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Polymethylene-interrupted fatty acids: Biomarkers for native and exotic mussels in the Laurentian Great Lakes

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ABSTRACT

Freshwater organisms synthesize a wide variety of fatty acids (FAs); however, the ability to synthesize and/or subsequently modify a particular FA is not universal, making it possible to use certain FAs as biomarkers. Herein we document the occurrence of unusual FAs (polymethylene-interrupted fatty acids; PMI-FAs) in select freshwater organisms in the Laurentian Great Lakes. We did not detect PMI-FAs in: (a) natural seston from Lake Erie and Hamilton Harbor (Lake Ontario), (b) various species of laboratory-cultured algae including a green alga (*Scenedesmus obliquus*), two cyanobacteria (*Aphanizomenon flos-aquae* and *Synechococystis* sp.), two diatoms (*Asterionella formosa, Diatoma elongatum*) and a chrysophyte (*Dinobryon cylindricum*) or, (c) zooplankton (*Daphnia* spp., calanoid or cyclopoid copepods) from Lake Ontario, suggesting that PMI-FAs are not substantively incorporated into consumers at the phytoplankton–zooplankton interface. However, these unusual FAs comprised 4–6% of total fatty acids (on a dry tissue weight basis) of native fat mucket (*Lampsilis siliquoidea*) and plain pocketbook (*L. cardium*) mussels and in invasive zebra (*Dreissena polymorpha*) and quagga (*D. bugensis*) mussels. We were able to clearly partition Great Lakes' mussels into three separate groups (zebra, quagga, and native mussels) based solely on their PMI-FA profiles. We also provide evidence for the trophic transfer of PMI-FAs from mussels to various fishes in Lakes Ontario and Michigan, further underlining the potential usefulness of PMI-FAs for tracking the dietary contribution of mollusks in food web and contaminant-fate studies.
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Introduction

Zebra and quagga mussels (*Dreissena* spp.) are freshwater bivalves native to the Caspian, Black and Azov Seas of Eastern Europe. They were introduced into the Laurentian Great Lakes through ballast water discharge from ocean-going ships and were first discovered in Lake St. Clair in 1988. Their adaptability, rapid life cycle, and high reproductive potential has ensured their continued invasion success and has led to their dominance over native mussels in the Great Lakes and elsewhere (Schloesser et al., 2006; Zanatta et al., 2002). The ecosystem impacts that zebra and quagga mussels (hereafter

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dreissenids) cause are extensive and, as yet, not fully understood (Connelly et al., 2007; Karatayev et al., 2007; Stoeckmann and Garton, 1997; Klerks et al., 1996; Holland, 1993; Wu and Culver, 1991). In addition, dreissenids bioaccumulate persistent organic contaminants (Gossiaux et al., 1998; Bruner et al., 1994a) and transfer these contaminants to higher trophic levels, e.g. watersnakes and fishes (Fernie et al., 2008; Bruner et al., 1994b). Fully understanding the extent to which dreissenids alter the function and stability of aquatic ecosystems requires the development of new methods designed to enable researchers to better track the passage of dreissenid-derived carbon to both native and introduced consumers at different trophic levels.

Fatty acids (FAs) are recognized as important tools in ecology (Arts et al., 2009), toxicology (Kainz and Fisk, 2009) and conservation biology (Gladyshev et al., 2009) because of their ability to act as both trophic markers (e.g., Koussoroplis et al., 2008, Napolitano, 1999; Desvilettes et al., 1997) and as indices of health and nutritional status (Arts et al., 2009, Arts and Wainman, 1999; Gulati and Demott, 1997). For example, they have recently been used in the Laurentian Great Lakes to track long-term systemic changes in pelagic food resources available to herring gulls (*Larus argentatus*) (Hebert et al., 2006, 2008) and to infer the health and nutritional status of cornerstone macro-invertebrates (e.g., *Mysis diluviana* [formerly *relicta*]; Johannsson et al., 2009; Schlechtriem et al., 2008). According to Dalsgaard et al. (2003), "the perfect trophic marker is a compound whose origin can be uniquely and easily identified, that is inert and non-harmful to the organisms, that is not selectively processed during food uptake and incorporation, and that is metabolically stable and hence transferred from one trophic level to the next in both a qualitative and quantitative manner. Such a marker would provide essential insight into the dynamics of ecosystems by presenting unique information on pathways of energy flows, i.e., crucial information on which all ecosystem models are eventually built. However, such markers are unfortunately rare, if nonexistent, and instead we have to be content with less ideal components, a category to which FA belong." Thus, FAs are usually partially successful as trophic markers, principally because they exhibit species-specific bioconversion rates and because many FAs are ubiquitous and abundant in aquatic food webs.

An exception to this convention might be a group of uncommon FA termed poly-methylene interrupted FAs (PMI-FAs; Mezek et al., 2009). PMI-FAs (which can now be synthesized in the laboratory, see Albu et al., 2011) contain more than one methylene group between double bonds in the aliphatic chain, whereas more common FAs (i.e., methylene-interrupted) contain only a single methylene group between double bonds. Although they occur rarely, PMI-FAs have been found in marine invertebrates (Budge et al., 2006, 2007; Saito, 2007; Garrido and Medina, 2002; Joseph, 1982; Pearce and Stillway, 1976; Paradis and Ackman, 1974; Ackman and Hooper, 1973; Jefferts et al., 1973), terrestrial seeds (Madrigal and Smith, 1975; Plattner et al., 1975; Smith et al., 1969) and plant oils (Fore et al., 1966). In the marine environment, it is thought that PMI-FAs are synthesized *de novo* primarily in bivalves and carnivorous gastropods (Saito, 2007; Budge et al., 2006, 2007; Joseph, 1982) and further accumulate, to varying extents (depending on diet), in large marine mammals such as the pacific walrus (*Odobenus rosmarus*) and bearded seal (*Erignathus barbatus*) that are closely associated with sea ice (Budge et al., 2007). PMI-FAs in marine environments are found in greater proportions in phospholipids (Freites et al., 2002) than in other lipid classes, and they occur in greater concentration in tissues more directly exposed to the immediate environment (Berge and Barnathan, 2005; Garrido and Medina, 2002; Freites et al., 2002). This latter observation is of benefit because it informs (a) which tissues might be expected to yield maximum concentrations of PMI-FAs in aquatic organisms and (b) might ultimately provide important clues as to the proximate synthesis source.

