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Article

Stable Isotope Analysis of Planktonic Lower Food Webs of Lakes Erie, Huron, Michigan and Superior

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Abstract: Historical plankton samples from the St. Lawrence Great Lakes were subjected to taxon-specific ^{15}N analysis to test the hypothesis that the changes recorded in zooplankton communities during the 21st Century are related to changes in the trophic positions of large-bodied carnivorous copepods. *Daphnia mendotae* was used as the reference herbivore for trophic-level comparisons. The results were that *Limnocalanus macrurus*, *Diaptomus (Leptodiaptomus) sicilis* as well as the cladoceran *Bythotrephes cederstroemi* show evidence of elevated carnivory compared to data from the 20th Century. The large diaptomid *Diaptomus (Leptodiaptomus) sicilis* has a stable isotope signature that is significantly more carnivorous in Lake Superior than in Lakes Michigan and Huron by approximately one-half trophic level. Differences were found in 10 cases out of 15 for *Limnocalanus* (Huron, Michigan Superior), 6 cases out of 15 for *Diaptomus* (Huron, Michigan) and in 1 out of 1 for *Senecella* (Superior). We did not find evidence to support the theory that large-bodied calanoid copepods may have improved their representation in the food webs of the upper Great Lakes by shifting their trophic position downward. Instead, large-bodied Calanoida have increased their trophic positions in parallel with their increased relative abundance. More research is thus needed to explain the driving forces for changing food web dynamics in the Great Lakes.

Keywords: Great Lakes; Nitrogen-15; zooplankton

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1. Introduction

The St. Lawrence Great Lakes have a storied history of food web alterations and species invasions, some purposeful and others accidental. These have resulted in demonstrations of both top-down, or predation-driven, changes as well as bottom-up, or nutrient-driven, changes to food web dynamics. In the 1970s a landmark environmental lawsuit pitted the State of Illinois against the City of Milwaukee, Wisconsin in the shadow of nascent U.S. Environmental Protection Agency policy about water quality [1]. Competing theories about the causality of the lake trophic condition were debated in a U.S. District Court and were ultimately adjudicated by the U.S. Supreme Court based on a legal technicality without resolving the actual causality.

More recent species invasions by invertebrate planktivores as well as zebra and quagga mussels in Lakes Huron and Michigan have added further layers of complexity to interpretations of ecosystem dynamics, including the proposition that the upper lakes (Huron, Michigan and Superior) are now converging on similar lower food webs among all three [2]. Adding to the historical debates about biota-driven alterations is the recognition that climate change can exert powerful effects on these ecosystems [3], thus compounding the challenges for the forensic scientific investigation of food web dynamics.

The crustacean zooplankton communities of the Great Lakes have been changing in the 21st Century [4–11]. In Lakes Huron and Michigan, the non-predatory cladoceran as well as the cyclopoid copepod biomass have decreased [10]. The mechanisms underlying these changes are hypothesized to be a decline in phytoplankton abundance associated with the oligotrophication of Lakes Huron and Michigan and increased invertebrate planktivory [6,8,9]. The changes have been accompanied by an increased representation of *Limnocalanus macrurus* and reported declines in total zooplankton biomass. Moreover, the reported biomass of *L. macrurus* was likely underestimated by a factor of two [12,13].

Not only are lower food webs converging toward one another in the upper lakes, but some studies suggest that the trophic positions of some species have been shifting based on stable isotope analysis [14]. Jackson et al. [15] reported that both *L. macrurus* and large-bodied diaptomid species in Lake Huron appeared to have reduced their trophic position from the 1990s to 2009 based on reduced $\delta\ ^{15}N$ enrichment compared to primary herbivores by more than 1‰. The data from 2009, however, were based on a single opportunistic sample obtained from the North Channel, whereas the data from the 1990s came from stations in the main basin.

A variation in $\delta^{15}N$ with the trophic level is diagnostic of the relative trophic position among organisms that depend on the same primary source of organic matter [16]. Carnivores are isotopically heavier than herbivores, which in turn are isotopically heavier than primary producers. Omnivores are isotopically intermediate between herbivores and carnivores. The differences result from the kinetic fractionation of nitrogen isotopes. Ammonotelic organisms preferentially metabolize and excrete ^{14}N faster than ^{15}N, which leads to the differential retention of ^{15}N with respect to an organism's food source. An increase in $\delta^{15}N$ values of about 3.4‰ indicates a separation between trophic levels based on laboratory studies [17]. Trophic fractionation ($\Delta\delta^{15}N$ across trophic levels) can vary by taxon, habitat, and diet. By using a recognized herbivore such as *Daphnia* as a reference baseline, the error variance in the trophic position can be reduced to ±0.20‰ [18].

This study was undertaken specifically to test the theory that zooplankton trophic positions are shifting within the Great Lakes, using multiple recent (collected after 2009) samples from the main basin of Lake Huron. Additionally, we performed similar analyses to test for possible changes in the trophic position of representative zooplankton taxa in Lakes Michigan and Superior, using a decades-long archive of zooplankton collected from the 1980s to the 2000s. Lastly, we assessed historical zooplankton data from Lake Erie for comparison with the upper lakes.

2. Materials and Methods

2.1. Comparison of Live and Preserved Zooplankton

To evaluate the suitability of formalin-preserved animals for stable isotope analyses, live zooplankton were collected from Baseline Lake, MI, USA (43.425 N, 83.895 W) by vertical net tows on 20 and 28 June 2017. The lake was one of the first sites for the application of egg ratio analysis to calculate birth and death rates in the study of population dynamics of *Daphnia* and *Leptodora* [19]. The samples were transported to the laboratory within 2 h of collection. On 20 June, the live sample was immediately concentrated on Nitex netting, and individuals of *D. pulicaria* and *L. kindti* were picked by watchmaker forceps and placed in tared tin capsules, with five replicates of 40 *Daphnia* or 10 *Leptodora* per capsule. The samples were dried for 72 h at 55 °C, reweighed, and prepared for stable C and N isotope analysis. Additionally, residual *Leptodora* from the live sample were resuspended in lake water and then preserved with 5% sucrose formalin. After 72 h, those preserved animals were rinsed in reverse osmosis water and treated for stable isotope analysis in identical fashion to the live samples.

