles identified through these experiments can be compared to metabolite profiles from in situ collected *Diporeia*, thereby potentially facilitating the identification of stressors affecting *Diporeia* in various areas of the Great Lakes. This research has also demonstrated the application potential of metabolomics in the study of aquatic ecology.

Acknowledgments

We would like to thank Daniel Ryan for the collection of samples; Carol Lembi, Charles Britton, and Kimberly Schulz for their assistance and advice on the diatom culture; and Jennifer Myer and Carolyn Foley for providing their assistance during experimental setup. This research was funded by the Great Lakes Fisheries Trust (grant 2008.886).

Literature Cited

Achiraman S., P. Ponmanickam, D. Ganesh, and G. Archunan. 2010. Detection of estrus by male mice: synergistic role of olfactory-vomeronasal system. Neurosci Lett 477:144–148.

Aznar F., D. Perdiguero, A. Pérez Del Olmo, A. Repullés, C. Agusti, and J. Raga. 2005. Changes in epizoic crustacean infestations during cetacean die-offs: the mass mortality of Mediterranean striped dolphins *Stenella coeruleoalba* revisited. Dis Aquat Organ 67:239–247.

Benjamini Y. and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 57:289–300.

Blomquist G.J., C.E. Borgeson, and M. Vundla. 1991. Polyunsaturated fatty acids and eicosanoids in insects. Insect Biochem 21:99–106.

Brannon J.M. and R. Karn. 1990. Cleanup of sediment extracts prior to PCB analysis. Bull Environ Contam Toxicol 44:542–548.

Bruner K.A., S.W. Fisher, and P.F. Landrum. 1994. The role of the zebra mussel, *Dreissena polymorpha*, in contaminant cycling. II. Zebra mussel contaminant accumulation from algae and suspended particles, and transfer to the benthic invertebrate, *Gammarus fasciatus*. J Great Lakes Res 20:735–750.

Buckner J.S., M.C. Mardaus, and D.R. Nelson. 1996. Cuticular lipid composition of *Heliothis virescens* and *Helicoverpa zea* pupae. Comp Biochem Physiol B 114:207–216.

Bychek E., G. Dobson, J. Harwood, and I. Guschina. 2005. *Daphnia magna* can tolerate short-term starvation without major changes in lipid metabolism. Lipids 40:599–608.

Cake M.H., G.W. Power, and I.C. Potter. 1995. The liver and muscle of early upstream migrant lampreys (*Geotria australis*) contain high levels of acetyl-CoA carboxylase and a carnitine palmitoyl transferase I that is sensitive to malonyl-CoA. Comp Biochem Physiol B 110:417–423.

Chen S., K. Wang, and Y.J.Y. Wan. 2010. Retinoids activate RXR/CAR-mediated pathway and induce CYP3A. Biochem Pharmacol 79:270–276.

Chich J.F., O. David, F. Villers, B. Schaeffer, D. Lutomski, and S. Huet. 2007. Statistics for proteomics: experimental design and 2-DE differential analysis. J Chromatogr B 849:261–272.

Chuang D.M., C. Hough, and V.V. Senatorov. 2005. Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases. Annu Rev Pharmacol Toxicol 45:269–290.

Cieniawski S. and C. Demaree. 2003. Post-remediation sediment sampling on the Raisin River near Monroe, Michigan: final results from 2001–2002 survey—PCB chemistry, caged fished and bioaccumulation results. Pp. 1–52 in Great Lakes National Program Office technical report. U.S. Environmental Protection Agency, Chicago.

Colinge J., D. Chiappe, S. Lagache, M. Moniatte, and L. Bougueleret. 2005. Differential proteomics via probabilistic peptide identification scores. Anal Chem 77:596–606.

Cook D. and M. Johnson. 1974. Benthic macroinvertebrates of the St. Lawrence Great Lakes. J Fish Res Board Can 3:763–782.

Cooke P.S., T. Sato, D.L. Buchanan, L. Robertson, and L. Hansen. 2001. Disruption of steroid hormone signaling by PCBs. Pp. 257–263 in L.W. Robertson and L.G. Hansen, eds. PCBs: recent advances in environmental toxicology and health effects. University Press of Kentucky, Lexington.