Herein, we provide the first report on the distribution of PMI-FAs among various freshwater organisms (algae, zooplankton, mussels, and fishes) based on a series of case studies from the Laurentian Great Lakes. One of our objectives was to assay natural seston samples and several species of laboratory-cultured algae from different taxonomic groups (two cyanobacteria, two diatoms, and a chrysophyte) to determine if PMI-FAs are present in substantive quantities at the level of planktonic primary producers. This is an important point because, if we cannot quantify PMI-FAs (in substantive quantities) in primary producers, it implies that these compounds will not be substantively incorporated into tissues of consumers across the phytoplankton–zooplankton interface. To further assess the potential efficacy of PMI-FAs as biomarkers, we quantified and compared PMI-FAs in both introduced and native freshwater mussels, as well as in several fishes from different locations in Lakes Ontario and Michigan. We provide a "weight of evidence" approach to highlight the potential use of PMI-FAs to track mollusk-derived carbon by comparing the putative source of PMI-FAs (i.e., mussels) with their concentrations in different fishes known or suspected to consume mollusks.

Materials and methods

Mussels

Freshly caught (~1–2 days prior to purchase) live specimens of the marine blue mussel (*Mytilus edulis*; $n=3$ samples of one individual each), originally caught in the waters off Prince Edward Island, Canada, were obtained from a local fish market in Burlington, Ontario. Zebra mussels (*Dreissena polymorpha*; total $n=9$ samples of one individual each) were collected from Hamilton Harbor in April 2007 ($n=3$) and August 2010 ($n=6$) and immediately frozen (−85 °C) for later analyses. Quagga mussels (*D. bugensis*) were collected from deep areas of Lakes Ontario ($n=15$ as above) and Huron ($n=8$ as above) in June to July 2007. Lipids, including PMI-FAs, were extracted from combined foot and mantle tissues for blue and dreissenid mussels. Two native mussels, fat mucket (*Lampsilis siliquoidea*; $n=12$ as above) and plain pocketbook (*L. cardium*; $n=18$), were collected from Lake St. Clair in 2003–2004 as part of a larger study to determine the effectiveness of natural refuge areas for native mussels (McGoldrick et al., 2009). Tissues of native mussels were separated into foot and mantle samples prior to extraction and quantification of PMI-FAs. Foot plus mantle PMI-FA concentrations were averaged (one sample of each tissue type per individual) prior to undertaking the among lakes/species comparisons (i.e., so that all mussel samples were comparable and more appropriately reflected what a fish predator consuming whole dreissinds would ingest) but were extracted separately to demonstrate how PMI-FA concentrations might be expected to differ between foot and mantle tissues (see below).

Seston and algae

Triplicate 5 l natural seston samples were obtained from 10 l Niskin bottles lowered to 1 m depth in Lake Erie (near Port Colbourne) and in Lake Ontario (Hamilton Harbor) during the first week of June 2008. The lakewater samples were filtered through a 64-μm Nitex® mesh to remove zooplankton and then collected on pre-combusted (450 °C) GF/F filters. Additional triplicate algal samples (collected on pre-combusted GF/F filters) were obtained from pure batch, stationary phase, ($>10^6$ cells ml^{-1}) cultures raised in defined media in the laboratory. These culture samples consisted of two cyanobacteria (*Aphanizomenon flos-aquae*, *Synechococystis* sp., 50 ml for each replicate), two diatoms (*Asterionella formosa* and *Diatoma elongatum*, 50 ml and 70 ml for each replicate, respectively, originally isolated from Lake Ontario), and a chrysophyte (*Dinobryon cylindricum*, 30 ml for each replicate, originally isolated from Glenmore reservoir, Alberta). The culture media consisted of CYANO (Jüttner an

Lüthi, 2008), Chu 10 (Nichols, 1973), and an amended version of WC (WCed) (Watson, 1999), for the cyanobacteria, diatoms, and chrysophyte, respectively. Finally, six replicates of 400 ml each were harvested from chemostat cultures of *Scenedesmus obliquus* (CPCC 5 Canadian Phycological Culture Centre, University of Waterloo, Waterloo, Canada; density ranged from 4.8 to 6.5×10^6 cells/mL) grown in Bold's basal medium. The *S. obliquus* cells were collected by centrifugation (1500 rpm at 21 °C) resulting between 31 and 52 mg dry weight after freeze-drying.

Zooplankton

Zooplankton were collected by towing a Wisconsin-style plankton net (115 μm mesh) vertically through the epilimnion, above the deepest basin (Rochester Basin) of Lake Ontario in July 2001. Zooplankton were individually separated (using forceps) into broad taxonomic groups (daphnids, calanoid copepods, and cyclopod copepods; $n=3$ in each case) under a dissecting microscope. These zooplankton samples, which consisted of ~100 individuals/sample, were extracted as described below; concentrated extracts were stored at −85 °C for subsequent PMI-FA analyses.

Fish

Four fish species were collected from various locations in Hamilton Harbor, Lake Ontario in July and September 2006. Fishes examined included: emerald shiner (*Notropis atherinoides*; $n=8$), largemouth bass (*Micropterus salmoides*; $n=10$), pumpkinseed (*Lepomis gibbosus*; $n=9$) and brown bullhead (*Ameiurus nebulosus*; $n=10$). Lake whitefish (*Coregonus clupeaformis*) were collected from the Bay of Quinte ($n=18$) and Lake Ontario in the offshore region off Point Petre ($n=9$) in November 2006. Lake whitefish were also sampled from three locations in Lake Michigan (Naubinway, $n=20$; Big Bay de Noc, $n=20$; and Bailey's Harbor, $n=20$) in October to November 2005. For all fishes, only skinless, boneless, dorsal muscle tissues (landmarked on either side of the dorsal fin) were assayed for PMI-FAs.