On 28 June, the live sample was split into two equal subsamples. One subsample was preserved with 5% sucrose-formalin and was set aside. The other (live) subsample was concentrated on Nitex netting from which five replicates of 20 *D. pulicaria* and five replicates of 10 *L. kindti* were picked by watchmaker forceps, followed by being treated as on 20 June. After 72 h, the formalin-preserved animals were rinsed in reverse osmosis water. Five replicates of 20 *D. pulicaria* and five replicates of 10 *L. kindti* were picked by watchmaker forceps and then treated the same as the live samples.

The samples were submitted to the Laboratory for Biotechnology and Bioanalysis at Washington State University, Pullman, WA, USA for C and N isotope analysis. As our results will demonstrate, ^{15}N data proved suitable but ^{13}C data did not. Further results were confined to nitrogen isotopes alone.

2.2. Analysis of Plankton Samples from the Great Lakes

All Great Lakes zooplankton samples were collected by 1 m diameter Puget Sound closing nets of 130 µm net apertures while the vessel lay at anchor. The samples were processed and analyzed identically with the methods used by [16]. We assembled a multi-year (1985 to 1997) time series of analyses for *D. mendotae* from a single reference station in southern Lake Michigan (Station M2: 43.000 N, 86.667 W, 100 m station depth) and examined the sample means for evidence of variation with time of day or long-term temporal trends. We supplemented this reference time series with additional samples of opportunity collected in 2009, 2013, 2014 and 2015 at other sites in Lakes Huron, Michigan and Superior.

Our preferred reference herbivore was *Daphnia mendotae*, but we also measured *D. pulicaria*, *D. retrocurva* and the non-daphnids *Bosmina longirostris*, *Holopedium gibberum* and veliger larvae. For trophic-level comparisons, we used mainly *Epischura lacustris*, *Diaptomus* (syn. *Leptodiaptomus*) *sicilis*, *Bythotrephes cederstroemi* and *Limnocalanus macrurus* plus occasional occurrences of *Senecella calanoides*, *Acanthocyclops vernalis*, *Mesocyclops edax*, *Polyphemus pediculus*, *Cercopagis pengoi*, *Leptodora kindti* and *Mysis relicta*.

In addition to stable isotopes, we quantified the mean dry mass and %N of our samples. We quantified the variability typical of the data to help judge the likely ecological significance of any statistical tests. When large numbers of comparisons are based on relatively small numbers of replicates, there is always a possibility of spurious conclusions. Standard deviations (SDs) or coefficients of variation (CVs: SD/mean) among replicate determinations were ln-transformed, and single factor analysis of variance (AOV) was applied to determine whether there were statistically significant differences among SDs or CVs based on two, three or four replicates. We next tested for an a priori expected negative correlation between CVs or SDs and the number of animals that were pooled to produce each replicate analysis across the observed range of pool sizes from 2 to 500.

We quantified the diurnal variability observed on 7 August 85 at three separate sampling times and compared it with the temporal variability observed across the full time series exclusive of 7 August 85. The mean values observed at each date and sampling time were ln-transformed prior to statistical comparisons. We also examined whether differences might exist among daphnid taxa drawn from different vertical strata on the same date and time. We used samples collected by the Puget Sound closing net (Research Nets, Inc., Bellevue, WA, USA) from 15–0 m, 40–15 m and 90–40 m shortly after midnight on 26 August 1986.

We continued the practice adopted by [16] of using *D. mendotae* as the preferred reference herbivore for imputing trophic-level comparisons whenever possible.

We next performed a statistical power analysis to define our criteria for ascribing ecological significance to statistically significant analytical differences between replicated samples. We established a balance between Type I and Type II errors by setting $\alpha = 0.1$ and $\beta = 0.75$. The object was to hold Type I errors reasonably low, while seeking a credible level of power to detect environmental differences if they indeed exist.

We estimated generic thresholds for ecologically significant differences between sample means (ΔX) as

$$\Delta X = \sigma(Z\alpha - Z\beta)/\sqrt{n} \quad (1)$$

where $Z\alpha$ and $Z\beta$ are values of the standard normal cumulative distribution under the specified power assumptions (1.28 and −0.67, respectively), σ is the standard deviation of the distribution of the variable being investigated and n is the sample size. For σ, we used the 90th percentile values for the SD of δ^{15}N (Table 1) to ensure that the variance would be well constrained and conservatively overestimated in most cases. For comparisons made between samples with three replicates each (85% of the cases), the mean differences between samples for δ^{15}N would have to exceed 0.54‰ to be regarded as statistically significant according to our power criteria. We reasoned that, otherwise, statistical vagaries stemming from small sample sizes and large numbers of comparisons could produce spurious instances of presumed statistical significance.

Table 1. Characteristic variability among replicates for elemental and isotope analyses.

Measurement	Metric	Mean (SE)	n	Range	90th Percentile
%N	CV	0.048 (0.004)	218	0.001–0.498	0.106
δ^{15}N	SD	0.213 (0.013)	219	0.000–1.114	0.481

For calculating lower and upper 95% confidence limits of SDs, we used conventional Excel™ functions:

$$\text{Lower limit} = \text{SD} \times \text{SQRT}((n - 1)/\text{CHIINV}((0.05/2), n - 1)) \quad (2)$$

$$\text{Upper limit} = \text{SD} \times \text{SQRT}((n - 1)/\text{CHIINV}(1 - (0.05/2), n - 1)) \quad (3)$$

For comparisons of trophic position among taxa across years and lakes, we subtracted the mean δ^{15}N of the putative herbivore, usually *D. mendotae*, from the δ^{15}N of each replicate alternative taxon, e.g., *L. macrurus*, to produce a Δδ^{15}N statistic following the method of [15]. The Δδ^{15}N values were compared by one-way AOV followed by post hoc Tukey's Honestly Significant Difference (HSD) test.

Geographic coordinates and station depths as well as all the original Great Lakes data used in this paper are included in a Supplemental Data File. Station locations are illustrated in Figure 1. Additional data have been deposited in an open access dataset as part of the University of Michigan Deep Blue data archive project [20].

