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Defining Seasonal Functional Traits of a Freshwater Zooplankton Community Using $\delta^{13}C$ and $\delta^{15}N$ Stable Isotope Analysis

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Abstract: Functional-based approaches are increasingly being used to define the functional diversity of aquatic ecosystems. In this study, we proposed the use of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes as a proxy of zooplankton functional traits in Lake Maggiore, a large, deep subalpine Italian lake. We analyzed the seasonal pattern of $\delta^{13}C$ and $\delta^{15}N$ signatures of different crustacean zooplankton taxa to determine food sources, preferred habitats, and trophic positions of species throughout one year. The cladocerans *Daphnia longispina galeata* gr., *Diaphanosoma brachyurum*, and *Eubosmina longispina* were grouped into a primary consumer functional group from their $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures, but while the former two species shared the same food sources, the latter exhibited a more selective feeding strategy. Cyclopoid copepods occupied a distinct functional group from the other secondary consumers, being the most ^{15}N enriched group in the lake. The $\delta^{15}N$ signature of calanoid copepods showed trophic enrichment in comparison to *Daphnia* and *Eubosmina* and linear mixing model results confirmed a predator-prey relationship. In our study, we have demonstrated that the use of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes represented an effective tool to define ecological roles of freshwater zooplankton species and to determine functional diversity in a lake.

Keywords: functional diversity; zooplankton; seasonality; stable isotope analysis; trophic interactions

1. Introduction

Functional-based approaches are increasingly being used to study aquatic ecosystems as an alternative to traditional taxonomy-based approaches. Functional diversity is a biodiversity measure based on the ecological role of the species present in a community. Species-specific functional traits, or "what they do" [1], allows species to be defined by their interactions within an ecosystem [2] in terms of their ecological roles and how they interact with the environment and with other species [3].

Many recent ecological studies [2,4,5] suggest the importance of species ecological roles, and not just the number of taxonomic species, in the relationship between biodiversity and ecosystem functioning. This is a central concept if we are to understand and predict the resilience of a community to perturbations. If two species are deemed to be functionally alike and to occupy a similar trophic niche [6,7], the loss of one of those species is not likely to have an impact on the resource pool, as the other will increase its activity accordingly. The loss of functional diversity in the ecosystem is mitigated, as the species lost does not possess unique functional traits. Thus, the sum of organism functional traits within an ecosystem can be said to represent an indirect measure of its functional diversity [1,8].

Although the importance of functional diversity is widely recognized, there is no consensus on how to quantify functional diversity within a community, as relationships between the various indices have not yet been established [9].

Freshwater zooplankton play a key role in aquatic ecosystems in the transferal of biomass and energy from phytoplankton to top predators, e.g., [10,11]. While studies on phytoplankton have increasingly highlighted the importance of functional traits and functional classification in ecological studies, only a few have attempted this approach with zooplankton. Barnett et al. [2] applied the measure of functional diversity [12] to crustacean zooplankton communities. Quantitative functional traits considered were the C:N ratio, mean body size, and preferred food size range, while qualitative traits described the preferred habitat, food selectivity, and trophic position of each species.

Analysis of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes is widely used to quantify food sources, trophic positions, and the interactions of organisms, as the $\delta^{13}C$ of a consumer can infer the assimilated source of dietary carbon [13,14] and $\delta^{15}N$ the trophic role [14,15]. Considering a functional-based perspective, $\delta^{13}C$ and $\delta^{15}N$ could identify ecological relations among taxa, such as competition and predation, ecological niche, habitat preference, and taxa redundancy, and help to define functional groups in an ecosystem. In this study, we propose the use of $\delta^{13}C$ and $\delta^{15}N$ stable isotope analysis to quantify some of the "qualitative functional traits" [2] for pelagic crustacean zooplankton taxa in Lake Maggiore, a large, deep subalpine lake in Italy. As species composition, diversity, and biomass of zooplankton can change significantly seasonally, especially in temperate lakes [16], the seasonal variation of $\delta^{13}C$ and $\delta^{15}N$ was used to define seasonal patterns in habitat preference, food sources, and taxa trophic position. This allowed an interpretation of taxa redundancy and a hypothesis of bottom-up and top-down mechanisms potentially driving the observed changes.