Lipid extraction and PMI-FA analyses

Lipids were passively extracted from both the seston and the batch cultured algae by placing the filters overnight in 5 ml of chloroform: methanol (2:1) at 4 °C. The lipid-bearing solvent was decanted and the process was repeated two more times over several hours during the next day. To obtain sufficient lipid for extraction from zooplankton (*Daphnia* spp. and copepods), we pooled ~100 individuals together, after which the tissues were freeze-dried, weighed to the nearest microgram (Sartorious ME5 microbalance) and then homogenized in chloroform/methanol (2:1). The chemostat-raised green alga *S. obliquus* provided larger amounts of tissue for lipid extraction (4.8–7.1% total lipid on a dry weight basis), therefore its lipid was extracted from freeze-dried samples using the method just described for zooplankton. For large-bodied organisms (adult mussels and fishes), freeze-dried tissues were homogenized by grinding them to a fine powder in liquid nitrogen using a mortar and pestle prior to weighing (Sartorious ME5 microbalance) following which they were extracted (three times) in chloroform:methanol (2:1) (Folch et al., 1957). All lipid extracts were subsequently dried under a stream of nitrogen and stored at −85 °C for later analyses.

Fatty acids were methylated to fatty acid methyl esters (FAME) using the sulphuric acid in methanol method (Christie, 1989). The PMI-FAs were analyzed by GC-MS (Agilent 6890N GC) equipped with a DB-23 polar capillary column (Agilent; #122-2361; 60 m × 0.25 mm id × 0.15 μm film thickness), and a mass selective quadrupole detector (Agilent 5973N). Helium was used as the carrier gas at a constant pressure (~180 kPa at 33 cm s^{-1}). Samples were injected in split or splitless mode depending on the requirements of individual samples, with an inlet temperature of 250 °C, at an initial oven temperature of 50 °C. After 1 min, the oven temperature was raised to 175 °C at a rate of 25 °C min^{-1}, then to 235 °C at 4 °C min^{-1} and held for 5 min. Transfer line temperature was 180 °C. Retention time locking was used to obtain FA elutions with very little retention time shifting of the peaks (methyl stearate was retention time locked to 14.0 min). Samples were analyzed in both SIM (selected ion monitoring) and SCAN mode and day-to-day instrument repeatability was quantified by comparing responses of standard solutions.

PMI-FAs were identified by comparing mass spectra, retention time matching and degree of unsaturation (Ag-Ion solid phase extraction), with FA profiles obtained by extracting fresh blue mussel tissue (previously reported to contain PMI-FA; Budge et al., 2007). As no laboratory standards for PMI-FAs existed when we conducted this study, and because the mass spectra of FAs with the same degree of unsaturation were very similar, we quantified the 6 PMI-FAs in our samples using the nearest-neighbor principle. In other words, we produced 4-point, peak area versus concentration, calibration curves for each of the following nearest-neighbor non-PMI-FAs; 20:2n-6, 20:3n-6 and 22:2n-6 (these FAs are part of Supelco's 37 Component FAME Mix; Catalog #47885-U). We then used these three standard curves to determine the concentrations of the 6 PMI-FAs. Non-PMI-FA neighbors with the same chain length and degree of saturation as the PMI-FAs were thus used to determine concentrations of the PMI-FAs in the species examined; except in the case of 22:3PMI where the non-PMI-FA 22:2n-6 was used (Table 1). PMI-FAs are reported on a concentration basis (i.e., μg PMI-FA/mg dry weight).

Differences in PMI-FA concentrations among species or locations were evaluated using ANOVA followed by post-hoc Tukey HSD (Honestly Significant Difference) tests (JMP ver. 7, SAS Institute Inc.) and, where two groups were compared, by simple *t*-tests (SigmaStat ver. 3.5). Critical alpha level for all statistical tests was set to 0.05. Discriminant function analysis (JMP ver. 7) was used to evaluate the ability of PMI-FAs to distinguish among mussels and among fishes.

Results and discussion

Case study 1: PMI-FA in marine, freshwater and invasive mussels

We confirmed our ability to detect PMI-FAs, as reported by Budge et al. (2007), by extracting and quantifying them from total lipid extracts of the marine-derived blue mussels. We also confirmed the existence, and quantified the concentrations, of PMI-FAs in freshwater-derived native and exotic mussel species (Fig. 1). To our knowledge, this is the first reported occurrence of these compounds in freshwater native and exotic mussels in the Great Lakes.

Total PMI-FA concentrations, on a relative basis, were greater in the marine blue mussel (~8% of total FAs on a dry mass basis)

Table 1
Polymethylene-interrupted fatty acid (PMI-FA) nomenclature and non-PMI-FA standards used to quantify PMI-FA using the nearest neighbor method.

Trivial name	PMI-FA (n-*x* nomenclature)[a]	PMI-FA (Δ^x nomenclature)[b]	Non-PMI-FA standards (n-*x* nomenclature)
20:2PMI1	20:2n-9	20:2Δ5,11	20:2n-6
20:2PMI2	20:2n-7	20:2Δ5,13	20:2n-6
20:3PMI	20:3n-6	20:3Δ5,11,14	20:3n-6
22:2PMI1	22:2n-9	22:2Δ7,13	22:2n-6
22:2PMI2	22:2n-7	22:2Δ7,15	22:2n-6
22:3PMI	22:3n-6	22:3Δ7,13,16	22:2n-6

[a] Where "m:pn-*x*" denotes a fatty acid with "m" carbon atoms, "p" ethylenic bonds (methylene-interrupted) if more than one, and "x" carbon atoms from and including the terminal group to and including the carbon atom nearest the first ethylenic bond.
[b] Where the double bonds are located on the *x*th carbon–carbon bonds, counting from the carboxylic acid end. Note: the position of the double bonds, based on preliminary GC-MS work, was provided by Suzanne M. Budge (Dalhousie University).