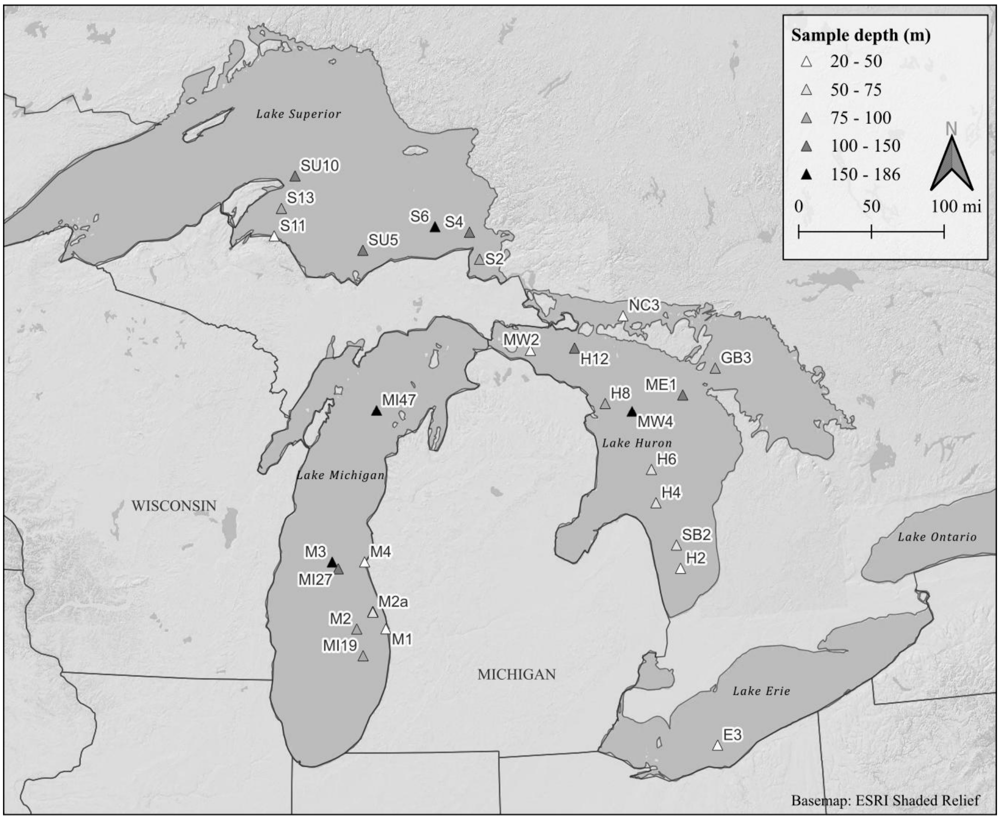

Figure 1. Locations of sampling stations referenced in this report.

3. Results

3.1. Comparison of Live and Preserved Zooplankton

We found differences in C and N isotope responses when comparing live and preserved zooplankton samples collected on 20 and 28 June 2017 from Baseline Lake, MI. The results of two-sample t-tests revealed that C isotope data from formalin-preserved samples were not representative of the live samples. The live versus preserved *Leptodora* from 20 June differed in %C ($p = 0.0007$), C:N ratio ($p = 0.0002$) and $\delta^{13}C$ ($p = 0.0004$). The live versus preserved samples from 28 June differed in %C for *Daphnia* ($p = 0.015$) but not for *Leptodora* ($p = 0.60$). However, the C:N ratios differed for both *Daphnia* ($p = 0.014$) and *Leptodora* ($p = 0.0002$), and the $\delta^{13}C$ differed for both *Daphnia* ($p = 0.0095$) and *Leptodora* ($p = 0.0002$) as well.

In contrast, $\delta^{15}N$ did not differ between live and preserved samples. The live versus preserved *Leptodora* collected on 20 June were not significantly different ($p = 0.13$) in $\delta^{15}N$. Likewise, the *Daphnia* and *Leptodora* collected on 28 June did not differ between live and preserved samples ($p = 0.28$ and $p = 0.08$, respectively). The %N content did not differ significantly for *Leptodora* on 20 June ($p = 0.07$) or 28 June ($p = 0.27$) or for *Daphnia* on 28 June ($p = 0.49$).

The standard errors of the mean ($SE = SD/\sqrt{n}$) compared to the mean differences revealed that the difference in $\delta^{15}N$ between *Leptodora* and *Daphnia* was 3.808‰ (SE = 0.053) on 20 June but only 1.952‰ (SE = 0.174) for live samples and 2.258 ‰ (SE = 0.069) for preserved samples on 28 Jun, which were not significantly different from each other ($p > 0.1$).

Based on these experiments we rejected the idea of using any carbon data from our archival Great Lakes formalin-preserved samples and restricted our analysis to N isotope data.

3.2. Analysis of Plankton Samples from the Great Lakes

After finding no differences ($p > 0.1$) among %N CVs calculated for two, three, or four replicates, grand means and associated statistics were calculated for the CVs (Table 1). For isotope data ($\delta^{15}N$), ln-transformed SDs were compared directly, and similarly, there were no statistically significant differences detected based on the number of replicates ($p > 0.1$), so grand means and associated statistics were calculated for the full dataset (Table 1).

For %N, the Pearson product-moment correlation coefficients (R) of CV against the number of animals pooled for each analytical sample were not significantly different from zero ($R = -0.01$). For $\delta^{15}N$, there was a small but statistically significant negative correlation between SD among the replicates and the number of animals pooled to produce each replicate ($R = -0.23$, $p < 0.001$, one-tailed test), which accounted for less than 5% of the variability. We ascribe the bulk of the measured variation among the replicate samples to random errors associated with sample preparation and instrument analysis.

3.2.1. Variations with Time of Day and Date

None of the variables reported in Table 2 displayed any pattern or trend with time of day or date (linear regressions, $p > 0.1$). Specifically, tests for a pattern with time of day for μgDW/ind produced $r^2 = 0.025$, and for $\delta^{15}N$, they produced $r^2 = 0.0002$. Tests for a pattern with date (1985–1997) for μgDW/ind produced $r^2 = 0.036$, and for $\delta^{15}N$, they produced $r^2 = 0.11$. There was, nonetheless, strong heterogeneity in the data overall based on one-way AOV for ^{15}N ($p < 0.0001$).

Although the numerical SD values for the full time series are greater than the SD values observed on 7 August 85 (n = 3), the 95% CI values for SD overlap so broadly that we cannot conclude that the variability observed over the years for *D. mendotae* is necessarily any greater than might be observed in a single day at a single site (Figure 2).