One use of stable isotopes is to determine the proportional contributions of several sources in a mixture. An example of a source proportion calculation includes the determination of various food sources in an animal's diet [17]. Linear mixing models are used to estimate proportions for two sources using isotopic signatures for a single element (e.g., $\delta^{13}C$), or for three sources using isotopic signatures for two elements (e.g., $\delta^{13}C$ and $\delta^{15}N$; [18]). In this study, we have used a linear mixing model [18,19] in order to discriminate the relative contribution of different preys in the diet of zooplankton consumers such as calanoid and cyclopoid copepods and the predatory cladoceran *Leptodora kindtii*. This further contributed to the understanding of food preference and taxa trophic position in the zooplankton community of Lake Maggiore.

2. Materials and Methods

Lake Maggiore (45°57′ N 8°32′ E 3°47′ W) is the second deepest (d_{max} 370 m) and largest (area 212.5 km^2, volume 37.5 km^3) subalpine lake in Italy. Being phosphorus-limited (TP_{max} ca. 10 $\mu g\ L^{-1}$), the lake is oligotrophic and has recovered from eutrophication of the late 1970s [20,21].

Except for September, vertical zooplankton hauls from the surface to a depth of 50 m were collected. This follows the standard routine sampling for deep subalpine lakes, in which samples are collected within the upper 50 m depth, as previous research on the vertical distribution of zooplankton showed that this is the water layer in which zooplankton live [22]. Monthly samples were collected from April to November 2009, when total zooplankton biomass was $\geq$3 $mg \cdot m^{-3}$, using a wide-mouth 450 µm mesh zooplankton net of diameter 0.58 m, filtering 13 m^3 lake water from three pelagic stations (G: 45°58′30″ N 8°39′09″ E, B: 45°54′28″ N 8°31′44″ E, L: 45°49′70″ N 8°34′70″ E) [16].

Zooplankton samples for the quantification of biomass were collected with a Clarke-Bumpus plankton sampler of a 126 µm mesh size and fixed in ethanol 96% to estimate the taxa-specific population density ($ind \cdot m^{-3}$) and standing stock biomass (dry weight, $mg \cdot m^{-3}$) [22]. Organisms for isotopic analyses were kept overnight in filtered (1.2 µm GF/C filters) lake water for gut clearance, before sorting into taxa and quantities suitable for isotopic analyses. The taxa analyzed were *Daphnia longispina galeata* gr., *Eubosmina longispina*, *Diaphanosoma brachyurum*, *Bythotrephes longimanus*,

Leptodora kindtii, adults of the calanoid copepods *Eudiaptomus padanus* and *Eudiaptomus gracilis*, and of the cyclopoid copepods *Mesocyclops leuckarti* and *Cyclops abyssorum*.

Samples were oven-dried for 24 h at 60 °C, before homogenizing and transferal into tin capsules of 5 × 9 mm in size. Depending on body mass, 50 to 700 individuals of each taxa were pooled to reach a minimum dry weight (DW) of 1 mg per sample. Three replicates of each taxa were run from each of the three sampling stations, as among-station differences were statistically non-significant ($p > 0.05$, Friedman Analysis of Variance, ANOVA test; [16]). The isotopic composition of organic carbon and nitrogen was determined from the analyses of CO_2 and N_2 by the G. G. Hatch Stable Isotope Laboratory at the University of Ottawa, Ontario, Canada, using a CE 1110 Elemental Analyser (Vario EL III manufactured by Elementar, Germany) and a DeltaPlus Advantage isotope ratio mass spectrometer (Delta XP Plus Advantage manufactured by Thermo, Bremen, Germany) coupled to a ConFlo III interface (Conflo II manufactured by Thermo, Bremen, Germany). The standard deviation of the analyses (SD) based on laboratory internal standards (C-55) was < 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$. Isotope ratios were expressed as the parts per thousand (‰) difference from a standard reference of PeeDee Belemnite for carbon and atmospheric N_2 for nitrogen:

$$\delta^{13}C, \delta^{15}N = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000 \quad (1)$$

where R is the isotopic ratio: $^{13}C/^{12}C$ and $^{15}N/^{14}N$.

Lipids can be $\delta^{13}C$ -depleted as a consequence of fractionation during lipid synthesis [23], which can lead to a misrepresentation of results as differences in predator-prey $\delta^{13}C$ could be greater than the expected 0.8‰ [24]. The C:N ratio was used as an indicator of lipid content. Invertebrates, including crustacean zooplankton, tend to have a C:N ratio of 4 [25], but C:N varies seasonally [26] and was as high as 7, so we used a revised version of the lipid normalizing procedure based on the C:N ratio [27], substituting the corrected parameters into Equations (2) and (3):

$$L = \frac{93}{1 + (0.246 \times (C \div N) - 0.75)^{-1}} \quad (2)$$

$$\delta^{13}C\prime = \delta^{13}C + D \times \left(I + \frac{390}{1 + \frac{287}{L}}\right) \quad (3)$$

where *L* is the proportion of lipid in the sample; C and N are the proportions of carbon and nitrogen in the sample, respectively; $\delta^{13}C'$ is the lipid normalized sample signature; $\delta^{13}C$ is the measured sample signature; D is the isotopic difference between the protein and lipid (7.018 ± 0.263); and I is a constant of 0.048 ± 0.013 [28].