Coutteau P., I. Geurden, M. Camara, P. Bergot, and P. Sorgeloos. 1997. Review on the dietary effects of phospholipids in fish and crustacean larviculture. Aquaculture 155:149–164.

Cowey C. and J. Forster. 1971. The essential amino-acid requirements of the prawn *Palaemon serratus*: the growth of prawns on diets containing proteins of different amino-acid compositions. Mar Biol 10:77–81.

Cristoni S., L. Molin, A. Lai, L.R. Bernardi, S. Pucciarelli, M. Agostini, C. Bedin, et al. 2009. MALDI-MS-NIST library approach for colorectal cancer diagnosis. Rapid Comm Mass Spectrom 23:2839–2845.

Cumming R.C., N.L. Andon, P.A. Haynes, M. Park, W.H. Fischer, and D. Schubert. 2004. Protein disulfide bond formation in the cytoplasm during oxidative stress. J Biol Chem 279:21749–21758.

D'abramo L.R. and S.S. Sheen. 1993. Polyunsaturated fatty acid nutrition in juvenile freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 115:63–86.

Dall W. and D. Smith. 1987. Changes in protein-bound and free amino acids in the muscle of the tiger prawn *Penaeus esculentus* during starvation. Mar Biol 95:509–520.

Dermott R. 2001. Sudden disappearance of the amphipod *Diporeia* from eastern Lake Ontario, 1993–1995. J Great Lakes Res 27:423–433.

Dermott R., M. Munawar, R. Bonnell, S. Carou, H. Niblock, T. Nalepa, and G. Messick. 2005. Preliminary investigations for causes of the disappearance of *Diporeia* spp. from Lake Ontario. Pp. 203–232 in Great Lakes Fisheries Commission technical report. Great Lakes Fisheries Commission, Ann Arbor, MI.

Egorova V., A. Nikitina, and N. Timofeeva. 2008. Effect of weaning terms and protein deficit in rat pup nutrition on activities of digestive enzymes. J Evol Biochem Physiol 44: 591–598.

Fitzgerald S.A. and W.S. Gardner. 1993. An algal carbon budget for pelagic-benthic coupling in Lake Michigan. Limnol Oceanogr 38:547–560.

Gonzalez-Felix M.L., A.L. Lawrence, and D.M. Gatlin. 2002. Growth, survival and fatty acid composition of juvenile *Litopenaeus vannamei* fed different oils in the presence and absence of phospholipids. Aquaculture 205:325–343.

Gossiaux D.C., P.F. Landrum, and S.W. Fisher. 1998. The assimilation of contaminants from suspended sediment and algae by the zebra mussel, *Dreissena polymorpha*. Chemosphere 36:3181–3197.

Gossiaux D.C., P.F. Landrum, and V.N. Tsymbal. 1993. A survey of Saginaw River and Saginaw Bay, Lake Huron, sediments using two bioassays with the amphipod *Diporeia* spp. J Great Lakes Res 19:322–332.

Helm P.A., S.B. Gewurtz, D.M. Whittle, C.H. Marvin, A.T. Fisk, and G.T. Tomy. 2008. Occurrence and biomagnification of polychlorinated naphthalenes and non- and mono-ortho PCBs in Lake Ontario sediment and biota. Environ Sci Technol 42:1024–1031.

Hornbuckle K., D. Carlson, D. Swackhamer, J. Baker, and S. Eisenreich. 2006. Polychlorinated biphenyls in the Great Lakes. Pp. 13–70 in R. Hites, ed. Persistent organic pollutants in the Great Lakes. Springer, Berlin.

Iozzo R.V. 1999. The biology of the small leucine-rich proteoglycans. J Biol Chem 274:18843–18846.

Johnson R.K. 1987. The life history, production and food habits of *Pontoporeia affinis* Lindström (Crustacea: Amphipoda) in mesotrophic Lake Erken. Hydrobiologia 144:277–283.

Kanazawa A. 2001. Sterols in marine invertebrates. Fish Sci 67: 997–1007.