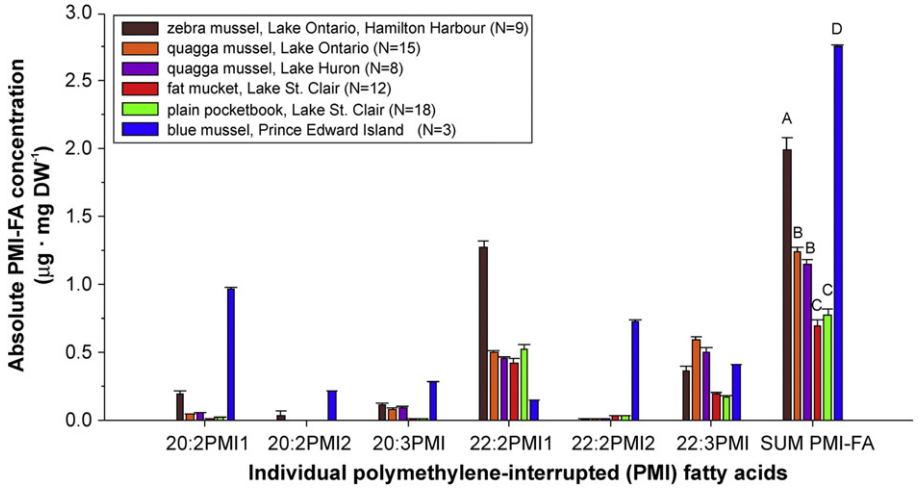

Fig. 1. Absolute concentrations (µg mg DW^{-1}; ± S.E.) of individual and total polymethylene-interrupted fatty acids (PMI-FAs) in tissues (mantle and foot combined) of marine blue mussels, native freshwater mussels from Lake St. Clair and invasive freshwater zebra (L. Ontario) and quagga mussels (L. Ontario and L. Huron). In the case of total PMI-FA concentrations means with the same capital letter are statistically indistinguishable (Tukey HSD test) from each other. See Table 1 for a list of the PMI-FAs.

compared with the freshwater mussel species (~4–6% of total FAs) (Fig. 1). Fried et al. (1993) similarly reported that PMI-FAs represented 2–3% of total FA in freshwater aquatic gastropods. We found 20:2PMI1 to be the dominant PMI-FA in blue mussels, whereas the dominant PMI-FAs in freshwater native and exotic mussels were 22:2PMI1 and 22:3PMI (Fig. 1, Table 1).

Zebra mussels had the highest total PMI-FA concentrations, on an absolute basis (µg PMI-FA mg DW^{-1}), of all freshwater mussels sampled; however, marine blue mussel total PMI-FA concentrations were even higher (Fig. 1; Tukey HSD). Within the four freshwater mussels examined, total PMI-FA concentrations were lowest in the two native species and intermediate in quagga mussels compared to zebra mussels (Fig. 1; Tukey HSD).

Using discriminant function analysis, we were able to clearly partition Great Lakes mussels into three separate groups (zebra, quagga, and native mussels) solely on the basis of their PMI-FA concentrations (Fig. 2). However, PMI-FA concentrations alone were insufficient, perhaps because of similar pathways of *de novo* synthesis and/or because PMI-FAs might be retained at a particular level within a species, to fully differentiate between quagga mussels from Lakes Ontario and Huron (5 out of 23 samples, or 22%, misclassified) or to discriminate between the two native freshwater mussel species (9 out of 30 samples, or 30%, misclassified) (Fig. 2).

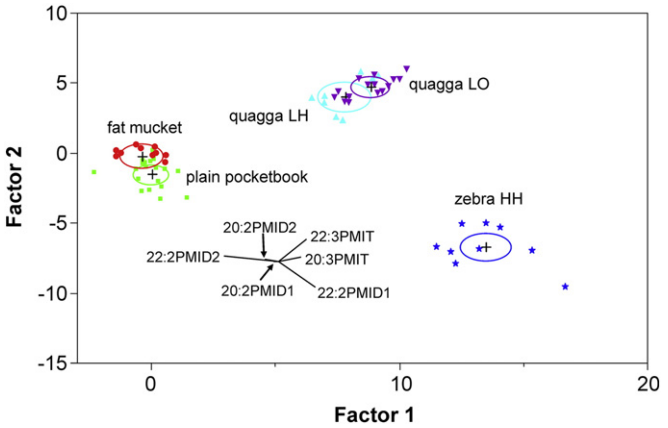

Fig. 2. Discriminant function analysis plot with individual data points representing polymethylene-interrupted fatty acid (PMI-FA) concentrations (µg mg DW^{-1}) in invasive zebra (stars) and quagga mussels (triangles) and in native fat mucket (circles) and plain pocketbook (squares) mussels. Ellipses (center indicated by a cross) represent the 95% confidence region estimated to contain the true mean for each of the four mussel species. Biplot rays provide the direction and strength of the discriminant weightings for each polymethylene-interrupted fatty acid (PMI-FA). A total of 62 samples is represented. There were 14 misclassifications among the groups depicted (see text for details). LO = Lake Ontario (downward-pointing triangles), LH = Lake Huron (upward-pointing triangles), HH = Hamilton Harbor.

Mean concentrations of the two most prominent (>0.05 µg mg DW^{-1}) PMI-FAs (i.e., 22:2PMI1 and 22:3PMI; see Table 1) and total PMI-FA concentrations were significantly greater (*t*-tests; $p<0.05$) in the foot versus mantle tissues of native mussels (Fig. 3). This reinforces previous findings that these compounds usually occur in greater concentrations in tissues more directly exposed to the immediate sedimentary environment (Berge and Barnathan, 2005; Freites et al., 2002; Garrido and Medina, 2002).