Zooplankton $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures were referred to that of the pelagic baseline, which was expressed by the primary consumer, *Daphnia longispina galeata* gr. This choice came from previous stable isotope studies in Lake Maggiore [16], showing that *Daphnia*'s $\delta^{13}C$ signature in the different seasons was closely correlated with the signature of seston ($r = 0.86$; $p < 0.01$; N = 13), confirming that *Daphnia* was an appropriate proxy for the pelagic baseline against which the carbon isotopic signals of other zooplankton can be compared. $\Delta^{13}C$ was used to detect seasonal changes in taxa specific feeding behavior and assess the origin of carbon sources fueling the pelagic food web.

The carbon fractionation between consumer and resource ($F = \delta^{13}C_{cons} - \delta^{13}C_{diet}$) is ≤ 0.8‰ (±1.1‰ S.D.) [24]. The $\delta^{15}N$ of consumers has been shown to be enriched 2.55‰ [29] for zooplankton, and was used to assess seasonal change in taxa-specific trophic position (T), as a consumer's carbon signature is related to the baseline ($F \leq 0.8 \pm 1.1$) by:

$$T = (E/\lambda) + 2 \quad (4)$$

where λ is the stepwise enrichment, E = 2.55‰ [29], and 2 is the value commonly assigned to the deviation of primary consumers from the pelagic isotopic baseline. A trophic level of T = 3 indicates that a consumer is feeding on a primary consumer, whereas T = 4 suggests that there is an intermediate prey.

When $\delta^{13}C$ of the predator lies between that of two different prey taxa, suggesting a simultaneous use of both sources, the percent carbon contribution (p; q) of each prey to the predator's diet was calculated by the 2-endmember linear mixing model (2-em LMM), [18,19] as:

$$p = (\delta^{13}C_{predator} - \delta^{13}C_{prey2})/(\delta^{13}C_{prey1} - \delta^{13}C_{prey2}); q = 1 - p \quad (5)$$

where p and q are the relative contributions (%) of prey1 and prey2 carbon signatures to the predator $\delta^{13}C$ carbon signature ($\delta^{13}C_{predator}$).

When three potential prey sources were assessed, their isotopic signatures were partitioned by applying a 3-end member mixing model [18] to calculate the fractional contribution (p; q; z) of each of the three food sources to the predator's diet as:

$$p = ((\delta^{15}N_{prey3} - \delta^{15}N_{prey2})(\delta^{13}C_{predator} - \delta^{13}C_{prey2}) - (\delta^{13}C_{prey3}\text{-}\delta^{13}C_{prey2})(\delta^{15}N_{predator} - \delta^{15}N_{prey2}))/$$
$$((\delta^{15}N_{prey3} - \delta^{15}N_{prey2})(\delta^{13}C_{prey1} - \delta^{13}C_{prey2}) - (\delta^{13}C_{prey3} - \delta^{13}C_{prey2})(\delta^{15}N_{prey1} - \delta^{15}N_{prey2}));$$
$$q = ((\delta^{13}C_{predator} - \delta^{13}C_{prey3}) - (\delta^{13}C_{prey1} - \delta^{13}C_{prey3})p/(\delta^{13}C_{prey2} - \delta^{13}C_{prey3});$$
$$z = 1 - p - q \quad (6)$$

where p, q, and z are the relative carbon contribution (%) of prey (prey 1, 2, and 3). As required by the mixing model, δ^{13}Cpredator and δ^{15}Npredator were corrected for trophic fractionation, by weighting the isotopic signature of the prey against their percentage contribution to total biomass on each sampling date.

The software IsoError 04 (https://www.epa.gov/eco-research/stable-isotope-mixing-models-estimating-source-proportion) [18] was used to perform all 3-end member LMM calculations. Statistical analyses (Shapiro-Wilkinson W-test, Spearman-Rank correlation, Hierarchical Cluster Analysis) were performed using the software Statistica 12 (version 12, TIBCO Statistica Company, Palo Alto, CA, USA) and Sigmaplot 11.0 (version 11, Systat Software Inc., San Jose, CA, USA).