Table 2. Standard deviations (SD) of ln-transformed mean δ^{15}N observed diurnally on 7 August 85 compared to the full multi-year time series in Table 1, excluding 7 August 85.

Time Period	Analyte	SD	95% CI of SD
7 August 85	δ^{15}N	0.071	0.037–2.796
1985 to 2015	δ^{15}N	0.331	0.245–0.793

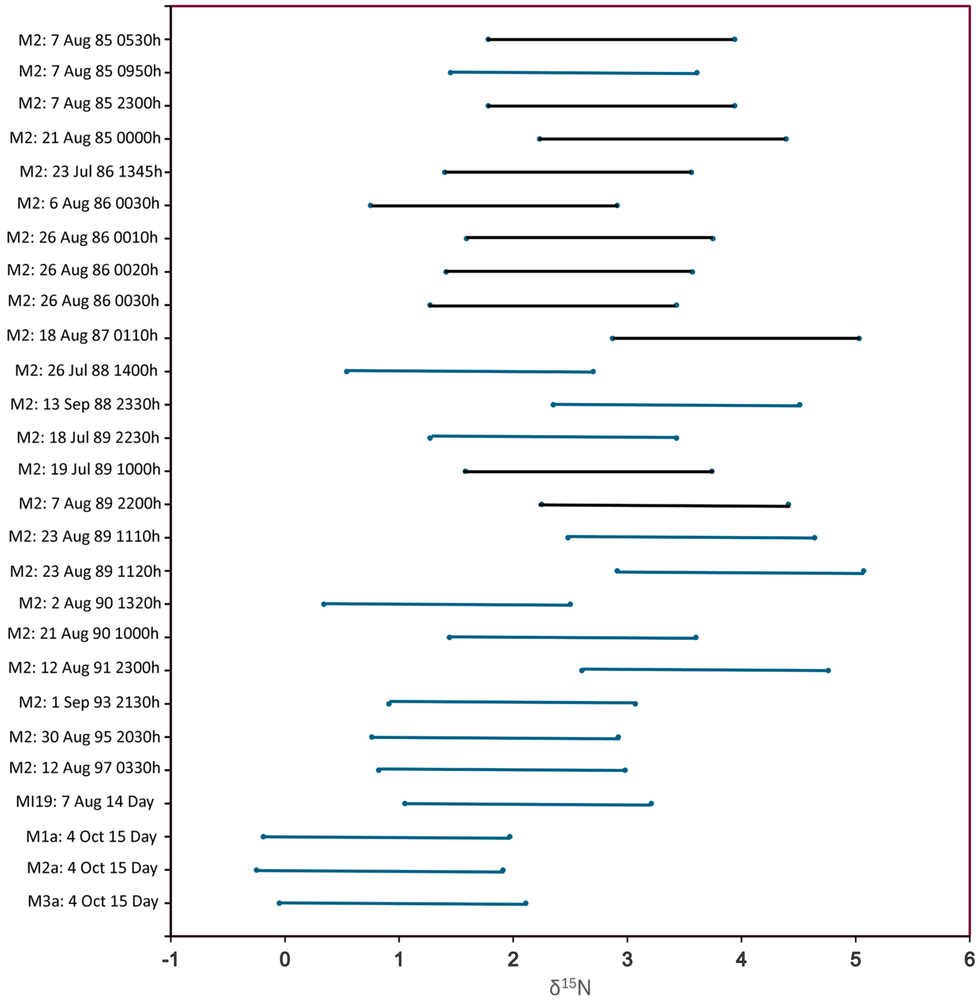

Figure 2. Isotope composition of *Daphnia mendotae* collected by vertical net tows from offshore southern Lake Michigan (M2, 100 m depth) from 1985 to 1997, from USEPA sampling station MI19 (92 m depth) in 2014 and from stations M1a, M2a and M3a (66 m depth) in 2015. Shown are the mean ±0.54‰.

3.2.2. Variations Among Sympatric Herbivores

The next question was whether there were significant differences among sympatric putative herbivores. Table 3 reports isotope data for three *Daphnia* species in Lake Michigan. In samples from 7 August 1985, when *Daphnia* species were abundant enough to obtain isotope signatures from all three, *D. pulicaria* measured nearly 1‰ δ^{15}N less positive than *D. mendotae*, more than the threshold (0.54‰) we identified with our statistical power analysis; for *D. retrocurva*, δ^{15}N was about 0.2‰ less than that of *D. mendotae*, which was less than the identified threshold. *D. pulicaria* did not appear in samples from later years, but in 1991, *D. mendotae* and *D. retrocurva* were indistinguishable with respect to ^{15}N at one nearshore station (M1), but *D. retrocurva* had 0.81‰ (SE = 0.09‰) elevated ^{15}N content compared to *D. mendotae* at nearshore station M4. The same was true of specimens collected in Lake Erie on 20 June 1995: *D. retrocurva* had δ^{15}N = 0.87‰ (SE = 0.13) elevated above *D. mendotae*.

Table 3. Isotope composition of sympatric *Daphnia* species collected by vertical net tows from a Lake Michigan reference station (M2, 100 m depth), from two nearshore stations (M1 and M4, 20 m depth) and from western Lake Erie (E3, 20 m depth). Values of $\Delta\delta^{15}N$ that exceeded our threshold criterion of ± 0.54‰ are printed in bold.