Case study 2: PMI-FAs in laboratory-cultured algae and wild-caught seston and zooplankton

We were unable to quantify PMI-FAs in seston samples from Lakes Ontario (Hamilton Harbor) and Erie or from the laboratory-cultured phytoplankton (either from five species raised in batch cultures or from the green alga raised in chemostats) at levels above our detection limits (which ranged from 0.2 to 10 ng depending on the PMI-FA). Similarly, we were unable to detect PMI-FA in *Daphnia* spp., calanoid or cyclopoid copepods from Lake Ontario. These results suggest that substantive quantities of PMI-FAs do not enter into the Animalia at the phytoplankton-zooplankton (plant–animal) interface. This finding supports the current hypothesis that *de novo* synthesis of these compounds in aquatic ecosystems takes place primarily in members of the phylum Mollusca (Budge et al., 2007).

Case study 3: PMI-FAs in five fishes from Hamilton Harbor

Much information regarding the feeding ecology of consumers can be determined from stomach-content analyses (Hyslop, 1980) and by more recent methods such as stable isotope (e.g., Cabana and Rasmussen, 2002) and FA signature analyses (Iverson et al., 2004). We quantified PMI-FAs in four fishes with different feeding habits. The fishes assayed for PMI-FAs ranged from the mainly pelagic-feeding emerald shiner to the more omnivorous, benthic-feeding brown bullhead (Scott and Crossman, 1973). The other two species (pumpkinseed, largemouth bass) are considered midwater littoral generalists (Scott and Crossman, 1973). During the ensuing discussion it must be stressed that, until more is known about species-specific conversion rates (i.e., how quickly different fish species catabolize or otherwise modify PMI-FAs), or until quantitative laboratory

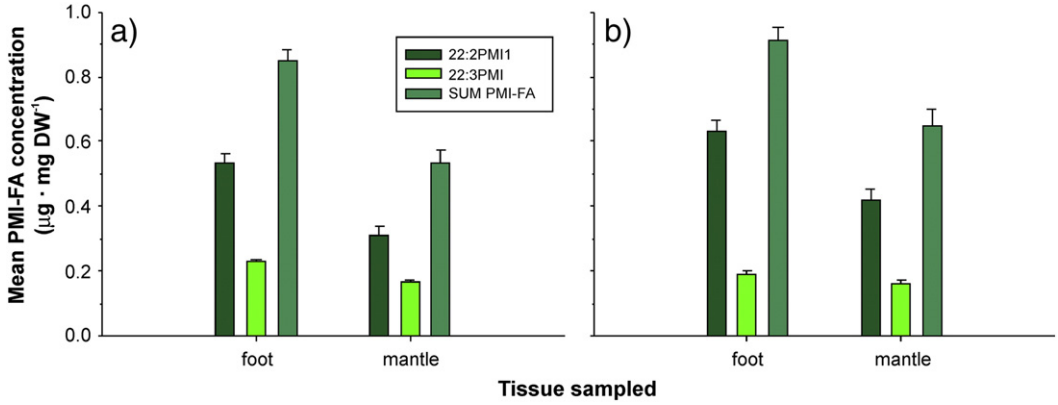

Fig. 3. Mean concentrations ± S.E. of the two dominant (>0.05 µg mg DW^{-1}) polymethylene-interrupted fatty acids (PMI-FAs)(i.e., 22:2PMI1 and 22:3PMI; see Table 1) and total PMI-FAs in foot versus mantle tissues of native mussels (a) fat mucket and (b) plain pocketbook) from the delta area of Lake St. Clair.

calibration studies can be performed, comparing between species should be done with caution.

With the exception of brown bullhead, mean concentrations of total PMI-FAs in fishes from Hamilton Harbor were low in comparison to total PMI-FA concentrations in native, and especially in exotic, mussels (Fig. 4), suggesting that these other fishes do not incorporate mussel carbon to the same degree. Total PMI-FA concentrations in the four fishes were statistically indistinguishable from each other ($p>0.05$, Tukey HSD). Despite the lack of statistical significance, the trend was consistent with the feeding habits of these fishes (Scott and Crossman, 1973); brown bullhead, a benthivorous feeder, had the highest total mean PMI-FA concentration whereas emerald shiner, the predominantly open-water pelagic feeder, and pumpkinseed a generalist feeder that consumes both open water and benthic prey (mainly a variety of insects), had the lowest total mean PMI-FA concentration (Fig. 4). Largemouth bass, a predatory midwater littoral generalist, had intermediate total mean PMI-FA concentrations.

Using discriminant function analysis, we attempted to partition the fishes into separate groups based on their PMI-FA profiles (Fig. 4). In total, 14 fish (37.8%) were misclassified indicating that PMI-FA profiles alone cannot be used to uniquely identify these particular fish species. However, consistent with their different mode of feeding, emerald shiner and brown bullhead were the most widely separated groups with the two midwater littoral generalists (largemouth bass and pumpkinseed) occupying an intermediate position (Fig. 4).

Biplot rays (Fig. 4), showing the direction and strength of the discriminant function weightings for each PMI-FA, revealed that brown bullhead were most strongly distinguished from emerald shiners by the weight and directionality attributed to 20:3PMI and 22:2PMI1 (in the direction of brown bullhead) and 22:3PMI and 20:2PMI1 (in the direction of emerald shiner) (Fig. 4). The latter two PMI-FAs exist in higher concentrations in exotic mussels compared to native mussels (Fig. 1), suggesting perhaps that emerald shiner incorporate (indirectly or directly) relatively more exotic mussel carbon than do brown bullhead. However, it must be noted that such inter-species comparisons must be interpreted cautiously because of the current uncertainty related to how different fishes process ingested PMI-FAs.