Cluster analysis of the seasonal variation in $\delta^{13}C$ and $\delta^{15}N$ for each taxa was performed with the software Sigmaplot 11.0. An Euclidean distance measure was used as the data were continuous [30].

3. Results

3.1. Seasonal Variation in $\delta^{13}C$ and the Determination of Consumer Resources

The seasonal variation of $\delta^{13}C$ for the different zooplankton species is shown in Figure 1. The variation of $\delta^{13}C$ in *Daphnia* was most depleted in spring, with a value of −36.3‰ ± 0.6 (SD), and became most enriched during the summer in August, with a value of −26.0‰ ± 0.1 (SD). *Diaphanosoma* was present in the lake in October and November and its $\delta^{13}C$ signature overlapped with a strong correlation (p = 0.06; R = 0.66) with the $\delta^{13}C$ signature of *Daphnia*. Also, the $\delta^{13}C$ signature of *Bosmina* overlapped with that of *Daphnia* in June, July, and October. This similarity in $\delta^{13}C$ indicates that herbivorous cladocerans are sharing the same type of resources. When more $\delta^{13}C$-depleted values were recorded for *Bosmina* (−32.0‰ ± 0.1, SD) than for *Daphnia* (−29.7‰ ± 0.1, SD), this might suggest a deviation in dietary sources over the winter months (November).

The $\delta^{13}C$ signature of copepods was more $\delta^{13}C$ -depleted than that of the pelagic baseline (annual mean of −26.4‰ ± 4.4, SD) all year round, with a $\delta^{13}C$ annual mean of −32.4‰ ± 4.6 (SD) for calanoid copepods and −34.5‰ ± 2.4 (SD) for cyclopoid copepods.

The procedure of lipid normalization for cyclopoid copepods decreased their average value of the $\delta^{13}C$ signature, but the relative values between species were unaffected. The seasonal trend of $\delta^{13}C$ signature for calanoid copepods generally traced the $\delta^{13}C$ pelagic baseline signature (F < 0.8). The $\delta^{13}C$

signature of *Bythotrephes* overlapped with the pelagic baseline (F < 0.8) from June to October, suggesting a tight dependence of this predatory cladoceran on pelagic food resources. However, in November, the $\delta^{13}C$ signature of *Bythotrephes* was less $\delta^{13}C$ -depleted (−26.8‰) and the least ^{13}C-depleted of all zooplankton taxa. The $\delta^{13}C$ signature of the other predatory cladoceran *Leptodora* was also related to the pelagic baseline (F < 0.8).

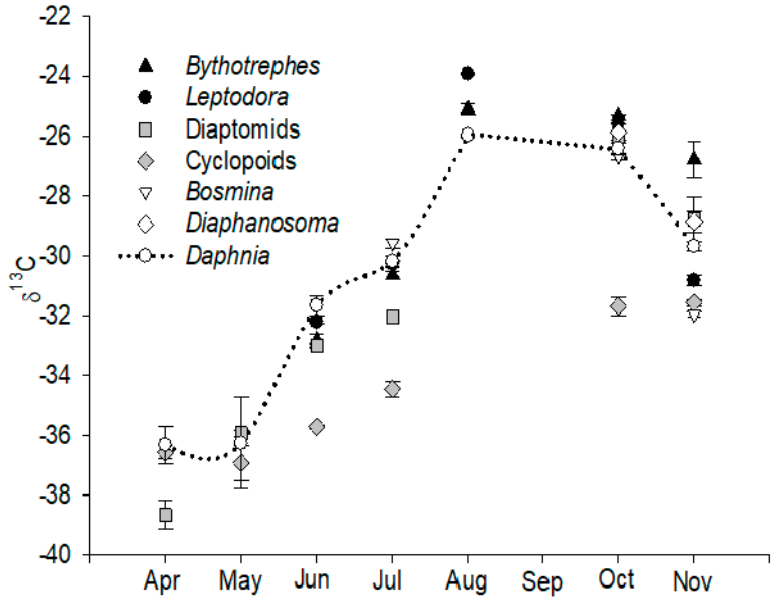

Figure 1. Seasonal changes in $\delta^{13}C$ signature (mean, ±SE) in pelagic zooplankton taxa of Lake Maggiore in 2009. White symbols refer to primary consumers, grey and black to secondary consumers, with the dotted line referring to the pelagic isotopic baseline.