Date	Sta	z (m)	Taxon	n	µgDW/ind (SE)	$\delta^{15}N$ (SE)	$\Delta\delta^{15}N$ (SE)
7 August 85 (0530 h)	M2	10–0	***D. mendotae***	3	12.6 (0.3)	2.86 (0.03)	
			D. pulicaria	3	17.9 (0.4)	1.92 (0.01)	**−0.95 (0.04)**
			D. retrocurva	1	4.8	2.73	−0.14
7 August 85 (2300 h)	M2	10–0	***D. mendotae***	3	16.5 (0.5)	2.86 (0.03)	
			D. pulicaria	3	57.7 (1.5)	2.04 (0.01)	**−0.82 (0.03)**
			D. retrocurva	3	4.3 (0.6)	2.63 (0.07)	−0.23 (0.08)
26 August 86	M2	15–0	***D. mendotae***	3	9.5 (0.2)	2.67 (0.03)	
			D. pulicaria	3	33.7 (1.7)	2.82 (0.06)	0.15 (0.07)
			D. retrocurva	3	3.3 (0.04)	3.57 (0.10)	**0.90 (0.10)**
26 August 86	M2	40–15	***D. mendotae***	3	11.4 (0.5)	2.49 (0.09)	
			D. pulicaria	2	37.0 (4.1)	2.17 (0.21)	−0.32 (0.23)
26 August 86	M2	90–40	***D. mendotae***	2	7.5 (1.2)	2.35 (0.05)	
			D. pulicaria	3	20.1 (5.3)	2.05 (0.16)	−0.30 (0.18)
			D. retrocurva	1	7.6	2.12	−0.23
12 August 91	M1	15–0	***D. mendotae***	3	10.4 (1.1)	6.09 (0.12)	
			D. retrocurva	3	4.1 (0.3)	6.29 (0.03)	0.20 (0.13)
13 August 91	M4	15–0	***D. mendotae***	3	5.1 (1.4)	5.80 (0.07)	
			D. retrocurva	3	2.0 (0.1)	6.61 (0.06)	**0.81 (0.09)**
20 June 95	E3	15–0	***D. mendotae***	3	12.0 (0.1)	4.62 (0.10)	
			D. retrocurva	3	9.2 (0.3)	5.49 (0.07)	**0.87 (0.13)**

If sympatric daphnid species differed significantly from *D. mendotae* in ^{15}N content, the general pattern was that *D. pulicaria* was more negative, meaning less ^{15}N content, and *D. retrocurva* tended to deviate in the positive direction, meaning slightly elevated ^{15}N content.

Near contemporaneous samples revealed significantly higher ^{15}N content in *D. mendotae* from nearshore sites than offshore sites (Table 4), suggesting that the differences may trace to the seston they were consuming [14].

In comparisons between *D. mendotae* and other sympatric non-daphnid putative herbivores (Table 5), the results were not as consistent as were comparisons among *Daphnia* species. *Holopedium* tended to be more enriched in ^{15}N than *D. mendotae* (six of seven cases) in Lakes Superior and Huron. In a sample from Lake Michigan on 7 August 2014 in which *D. mendotae* co-occurred with *Bosmina longirostris* and dreissenid veligers, the three taxa were indistinguishable from each other based on our power analysis criteria.

Table 4. Isotope composition of *Daphnia mendotae* collected by vertical net tows through the entire water column from two offshore Lake Michigan stations and two nearshore stations.

Date	Sta	Lat (N)	Long (W)	z (m)	n	µgDW/ind (SE)	$\delta^{15}N$ (SE)
12 August 91	M2	43.000	86.667	100	3	10.2 (0.9)	3.68 (0.14)
13 August 91	M3	43.667	87.000	165	3	12.3 (0.2)	3.20 (0.03)

Table 4. *Cont.*

Date	Sta	Lat (N)	Long (W)	z (m)	n	µgDW/ind (SE)	δ^{15}N (SE)
12 August 91	M1	43.000	86.272	20	3	10.4 (1.1)	6.09 (0.12)
13 August 91	M4	43.667	86.555	20	3	5.1 (1.4)	5.80 (0.07)

Table 5. Isotope composition of *Daphnia mendotae* and sympatric non-daphnid presumptive herbivores in Lakes Superior, Huron and Michigan.

Date	Sta	Lat (N)	Long (W)	Taxon	n	µgDW/ind (SE)	δ^{15}N (SE)	$\Delta\delta^{15}$N (SE)
2 August 97	S11	46.915	87.843	*Daphnia*	1	7.3	−3.44	
				Holopedium	1	9.2	−2.37	1.07
2 August 97	S13	47.190	87.843	*Daphnia*	3	9.5 (2.4)	−2.07 (0.12)	
				Holopedium	2	13.3 (1.8)	−1.41 (0.15)	0.66 (0.19)
30 July 97	S2	46.667	84.867	*Daphnia*	3	6.4 (0.7)	−2.01 (0.10)	
				Holopedium	3	5.6 (0.3)	−1.55 (0.08)	0.46 (0.13)
31 July 97	S4	45.938	85.003	*Daphnia*	3	5.9 (0.2)	−1.78 (0.07)	
				Holopedium	3	4.6 (0.6)	−1.26 (0.07)	0.52 (0.10)
22 August 13	SU5	46.775	86.556	*Daphnia*	2	15.8 (2.2)	−1.76 (0.20)	
				Holopedium	3	27.9 (2.3)	−2.34 (0.08)	−0.58 (0.21)
22 August 13	SU10	47.514	87.546	*Daphnia*	1	6.9	−2.59	
				Holopedium	2	21.5 (1.5)	−0.75 (0.08)	1.83
9 October 09	MW2	45.745	84.180	*Daphnia*	3	13.3 (2.9)	2.17 (0.16)	
				Holopedium	3	24.7 (8.4)	3.74 (0.19)	1.57 (0.25)
7 August 14	MI19	42.733	86.583	*Daphnia*	3	8.8 (2.8)	2.13 (0.06)	
				Bosmina	3	2.6 (0.2)	2.37 (0.02)	0.24 (0.06)
				veligers	3	0.83 (0.03)	2.20 (0.06)	0.07 (0.08)

3.2.3. Differences Between Herbivores and Other Zooplankton Taxa

We next examined the differences in the isotope composition of herbivores, using *D. mendotae* whenever possible, and other members of the zooplankton community. The results for Lakes Erie (Table 6), Huron (Figure 3 and Supplemental Data Table S3), Michigan (Figure 4 and Supplemental Table S4) and Superior (Figure 5 and Supplemental Data Table S5) all revealed highly significant differences ($\Delta\delta^{15}$N) between putative herbivores and other taxa with $\Delta\delta^{15}$N values approaching 10‰, or three trophic levels, in some cases.

Table 6. Isotope composition of presumptive primary herbivores and sympatric taxa in Lake Erie (E3, 20 m depth) collected by vertical net tows (15–0 m) in 1995.