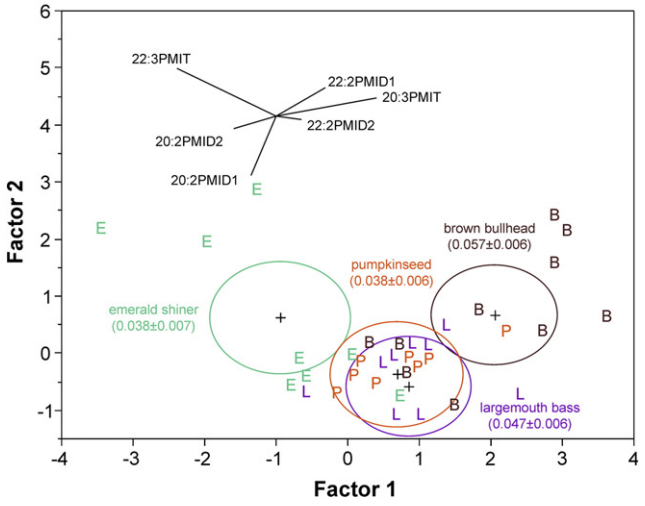

Fig. 4. Discriminant function analysis plot with individual data points representing polymethylene-interrupted fatty acid (PMI-FA) concentrations (µg mg DW^{-1}) in skinless, boneless, dorsal muscle tissue of four fishes from Hamilton Harbor, Lake Ontario including; emerald shiner = E, pumpkinseed = P, largemouth bass = L and brown bullhead = B. Ellipses (center indicated by a cross) represent the 95% confidence region estimated to contain the true mean of the four fishes. Mean PMI-FA concentration and standard error in brackets below each fish name. Biplot rays provide the direction and strength of the discriminant function weightings for each polymethylene-interrupted fatty acid (PMI-FA). A total of 37 fish samples is represented. There were 14 misclassifications among the species (see text for details).

Case study 4: Lake whitefish from Lake Ontario and Lake Michigan

Lake whitefish are opportunistic feeders that, historically, preyed extensively on the deep-water burrowing amphipod *Diporeia* spp.; however, the abundance of this prey has declined precipitously in many areas of the Great Lakes in recent years (Walsh et al., 2008; Nalepa et al., 2006, 2007). *Diporeia* were an important diet item because they (a) were historically abundant (Nalepa et al., 2006, 2007), (b) are rich in energy, i.e., high total lipid concentrations (Cavaletto et al., 1997), and (c) have high concentrations of essential fatty acids (Kainz et al., 2010) that are known to promote fish health, overall condition, and enhance reproductive success (Arts and Kohler, 2009; Tocher, 2003). As *Diporeia* abundance declines, lake whitefish are forced to compensate by consuming different prey including exotic mussels (Pothoven and Madenjian, 2008). Thus, we hypothesized that PMI-FA concentrations would be higher in lake whitefish from areas where they are known (through gut content verification) or suspected (due to known declines in *Diporeia*) to incorporate more mussels in their diet.

Dermott (2001) noted that *Diporeia* were absent (<40 m depth) from the eastern half of Lake Ontario by summer 1995, and Watkins et al. (2007) noted that *Diporeia* had almost completely disappeared from depths<90 m by 2003 and that quagga mussels had largely displaced zebra mussels in the lake. Since that time, *Diporeia* have continued to decline and are now absent above 120–150 m depths in Lake Ontario except in the area just northwest of the Niagara River and down the very middle of the lake (R. Dermott, Fisheries and Oceans, Canada, pers. comm.). Consequently, dreissenid mussels now comprise an important part of lake whitefish diet in this lake (Hoyle et

al., 2008). In contrast, *Diporeia* were found in high numbers in certain parts of Lake Michigan (e.g., northwest coastal areas such as Bailey's Harbor and Big Bay de Noc, and south to Sheboygan), although they were largely absent in northern Lake Michigan including the Naubinway sampling station leading up to the time that our lake whitefish tissues were collected (Nalepa et al., 2006, 2009b).

Consistent with these observations, lake whitefish from Lake Ontario (Point Petre and Bay of Quinte) had significantly higher total PMI-FA concentrations than did lake whitefish from Lake Michigan (Naubinway, Baileys Harbor and Big Bay de Noc) (Fig. 5a; *t*-test, $t = 1.99$, $p < 0.05$). However, lake whitefish from the offshore station in Lake Ontario (Point Petre) had significantly higher total PMI-FA concentrations than did lake whitefish from the Bay of Quinte (Fig. 5b; Tukey HSD). This is perhaps not surprising since the Bay of Quinte, in comparison to Point Petre, is a shallow and more productive system that is still recovering from historically high phosphorus loadings (Millard and Sager, 1994). This more eutrophic environment, with its higher connectivity to benthic habitats, should provide lake whitefish with a diverse, abundant, and highly productive benthic prey base. However, the situation is complex because, although the Point Petre and Bay of Quinte lake whitefish do represent separate spawning stocks, they also presumably intermingle to varying extents during the non-spawning (spring to fall) period, especially when warm water temperatures in the Bay of Quinte drive them out to deeper cooler waters.

Lake whitefish from Naubinway had significantly higher total PMI-FA concentrations than did lake whitefish from the other two stations (Big Bay de Noc and Bailey's Harbor) in Lake Michigan (Fig. 5c; Tukey HSD). This pattern matches documented densities of *Diporeia* at these three Lake Michigan stations leading up to when lake whitefish were collected for this study (Nalepa et al., 2006, 2009b), and is consistent with observations (see above) of lake whitefish consuming dreissenids when *Diporeia* decline. Lake whitefish from Naubinway and Point Petre in Lakes Michigan and Ontario, respectively, and brown bullhead from Hamilton Harbor had similar total PMI-FA concentrations (Figs. 4 and 5; Tukey HSD), suggesting that they all consumed dreissenids directly and/or consumed prey that had themselves consumed dreissenids.