3.2. Seasonal Variation in $\delta^{15}N$ and Determination of Trophic Levels

The $\delta^{15}N$ seasonal pattern was the same for all zooplankton taxa, with more N-enriched values in early spring and late autumn, and less $\delta^{15}N$-enriched values during the warm months (Figure 2). The seasonal variation in $\delta^{15}N$ was more pronounced in primary than in secondary consumers, as the predatory taxa *Bythotrephes*, *Leptodora*, and cyclopoid copepods had a small $\delta^{15}N$ range (NR) of 1.27‰, 1.32‰, and 1.74‰, respectively, whilst *Daphnia*, *Bosmina*, *Diaphanosoma*, and *Eudiaptomus* had a wider NR range of 3.75‰, 3.32‰, 3.57‰, and 2.50‰, respectively.

Cyclopoid copepods were the most $\delta^{15}N$-enriched group, with $\delta^{15}N$ ranging between 7.92‰ in October and 9.67‰ in May, and with a F_{max} of 6.6‰.

On average, predatory cladocerans were more $\delta^{15}N$-enriched than the pelagic isotopic baseline. The $\delta^{15}N$ signature of *Daphnia*, *Bosmina*, and *Diaphanosoma* overlapped (≤5‰), with $\delta^{15}N$ of the latter two taxa significantly correlated with *Daphnia* ($p < 0.001$; Spearman Rank Correlation coefficient R > 0.9; N = 8, 12, respectively).

Calanoid copepods had enriched $\delta^{15}N$ signatures, ranging from 5.81‰ in October to 9.38‰ in April. $\Delta^{15}N$ enrichment with respect to the pelagic baseline varied between a maximum of 4.45‰ in October and a minimum of 2.65‰ in April, suggesting a change in trophic feeding level and differential exploitation of food resources.

Using mixing models to quantify the contribution of different prey to predators' diet, the contribution of the zooplankton preys assimilated by the consumer did not always match the zooplankton prey biomass present in the lake (Figure 3a,b). For example, in July, the estimated proportion of *Daphnia* and *Bosmina* in the diet of calanoid copepods was 53.2% and 46.8%, respectively, when *Daphnia* was present in the lake with a biomass of 80%. In October and November, the diet of

calanoids copepods maintained a similar estimated contribution of *Daphnia* 57.7% and *Bosmina* 42.3%, when *Daphnia* was present in the lake with a biomass of 16.9% of total zooplankton biomass.

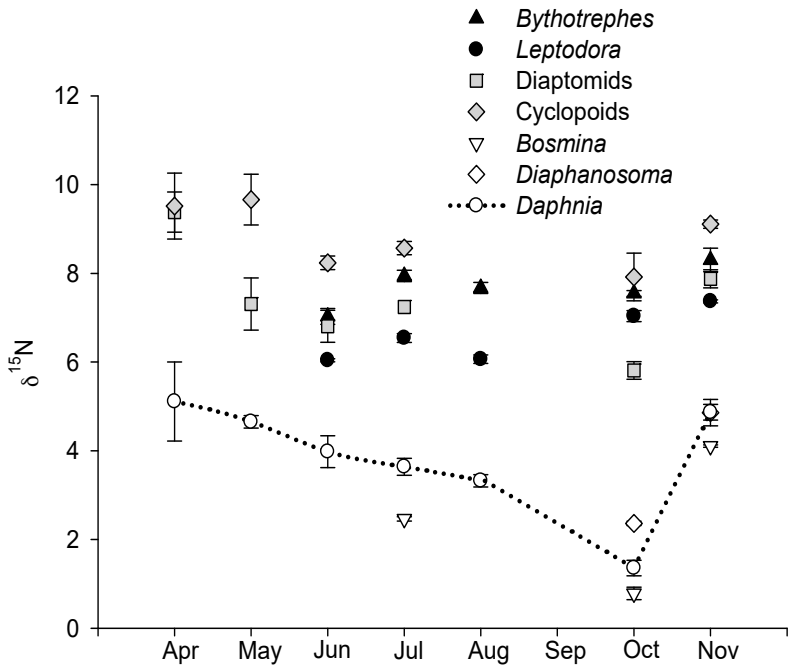

Figure 2. Seasonal changes in δ^{15}N signature (mean, ±SE) in pelagic zooplankton taxa of Lake Maggiore in 2009. White symbols refer to primary consumers, grey and black to secondary consumers, with the dotted line referring to the pelagic isotopic baseline.