Kanazawa A., S. Teshima, and S. Tokiwa. 1977. Nutritional requirements of prawn. VII. Effect of dietary lipids on growth. Bull Jpn Soc Sci Fish 43:849–856.

Karpievitch Y.V., A.D. Polpitiya, G.A. Anderson, R.D. Smith, and A.R. Dabney. 2010. Liquid chromatography mass spectrometry–based proteomics: biological and technological aspects. Ann Appl Stat 4:1797–1823.

Klatt P. and S. Lamas. 2000. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. Euro J Biochem 267:4928–4944.

Kukkonen J.V., P.F. Landrum, S. Mitra, D.C. Gossiaux, J. Gunnarsson, and D. Weston. 2004. The role of desorption for describing the bioavailability of select polycyclic aromatic hydrocarbon and polychlorinated biphenyl congeners for seven laboratory-spiked sediments. Environ Toxicol Chem 23:1842–1851.

Kuo Y.M., M.S. Sepúlveda, I. Hua, H.G. Ochoa-Acuña, and T.M. Sutton. 2010. Bioaccumulation and biomagnification of polybrominated diphenyl ethers in a food web of Lake Michigan. Ecotoxicology 19:623–634.

Landrum P.F., S. Driscoll, E. Tigue, D. Gossiaux, M. Gedeon, and M. Adler. 1998. Toxicokinetics of polychlorinated biphenyl congeners by *Diporeia* spp.: effects of temperature and organism size. NOAA Tech Mem 106:5–21.

Landrum P.F. and T.F. Nalepa. 1998. A review of the factors affecting the ecotoxicology of *Diporeia* spp. J Great Lakes Res 24:889–904.

Landrum P.F., E.A. Tigue, S.K. Driscoll, D.C. Gossiaux, P.L. Van Hoof, M.L. Gedeon, and M. Adler. 2001. Bioaccumulation of PCB congeners by *Diporeia* spp.: kinetics and factors affecting bioavailability. J Great Lakes Res 27:117–133.

Lasser G.W. and W.V. Allen. 1976. The essential amino acid requirements of the Dungeness crab, *Cancer magister*. Aquaculture 7:235–244.

Lauritano C., M. Borra, Y. Carotenuto, E. Biffali, A. Miralto, G. Procaccini, and A. Ianora. 2011. First molecular evidence of diatom effects in the copepod *Calanus helgolandicus.* J Exp Mar Biol Ecol 404:79–86.

Lee R.F. and D.L. Puppione. 1978. Serum lipoproteins in the spiny lobster, *Panulirus interruptus.* Comp Biochem Physiol B 59:239–243.

Lozano S.J., J.V. Scharold, and T.F. Nalepa. 2001. Recent declines in benthic macroinvertebrate densities in Lake Ontario. Can J Fish Aquat Sci 58:518–529.

Ma X., K.A. Bruner, S.W. Fisher, and P.F. Landrum. 1999. Absorption of hydrophobic contaminants from ingested *Chlamydomonas rheinhardtii* and *Chlorella vulgaris* by zebra mussels, *Dreissena polymorpha.* J Great Lakes Res 25:305–317.

Maity S., A. Jannash, J. Adamec, M. Gribskov, T. Nalepa, T. Höök, and M.S. Sepúlveda. 2012*a*. Metabolite profiles in starved *Diporeia* spp. using liquid chromatography–mass spectrometry (LC-MS) based metabolomics. J Crustac Biol 32:239–248.

———. 2012*b*. Starvation causes disturbance in amino acid and fatty acid metabolism in *Diporeia.* Comp Biochem Physiol B 161:348–355.

Makarewicz J.C., T.W. Lewis, and P. Bertram. 1999. Phytoplankton composition and biomass in the offshore waters of Lake Erie: pre-and post-*Dreissena* introduction (1983–1993). J Great Lakes Res 25:135–148.

Martin-Creuzburg D. and E.V. Elert. 2004. Impact of 10 dietary sterols on growth and reproduction of *Daphnia galeata.* J Chem Ecol 30:483–500.