Ebener et al. (2010) used mark-recapture techniques to demonstrate that a large part of the Big Bay de Noc lake whitefish stock move into the waters of the Wisconsin-side of Lake Michigan during the non-spawning season and that the Naubinway stock is largely segregated from the Big Bay de Noc and Bailey's Harbor stocks. Consistent with this finding, discriminant function analysis of individual PMI-FA concentrations (this study) revealed that lake whitefish from Big Bay de Noc and Bailey's Harbor were more similar to each other than were fish from Naubinway, which formed a separate group (Fig. 6). Of the 60 lake whitefish individuals that comprised this analysis, 15 fish were misclassified as follows: 1 Bailey's Harbor fish was classed as a fish from Naubinway and 1 Naubinway fish was classed as a fish from Big Bay de Noc. The remaining 13 misclassifications were the result of Bailey's Harbor fish being classified as fish from Big Bay de Noc and vice versa. Biplot rays, showing the direction and strength of the discriminant function weightings for each PMI-FA (Fig. 6), revealed that 20:2PMI1, and

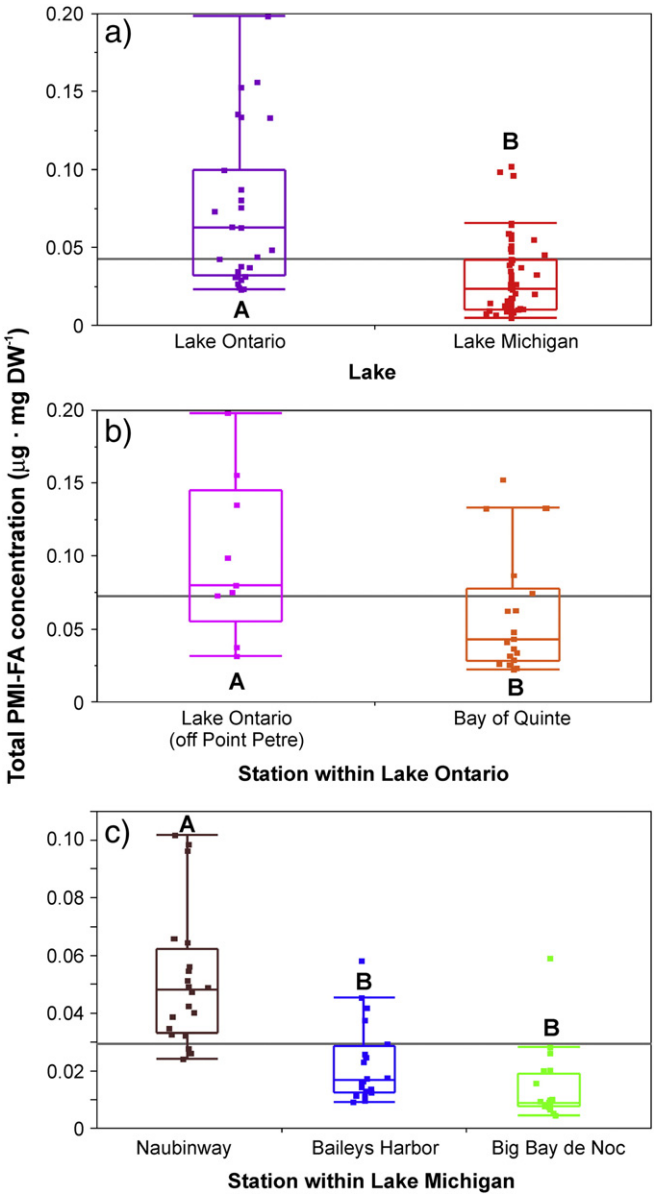

Fig. 5. Box-whisker plots of total polymethylene-interrupted fatty acid (PMI-FA) concentrations (DW = dry weight) in skinless, boneless, dorsal muscle tissue of lake whitefish from (a) Lakes Ontario ($n = 27$) and Michigan ($n = 60$), (b) two sampling locations in Lake Ontario ($n = 9$ for Point Petre; $n = 18$ for Quinte) and (c) three sampling locations in Lake Michigan ($n = 20$ for each station). Means, within each subfigure, with the same capital letter are statistically indistinguishable (Tukey HSD test). The horizontal line on each subfigure is the grand mean.

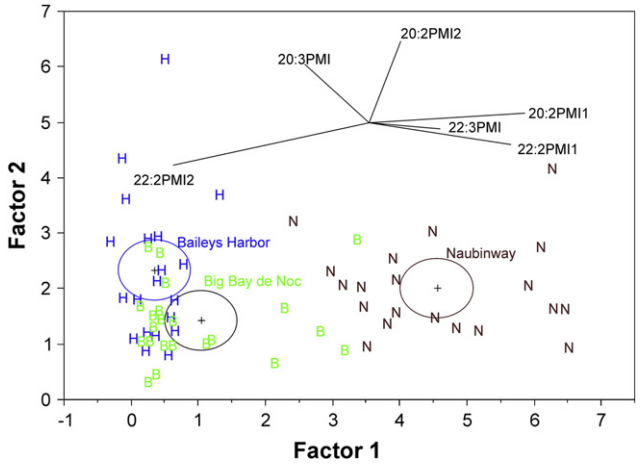

Fig. 6. Discriminant function analysis plot with individual data points representing polymethylene-interrupted fatty acid (PMI-FA) concentrations (µg mg DW^{-1}) in skinless, boneless, dorsal muscle tissue of lake whitefish from three sampling locations in Lake Michigan. H = Baileys Harbor, B = Big Bay de Noc, N = Naubinway. Ellipses (center indicated by a cross) represent the 95% confidence region estimated to contain the true mean of each group. Biplot rays provide the direction and strength of the discriminant function weightings for each polymethylene-interrupted fatty acid (PMI-FA). A total of 60 fish samples is represented ($n = 20$ for each station). There were a total of 15 misclassifications among the stations (see text for details).

especially 22:2PMI1 and 22:3PMI (the most abundant PMI-FA in dreissenid and native mussels; Fig. 1), were the most influential PMI-FAs for separating lake whitefish from Naubinway into a unique group, in agreement with Ebener et al. (2010), and further confirming their higher reliance on mussel prey.