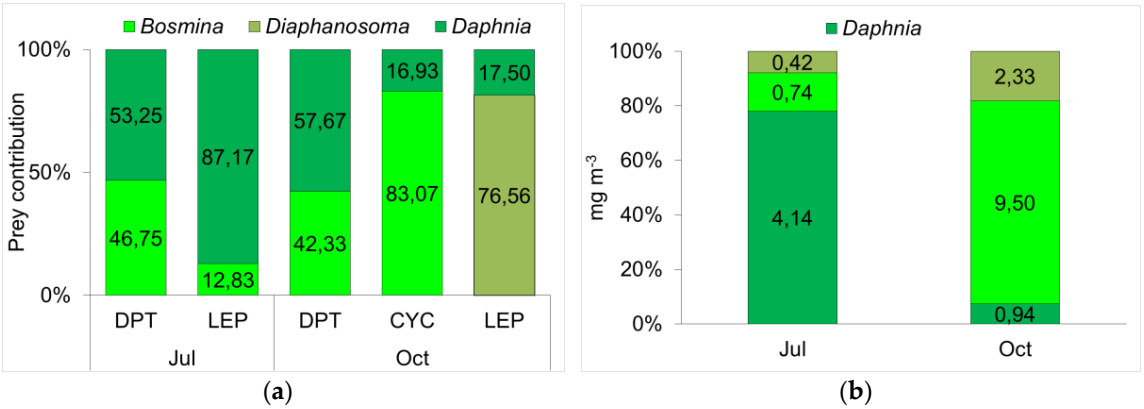

Figure 3. (**a**) Comparison between (**a**) contribution of different preys to the predators' diet calculated with the 2-em-LMM (JUL) and the 3-em-LMM (OCT-NOV); (**b**) biomasses (mg m^{-3}) of the potential preys in the sampling moment. Numbers within the bars correspond to calculated percentage values. DPT = calanoids; CYC = cyclopoids; LEP = *Leptodora*.

The diet of *Leptodora* in July was partitioned between *Daphnia* and *Bosmina* with 87.2% and 12.8%, respectively, while in autumn, the 3-linear mixing model indicated a higher consumption of *Diaphanosoma* (76.6%) than *Daphnia* (5.9%) and *Bosmina* (17.5%).

The diet of cyclopoid copepods in October and November was estimated by the mixing model to be 83.1% *Bosmina* and 16.9% *Daphnia*, when *Bosmina* was present in the lake, representing 75% of the total zooplankton biomass.

3.3. Determination of Functional Roles from $\delta^{13}C$ and $\delta^{15}N$

Cluster analysis of the seasonal variation in $\delta^{13}C$ for each taxon (Figure 4a) identified three major groups. The first split of the ordination separates the cyclopoid copepods from the other taxa, most likely as a result of the group utilizing deeper carbon sources in the pelagic zone. Lipid correction applied to $\delta^{13}C$ did not affect the seasonal pattern observed in the taxa. The second and further split in the cluster analysis grouped together the primary herbivorous cladocera *Daphnia longispina galeata* gr., *Diaphanosoma brachyurum*, and *Eubosmina longispina*, and the secondary consumers *Bythotrephes longimanus*, *Leptodora kindtii*, and calanoid copepods.

Cluster analysis of the seasonal variation in $\delta^{15}N$ for each taxon (Figure 4b) clearly grouped the taxa into two functional groups, the primary consumers, *D. longispina galeata* gr., *D. brachyurum*, and *E. longispina*, and the secondary consumers *B. longimanus*, *L. kindtii*, and copepods.