Miyajima L.S., G.A. Broderick, and R.D. Reimer. 1976. Identification of the essential amino acids of the freshwater shrimp, *Macrobrachium ohione.* Proc Annu Meet World Maric Soc 7:699–704.

Nalepa T.F. 1989. Estimates of macroinvertebrate biomass in Lake Michigan. J Great Lakes Res 15:437–443.

———. 1991. Status and trends of the Lake Ontario macrobenthos. Can J Fish Aquat Sci 48:1558–1567.

Nalepa T.F., D.L. Fanslow, A.J. Foley III, G.A. Lang, B.J. Eadie, and M.A. Quigley. 2006. Continued disappearance of the benthic amphipod *Diporeia* spp. in Lake Michigan: is there evidence for food limitation? Can J Fish Aquat Sci 63:872–890.

Nalepa T.F., D.L. Fanslow, and G.A. Lang. 2009. Transformation of the offshore benthic community in Lake Michigan: recent shift from the native amphipod *Diporeia* spp. to the invasive mussel *Dreissena rostriformis bugensis.* Freshw Biol 54:466–479.

Nalepa T.F., D.J. Hartson, D.L. Fanslow, G.A. Lang, and S.J. Lozano. 1998. Declines in benthic macroinvertebrate populations in southern Lake Michigan, 1980–1993. Can J Fish Aquat Sci 55:2402–2413.

Nalepa T.F., L.C. Mohr, B.A. Henderson, C.P. Madenjian, and P.J. Schneeberger. 2005. Lake whitefish and *Diporeia* spp. in the Great Lakes: an overview. Pp. 3–20 in L.C. Mohr and T.F. Nalepa, eds. Great Lakes Fishery Commission technical report 66. Great Lakes Fishery Commission, Ann Arbor, MI.

Oh C., X. Huang, F.E. Regnier, C. Buck, and X. Zhang. 2008. Comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry peak sorting algorithm. J Chromatogr A 1179:205–215.

Owens R.W. and D.E. Dittman. 2003. Shifts in the diets of slimy sculpin (*Cottus cognatus*) and lake whitefish (*Coregonus clupeaformis*) in Lake Ontario following the collapse of the burrowing amphipod *Diporeia.* Aquat Ecosyst Health Manag 6:311–323.

Pocar P., T. Klonisch, C. Brandsch, K. Eder, C. Frehlich, C. Hoang-Vu, and S. Hombach-Klonisch. 2006. AhR-agonist-induced transcriptional changes of genes involved in thyroid function in primary porcine thyrocytes. Toxicol Sci 89:408–414.

Quigley M. and H. Vanderploeg. 1991. Ingestion of live filamentous diatoms by the Great Lakes amphipod, *Diporeia* sp.: a case study of the limited value of gut contents analysis. Hydrobiologia 223:141–148.

Quinlivan E.P., A.D. Hanson, and J.F. Gregory. 2006. The anal-

ysis of folate and its metabolic precursors in biological samples. Anal Biochem 348:163–184.

Ralston-Hooper K., A. Hopf, C. Oh, X. Zhang, J. Adamec, and M.S. Sepúlveda. 2008. Development of GCxGC/TOF-MS metabolomics for use in ecotoxicological studies with invertebrates. Aquat Toxicol 88:48–52.

Rees J., K. Curé, S. Piyatiratitivorakul, P. Sorgeloos, and P. Menasveta. 1994. Highly unsaturated fatty acid requirements of *Penaeus monodon* postlarvae: an experimental approach based on *Artemia* enrichment. Aquaculture 122:193–207.

Ryan D. 2010. Spatial variation in condition and consumptive demand of *Diporeia* spp. in the Great Lakes region. MS thesis. Purdue University, Lafayette, IN.

Sánchez-Paz A., F. García-Carreño, A. Muhlia-Almazán, A.B. Peregrino-Uriarte, J. Hernández-López, and G. Yepiz-Plascencia. 2006. Usage of energy reserves in crustaceans during starvation: status and future directions. Insect Biochem Mol Biol 36:241–249.