Date	Sta	Taxon	n	µgDW/ind (SE)	δ^{15}N (SE)	$\Delta\delta^{15}$N (SE)
20 June 95	E3	*D. mendotae*	3	12.0 (0.1)	4.62 (0.10)	
		Epischura	3	9.8 (0.8)	8.72 (0.13)	4.10 (0.16)
		Bythotrephes	4	69.8 (19.0)	8.12 (0.06)	3.50 (0.12)
		Limnocalanus	3	25.0 (1.5)	11.09 (0.04)	6.46 (0.11)
		Mesocyclops	3	5.7 (0.2)	10.68 (0.09)	6.05 (0.14)

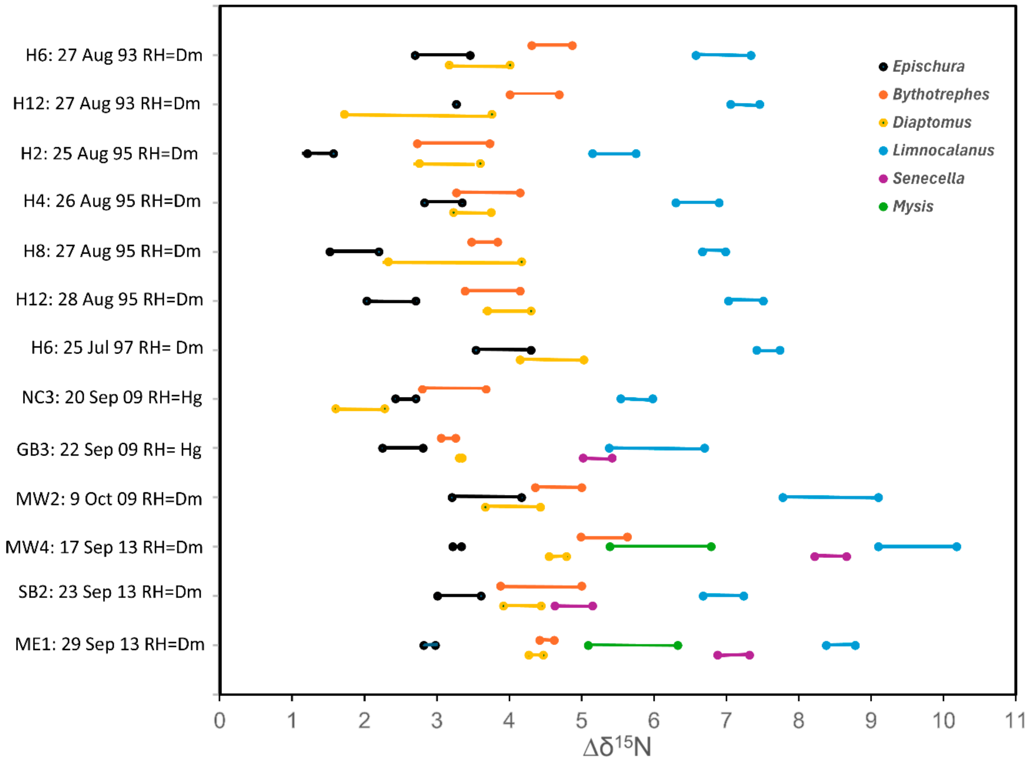

Figure 3. Lake Huron: Differences in ^{15}N content ($\Delta\delta^{15}$N) of the specified taxa from that of the reference herbivore (RH) from the corresponding station and date. Dm = *Daphnia mendotae*, Hg = *Holopedium gibberum*. Shown are means ±2SE.

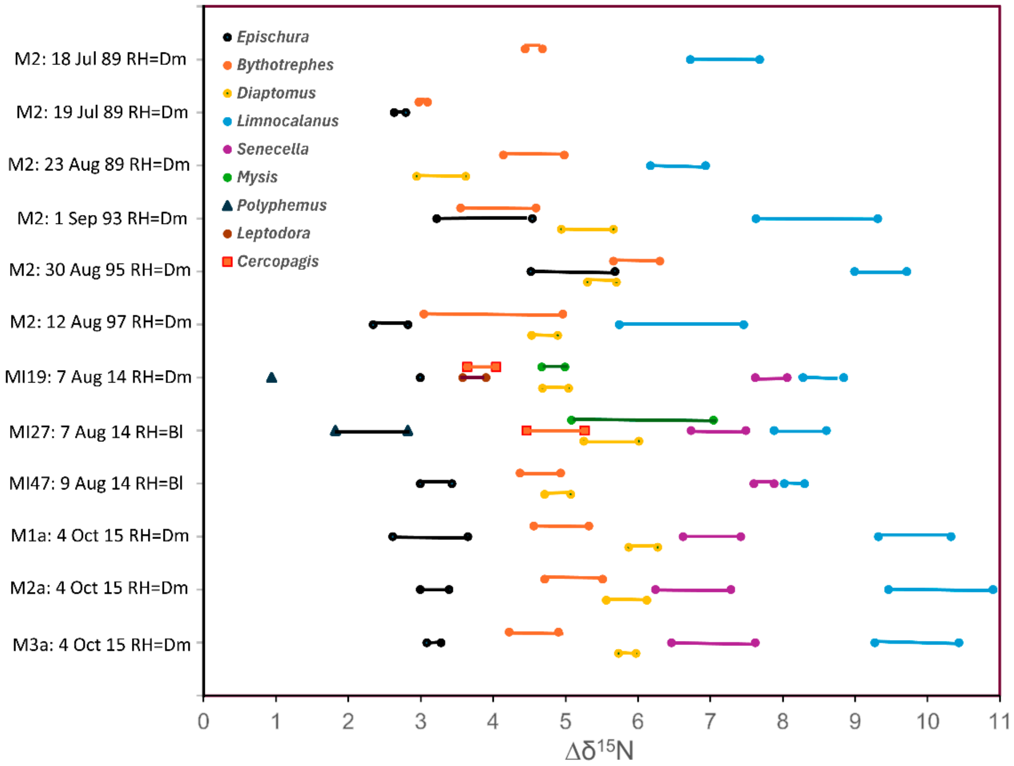

Figure 4. Lake Michigan: As in Figure 2; Bl = *Bosmina longirostris*.