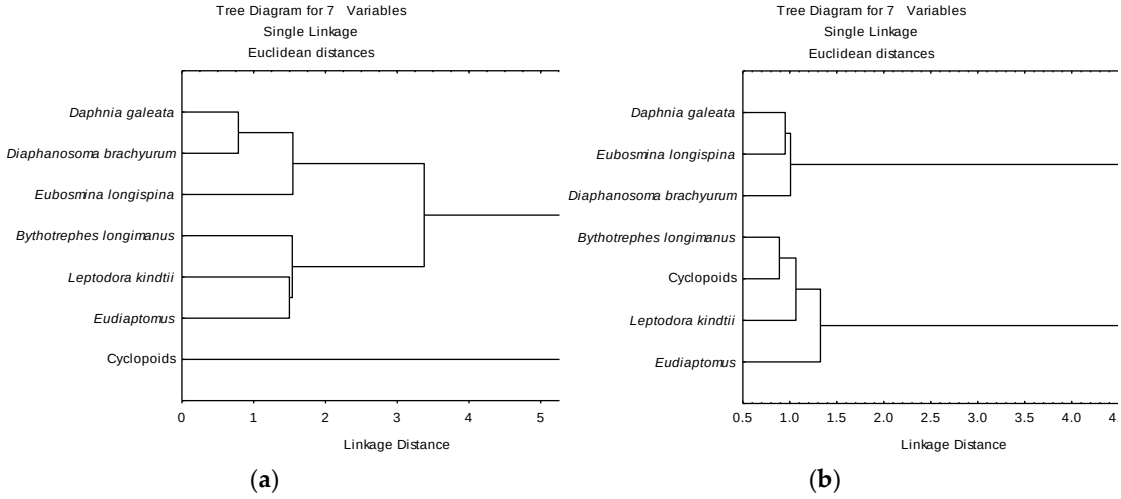

Figure 4. Cluster analysis of the seasonal variation of (**a**) $\delta^{13}C$ and (**b**) $\delta^{15}N$ for each zooplankton taxa using the Euclidean measure of distance.

4. Discussion

Functional biodiversity measures are based on the functional traits of the species in a community, rather than species richness, as it is interactions that determine the response of the ecosystem to a perturbation [31–33]. A methodology to determine the functional diversity within an ecosystem is quantifying the functional relationships between species as the distance measure of the branch length of the connecting functional dendrogram [12]. But it is not just the distance measure or clustering algorithm used that is important, it is the choice of which functional traits to include that is crucial [34].

In this study, we propose to use $\delta^{13}C$ signature as a proxy for determining habitat preference and foraging zone by inferring pelagic vs. littoral feeding preferences. We further analyzed the relationship between $\delta^{13}C$ taxa signature and phytoplankton succession during the studied year of 2009.

Confounding factors in a dynamic ecosystem like Lake Maggiore include seasonality and predation pressure variation. Seasonal changes in the littoral and pelagic $\delta^{13}C$ isotopic baseline were identified by a temporal shift towards less ^{13}C-depleted values in the summer, which is a trend commonly observed in thermally-stratified lakes [35,36]. In lacustrine systems, $\delta^{13}C$ and $\delta^{15}N$ of suspended particulate matter (seston) varies seasonally [37] because of differences in the allochthonous input, phytoplankton species composition, and primary productivity [38,39]. In Lake Maggiore, it has been demonstrated that $\delta^{13}C$ of the primary consumer *Daphnia longispina galeata* gr. tracks the isotopic composition of seston ($50 \leq$ size ≤ 126 µm) and of the pelagic baseline [16,35]. The choice of *Daphnia* as a useful isotopic baseline is supported by other studies [27], considering this taxa as a short-lived organism suited for fine scale temporal integration of pelagic $\delta^{13}C$ or $\delta^{15}N$ signatures.

If we consider the phytoplankton component of seston, there is variation in the fractionation of $\delta^{13}C$ between phytoplankton groups, with Chrysophycee and Bacillariophycee being more depleted in $\delta^{13}C$ than Cyanobacteria [37]. In Lake Maggiore, Bacillariophycee are the most dominant phytoplankton group with a high biomass throughout the year [37,40]. In the year of our study (2009), a peak in the Bacillariophycee biomass of *Aulacoseira* sp., *Asterionella* sp., and *Fragilaria* sp. was recorded in spring [37], when the cladoceran *Daphnia longispina galeata* gr. had the most $\delta^{13}C$-depleted values, while when Cyanobacteria biomass increased in the lake during the summer and autumn, *Daphnia longispina galeata* gr. had more $\delta^{13}C$-enriched values. This is suggestive of the opportunist nature of *Daphnia longispina galeata* gr., feeding on the most abundant component of the phytoplankton.

Rather than simply attributing organisms to different trophic groups per se from isolated values of $\delta^{15}N$, we analyzed the seasonal variation in $\delta^{13}C$ and $\delta^{15}N$ fractionation to identify changes in trophic interactions and feeding niche change. *Daphnia longispina galeata* gr., *Diaphanosoma brachyurum*, and *Eubosmina longispina* can be grouped into a "primary consumer" functional group from their $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures and known trophic interactions. Determination of functional groups is important and consensus is being placed on the importance of how species are likely to react to a perturbation, which is particularly crucial in considering the consequences of species loss. In the cluster analysis we performed, the grouping of the $\delta^{13}C$ and $\delta^{15}N$ isotopic signature of the herbivorous cladocerans *Daphnia longispina galeata* gr. and *Diaphanosoma brachyurum*, indicates a use of the same seasonal carbon pool. This is also confirmed by the similar values of the two cladoceran taxa in their $\delta^{13}C$ signature. The position of *Eubosmina* in the cluster analysis might show a slightly different use of food resources, a hypothesis confirmed by its $\delta^{13}C$ depletion values observed in November. These results indicate a deviation in dietary sources and a possible shift towards a more selective feeding strategy for phytoplankton [29,41] over the winter, as phytoplankton tends to be more $\delta^{13}C$-depleted than detritus [28,41,42].