Scavia D. and G.L. Fahnenstiel. 1987. Dynamics of Lake Michigan phytoplankton: mechanisms controlling epilimnetic communities. J Great Lakes Res 13:103–120.

Sheen S.S. and S.W. Wu. 1999. The effects of dietary lipid levels on the growth response of juvenile mud crab *Scylla serrata*. Aquaculture 175:143–153.

Silkworth J. and L. Antrim. 1985. Relationship between Ah receptor-mediated polychlorinated biphenyl (PCB)-induced humoral immunosuppression and thymic atrophy. J Pharmacol Exp Ther 235:606–611.

Smith W.L. and P. Borgeat. 1985. The eicosanoids: prostaglandins, thromboxanes, leukotrienes, and hydroeicosanoic acids. Pp. 325–360 in D.E. Vance and J.E. Vance, eds. Biochemistry of lipids and membranes. Cummings, Menlo Park, CA.

Spokas E.G., J. Rokach, and P. Wong. 1999. Leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids. Methods Mol Biol 120:213–248.

Sundelin B., R. Rosa, and A.K.E. Wiklund. 2008. Reproduction disorders in the benthic amphipod *Monoporeia affinis*: an effect of low food resources. Aquat Biol 2:179–190.

Tornabene T., M. Kates, and B. Volcani. 1974. Sterols, aliphatic hydrocarbons, and fatty acids of a nonphotosynthetic diatom, *Nitzschia alba*. Lipids 9:279–284.

Unnebrink K. and J. Windeler. 2001. Intention to treat: methods for dealing with missing values in clinical trials of progressively deteriorating diseases. Stat Med 20:3931–3946.

Vanderploeg H.A., T.F. Nalepa, D.J. Jude, E.L. Mills, K.T. Holeck, J.R. Liebig, I.A. Grigorovich, and H. Ojaveer. 2002. Dispersal and emerging ecological impacts of Ponto-Caspian species in the Laurentian Great Lakes. Can J Fish Aquat Sci 59:1209–1228.

Watkins J.M., R. Dermott, S.J. Lozano, E.L. Mills, L.G. Rudstam, and J.V. Scharold. 2007. Evidence for remote effects of dreissenid mussels on the amphipod *Diporeia*: analysis of Lake Ontario benthic surveys, 1972–2003. J Great Lakes Res 33:642–657.

Watkins J.M., L.G. Rudstam, E.L. Mills, and M.A. Teece. 2012. Coexistence of the native benthic amphipod *Diporeia* spp. and exotic dreissenid mussels in the New York Finger Lakes. J Great Lakes Res 38:226–235.

Weihrauch D., S. Morris, and D.W. Towle. 2004. Ammonia excretion in aquatic and terrestrial crabs. J Exp Biol 207: 4491–4504.

Wiklund A.K.E. and B. Sundelin. 2001. Impaired reproduction in the amphipods *Monoporeia affinis* and *Pontoporeia femorata* as a result of moderate hypoxia and increased temperature. Mar Ecol Prog Ser 222:131–141.

Wishart D.S., D. Tzur, C. Knox, R. Eisner, A.C. Guo, N. Young, D. Cheng, et al. 2007. HMDB: the Human Metabolome Database. Nucl Acids Res 35:D521–D526.

Wong C.S., S.A. Mabury, D.M. Whittle, S.M. Backus, C. Teixeira, D.S. Devault, C.R. Bronte, and D.C. Muir. 2004. Organochlorine compounds in Lake Superior: chiral polychlorinated biphenyls and biotransformation in the aquatic food web. Environ Sci Technol 38:84–92.

Wright P.A. 1995. Nitrogen excretion: three end products, many physiological roles. J Exp Biol 198:273–281.

Zhang X., J.M. Asara, J. Adamec, M. Ouzzani, and A.K. Elmagarmid. 2005. Data pre-processing in liquid chromatography–mass spectrometry–based proteomics. Bioinformatics 21:4054–4059.

Zsila F., Z. Bikádi, I. Fitos, and M. Simonyi. 2004. Probing protein binding sites by circular dichroism spectroscopy. Curr Drug Dis Technol 1:133–153.