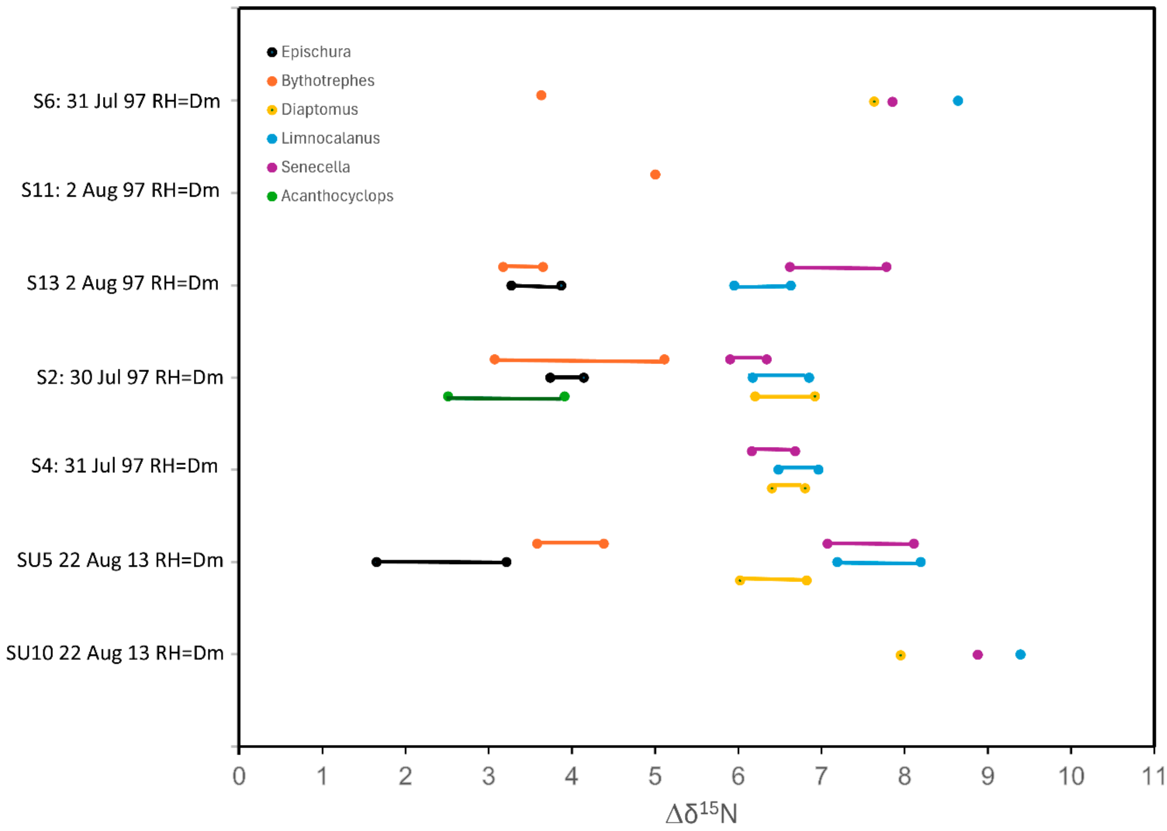

Figure 5. Lake Superior: As in Figure 2.

Zooplankton collections from the North Channel (NC3) and Georgian Bay (GB3) of Lake Huron in 2009 did not contain enough *D. mendotae* to obtain stable isotope samples, so *Holopedium* was used instead. However, comparing the δ^{15}N of *Holopedium* with the δ^{15}N of *D. mendotae* from the main body of Lake Huron that same year (Table 7), the *Holopedium* were significantly enriched with ^{15}N (*t*-test $p = 0.003$) by 1.57‰ on average. Consequently, those two stations were removed from the further analysis of trophic positions among sympatric taxa.

Table 7. Isotope composition of *Daphnia mendotae* and *Holopedium gibberum* from a station in Lake Huron (MW2: 45.745 N, 84.180 W, 42 m depth) collected by a vertical net tow on 9 October 2009.

Taxon	n	µgDW/ind	δ^{15}N
Daphnia	80	11.4	2.07
Daphnia	80	18.9	2.49
Daphnia	80	9.6	1.96
Holopedium	60	36.1	3.46
Holopedium	60	29.8	4.10
Holopedium	60	8.3	3.65

3.2.4. Comparisons with Lake Erie

We were able to compare the trophic positions of three taxa, *Bythotrephes*, *Epischura* and *Limnocalanus*, from Lake Erie in 1995 with the same taxa in Lakes Huron, Michigan and Superior during the 1990s. *Bythotrephes* was not significantly different from any of the upper lakes except Michigan in 1995 ($p = 0.001$), but only by 0.57‰, barely more than our threshold value of 0.54‰. Similarly, *Epischura* from Lake Erie were no different from the three upper lakes in any year except Michigan in 1997 ($p = 0.015$), when the Lake Erie animals were 1.51‰ richer in ^{15}N than those of Lake Michigan. The trophic position of *Limnocalanus* from Lake Erie differed only from that of Lake Michigan in 1993 ($p = 0.02$) and 1995 ($p = 0.001$), with animals from Lake Michigan being richer in ^{15}N by 2.00‰ and 2.88‰, respectively.

3.2.5. Comparisons Between 20th and 21st Centuries

We applied one-way AOV to $\Delta\delta^{15}$N values of individual taxa in Lakes Huron, Michigan and Superior (Figures 3–5) during two time periods: the 1990s, including 1989, and the 2000s. For *Epischura*, there were no significant differences among the lakes in the 1990s ($p = 0.26$) or the 2000s ($p = 0.33$), nor were there any significant differences among lakes for *Bythotrephes*, *Limnocalanus* and *Senecella* ($p > 0.3$ in all cases). *Diaptomus sicilis* proved to be an exception. There were strong differences among the lakes both in the 1990s ($p = 0.0003$) and in the 2000s ($p = 0.0007$). Tukey HSD comparisons revealed that Michigan and Huron were indistinguishable in the 1990s, but both were significantly different from Lake Superior ($p < 0.01$). In the 2000s, there were significant differences among all three lakes ($p < 0.05$ in all pairwise comparisons).

Finally, we tested the hypothesis that omnivores and predators in the upper Great Lakes have shifted in trophic positions from the late 20th Century to the early 21st Century (Table 8). The trophic position of *Bythotrephes* remained consistent in Lakes Michigan and Superior, as well as in four of six comparisons for Lake Huron. In the two cases where significant differences emerged, the δ^{15}N of *Bythotrephes* increased by slightly more than 1‰. *Epischura* reduced its trophic position with respect to *D. mendotae* in Lake Superior and in three cases out of eight in Lake Michigan but increased in two cases out of six in Lake Huron. In contrast, when changes were statistically significant, *Diaptomus sicilis*, *Limnocalanus* and *Senecella* all showed consistent increases in the trophic position with respect to *D. mendotae* in the upper lakes, with one exception (1995 vs. 2014 for *Limnocalanus* in Lake Michigan).