General discussion

We detected, for the first time, the existence of PMI-FAs in freshwater organisms of the Laurentian Great Lakes. Among the species surveyed, the highest concentrations of PMI-FAs were found in both exotic (zebra and quagga) and native (fat mucket and plain pocketbook) mussels in agreement with previous reports where PMI-FAs have been quantified in marine mussels (Budge et al., 2007; Zhukova, 1991). Within the mussels, foot tissue had higher concentrations of PMI-FAs than did mantle tissues, reinforcing the findings of others that tissues in intimate contact with sediments have higher PMI-FA concentrations than more "internal" tissues (Berge and Barnathan, 2005; Freites et al., 2002; Garrido and Medina, 2002). Although we know that PMI-FAs exist in high concentrations in Mollusca, their exact origin and function are unknown. We do not yet know if PMI-FAs are *de novo* synthesized by the mollusks themselves, and/or by the microbes associated with the mollusks, and/or if they are derived from the diet of mollusks. Further, we do not yet know what role they play in mollusk physiology or biochemistry. It has been reported that PMI-FAs in marine mussels are synthesized from eicosadienoic acid (20:2n-6) by desaturation and subsequent elongation (Garrido and Medina, 2002) and that certain PMI-FAs detected in terrestrial mammals, and originating from conifer oils, can be metabolized to essential fatty acids (Tanaka et al., 2007). However, information on PMI-FA synthesis pathways and/or on their conversion to other compounds (e.g., essential fatty acids) is currently unavailable for freshwater mussels.

We did not find any evidence of PMI-FAs in either field-collected seston samples or laboratory-cultured algae, nor could we detect PMI-FAs in zooplankton (*Daphnia* spp., calanoid and cyclopoid copepods). Taken together, these observations suggest that PMI-FAs likely do not cross the plant–animal interface, in substantive quantities, at the phytoplankton–zooplankton interface. This is an important finding because it strengthens the case for using PMI-FAs as a biomarker (biochemical tracer) for mollusk-derived carbon. We hasten to add, however, that our preliminary synoptic survey is far from complete raising the possibility that other benthic organisms, and especially other mollusks (e.g., gastropods), may contain measurable quantities of PMI-FAs.

We quantified PMI-FAs in four fishes from Lake Ontario (Hamilton Harbor) and in lake whitefish from Lakes Ontario and Michigan. Both the total and the individual PMI-FA concentrations were broadly consistent with what we would expect based on presumed consumption of mollusks by the various groupings of fish provided here. Although our weight-of-evidence type study provides preliminary information regarding the utility of using PMI-FAs to track mussel-derived carbon through various trophic pathways (e.g., to fish or birds) many questions remain. For example, we reported lower values of PMI-FAs in fishes (from 0.1% to 0.3% of total quantified fatty acids; data not shown) compared to mussels, where they constituted 4% to 6% of total quantified fatty acids. Clearly, the fishes we surveyed eat more than just mussels, so it is not surprising that the PMI-FA signal becomes somewhat muted when we examine consumers at higher trophic levels. In addition, we do not yet know how fish and other organisms (e.g., birds) metabolize or otherwise modify PMI-FAs once they are ingested. Further, PMI-FAs can enter the rest of the food web through direct ingestion, for example, when fishes directly consume sessile adult mussels and/or when invertebrates (e.g., *Diporeia* spp. and other amphipods) scavenge dead or dying mussels, or indirectly when larger fishes prey on smaller ones (that have themselves eaten mussels and/or invertebrates that have eaten mussels). For example, Cloe et al. (1995) reported that zebra mussel shells were crushed and their contents ingested by bull chub (*Nocomis raneyi*), and Nalepa et al. (2009a), Hoyle et al. (2003) and Owens and Dittman (2003) provided evidence that lake whitefish actively consumed dreissenids, particularly when native prey (particularly *Diporeia*) became rare. Answers to these questions require more detailed study (e.g., controlled feeding studies using radiotracers) and are beyond the scope of the present survey.

Since dreissenids invaded the Great Lakes in 1986, information on where and how their carbon is flowing through food web compartments is continually coming to light via classical diet studies. For example, it is now known that fishes such as the exotic round goby (*Neogobius melanostomus*) and native lake whitefish both consume dreissenids (Madenjian et al., 2010) as do, to a lesser extent, native burbot (*Lota lota*) (Jacobs et al., 2010). There is also good evidence that double-crested cormorants (*Phalacrocorax auritas*) prey extensively on dreissenid-consuming round goby in areas of the Great Lakes where these fish are abundant (Johnson et al., 2010). Extrapolating from these studies, it becomes clear that having at our disposal a reliable biomarker of mussel carbon could offer even more detailed insights into exactly how exotic mussels impact the ecosystems they invade, not only from an ecological perspective, but also from a contaminant-transfer perspective. Combining biochemical tracers like PMI-FAs, contaminants, and stable isotopes, both for present-day and retrospective studies, offers a more powerful array of tools to quantify the ecological and chemical consequences of exotic species introductions. For example, Hebert et al. (2009) used combined tracers (fatty acids, stable isotopes and persistent contaminants) to map food web pathways utilized by herring gulls (*Larus argentatus*) breeding along the shores of the St. Lawrence River, Canada.

In summary, our preliminary synoptic survey suggests that PMI-FAs appear to have most, if not all, of the hallmarks of a good biochemical tracer in that they, (a) are metabolically relatively stable and apparently non-harmful to the organisms that harbor them, (b) originate from a unique source, and (c) can be readily identified and quantified as they are transferred from one trophic level to the next (Dalsgaard et al., 2003). Thus, we conclude that, although more verification is required, PMI-FAs appear to be a promising biomarker for mussel carbon in the Great Lakes and, presumably, in other aquatic ecosystems.

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