Table 8. Tukey's HSD pairwise comparisons of δ^{15}N in taxa measured in the 20th Century versus the 21st Century. ns = no significant difference. The magnitudes and directions of statistically significant changes in the ^{15}N content are reported as $\Delta\delta^{15}$N (‰).

Lake	**Years**	***Bythotrephes***		***Epischura***		***Diaptomus***		***Limnocalanus***		***Senecella***	
		p	$\Delta\delta^{15}$N	p	$\Delta\delta^{15}$N	p	$\Delta\delta^{15}$N	p	$\Delta\delta^{15}$N	p	$\Delta\delta^{15}$N
Huron	1993 vs. 2009	ns		ns		ns		ns			
	1995 vs. 2009	ns		0.001	1.51	ns		0.007	1.97		
	1997 vs. 2009	0.001	1.14	ns		ns		ns			
	1993 vs. 2013	ns		ns		0.001	1.18	0.039	1.28		
	1995 vs. 2013	0.001	1.09	0.001	0.99	0.001	0.93	0.001	1.92		
	1997 vs. 2013	ns		ns		ns		ns			
Michigan	1989 vs. 2014	ns		ns		0.001	1.85	0.001	1.42		
	1993 vs. 2014	ns		ns		ns		ns			
	1995 vs. 2014	ns		0.001	−1.96	ns		0.002	−1.03		
	1997 vs. 2014	ns		ns		ns		0.001	1.72		
	1989 vs. 2015	ns		ns		0.001	2.62	0.001	3.05		
	1993 vs. 2015	ns		0.02	−0.71	0.02	0.61	0.001	1.48		
	1995 vs. 2015	ns		0.001	−1.94	ns		ns			
	1997 vs. 2015	ns		ns		0.001	1.19	0.001	3.35		
Superior	1997 vs. 2013	ns		0.001	−1.33	ns		0.006	1.67	0.002	1.37

4. Discussion

Our results (Table 8) are inconsistent with the theory that large calanoid copepods may have improved their representation in the food webs of the upper Great Lakes by shifting their trophic position downward. In contrast, they show evidence of elevated carnivory. In light of the evidence displayed in Table 7, our original hypothesis [15] resulted from using *Holopedium* rather than *Daphnia mendotae* as the reference putative herbivore for zooplankton collections in the North Channel of Lake Huron in 2009.

We examined the isotope composition of sympatric *Daphnia* species as well as those of sympatric non-daphnids (Tables 3 and 5), recognizing that species-specific differences might exist based on the ingestion of non-algal protists from a common seston pool. Our results demonstrate that such differences can indeed occur, particularly for the cases of *Daphnia* and *Holopedium*. For this reason, we elected to use *D. mendotae* as our reference putative herbivore whenever its abundance made that possible. Previous work had demonstrated that using a single, consistent reference herbivore improves the precision of inferences about trophic position [18]. Other than our few analyses of *Mysis*, all the taxa we examined are holoplanktonic and therefore ultimately dependent on the local suspended seston pool. The seston composition can vary spatially, and those differences can be reflected in the isotope composition of the reference putative herbivore, as Table 4 demonstrates. We recognize that predators likely feed on multiple herbivores and omnivores. That diet breadth combined with the evidence of Tables 3 and 5 may introduce additional uncertainty in the trophic positions deduced from isotope fractionations. In the absence of certainty about quantitative diet composition, we chose to keep our reference herbivore species as consistent as possible in order to provide a baseline for future investigations.

The study most immediately relevant to ours was based on eight sampling dates between June and October 2011 and six sampling dates between May and November 2012 at an offshore site in Lake Michigan near Milwaukee, WI [14]. They reported that the mean δ^{15}N of *D. mendotae* was 3.4‰ enriched with respect to epilimnetic seston, and like us, they used *D. mendotae* as a representative herbivore to identify trophic positions. They did not measure other daphnids nor *Holopedium*, but they found no significant differences between *D. mendotae* and *B. longirostris*. Like us (Figure 3), they found *Epischura* to occupy the trophic position of the omnivore, but unlike us, they placed *Bythotrephes* as an omnivore as well. All our samples from Lake Michigan except for those for 19 July 89 registered *Bythotrephes* as a carnivore, which is consistent with its well-known feeding behavior. They likewise reported that the nitrogen isotope content placed *Leptodiaptomus* at the same trophic position as other known carnivores and that *Limnocalanus* was a full trophic level above it.

There was consistent evidence, with only one exception, that both *Bythotrephes* and *Limnocalanus* increased their trophic position by increased carnivory from the 1990s to the 2000s. *Diaptomus sicilis* likewise increased its trophic level, but the most striking finding about the species was that it was far more carnivorous, by almost one-half trophic level, in Lake Superior compared with Lakes Huron and Michigan in both time periods. Most if not all Diaptomidae are herbivorous in their juvenile stages, but omnivory can appear in the copepodid stages [14], and *D. sicilis* is known to prey on veligers [21]. All of the copepods we analyzed in this study were C6 adults, so evidence of carnivory is unsurprising.

Small but consistent differences in trophic level signatures existed among the daphnids *D. pulicaria*, *D. mendotae* and *D. retrocurva*, with *D. pulicaria* being slightly less enriched and *D. retrocurva* being slightly more enriched in ^{15}N than *D. mendotae*. *Holopedium gibberum* in sympatry with *D. mendotae* was consistently more enriched in ^{15}N by sometimes more than 1‰, confirming that *Holopedium* is not a strict herbivore [22], possibly by including ciliates in its diet.

Particularly notable in this study were the elevated trophic positions recorded for *D. sicilis*, *Limnocalanus* and *Senecella* from the 20th to 21st Centuries. These changes parallel the increased relative abundance of large-bodied zooplankton in the Upper Lakes [4–11]. The reason why an elevated trophic level position is correlated with an increased relative abundance of large-bodied Calanoida in the food webs of the upper Great Lakes, however, remains unresolved by this study but continues to be worthy of investigation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/limnolrev24040029/s1, Supplemental_Data.xlsx.

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