A redundant species can be generically defined as one that co-exists with an overlapping functional role and trophic niche to other species [5] in a community.

Bythotrephes is a cladoceran known to actively predate on *Daphnia* [43], which was indicated by its trophic $\delta^{15}N$ enrichment in comparison to *Daphnia*. However, in our study, when *Daphnia* was scarce in the lake in October and November, *Bythotrephes* seemed to switch its feeding preference to *Diaphanosoma* as its prey. This diet switching from *Daphnia* to *Diaphanosoma* during periods of *Daphnia* scarcity was also observed for *Leptodora*, as the $\delta^{15}N$ of *Leptodora* did not indicate that they fed on calanoid or cyclopoid copepods, rather that they preferentially exploit cladocerans.

Thus, the results in our study might indicate that *Daphnia longispina galeata* gr. and *Diaphanosoma brachyurum* are redundant species in the zooplankton community of Lake Maggiore, as their trophic roles are overlapping and interchangeable. Our hypothesis is further supported by the well-known interspecific competition of these two taxa for the same food sources in lakes [44].

The $\delta^{13}C$ signature of calanoid copepods in our study was more depleted than the *Daphnia* $\delta^{13}C$ signature. This has been observed in previous studies on freshwater zooplankton [28,38,41], and has been attributed to the calanoid copepods omnivorous diet of seston and small zooplankton like *Eubosmina* and rotifers [45–48].

The $\delta^{15}N$ isotopic signature of calanoid copepods showed a trophic stepwise enrichment in comparison to *Daphnia* and *Eubosmina*, indicative of a predator-prey relationship. This was confirmed by the results of the linear mixing model [18], showing that calanoid copepods preyed on *Daphnia* and *Eubosmina* in similar percentages in spring and in autumn. Although herbivorous during some life stages, copepods are one of the main groups of invertebrate predators in limnetic and littoral inland waters [45].

In our study, cyclopoid copepods occupied a distinct functional group from the other secondary consumers, being the most ^{15}N -enriched zooplankton group in the lake during the year. We recorded highly $\delta^{13}C$ depleted values for cyclopoid copepods, and a high fractionation with respect to the pelagic baseline (F ~ 7.6), suggesting that cyclopoids may be relying upon deeper carbon sources

than those exploited by *Daphnia*. In deep lakes, the carbon isotopic signature is strongly influenced by depth, with organisms living in deeper layers characterized by more negative values than those living closer to the surface and in the littoral zone [42,49,50]. Moreover, in oligotrophic clear lakes, especially in periods of water column stratification, it is common to have a gradient of $\delta^{13}C$ POM (Particulate Organic Matter) with depth [51,52]. Matthew and Mazumder (2006) conducting a study in the oligotrophic Council Lake, recorded a $\delta^{13}C$ of POM decrease with depth in the water column, and zooplankton taxa at deeper depths also had a lower $\delta^{13}C$. Our hypothesis is reinforced by the cluster analysis with $\delta^{13}C$ values, which clearly show that cyclopoid copepods represented a distinct group, indicating a different utilization of carbon sources than other zooplankters, possibly found in deeper parts of the pelagic zone [49,53–55].

Because seasonal changes in the $\delta^{13}C$ isotopic baseline were identified by a temporal shift towards less ^{13}C depleted values in the littoral in the summer, this enabled the determination of the preferred foraging habitat of zooplankton taxa. The seasonal trend of cyclopoid copepods $\delta^{13}C$ values could also be related to the observed presence of the plankivorous fish roach (*Rutilus rutilus*) in the lake during the summer, as a predation-avoidance strategy by cyclopoid copepods from the increase in predator pressure in the pelagic. The $\delta^{13}C$ -enrichment observed in cyclopoid copepods in autumn may be explained by their return to the upper part of the pelagic zone when roach have migrated to the littoral zone to spawn [35].

The variation in $\delta^{15}N$ of *Bythotrephes* could also be related to the presence or absence of plankivorous fish in the pelagic zone, as they exhibit $\delta^{15}N$ depletion for the entirety of the period when plankivorous fish are feeding in the pelagic [35]. $\delta^{15}N$ enrichment increases when the predation-pressure decreases. As the variation of $\delta^{15}N$ between prey-predator decreases with niche occupation of increasing trophic levels, it is likely to be led by a top-down mechanism.

Cluster analysis with $\delta^{15}N$ seasonal variation clearly split the zooplankton taxa into two functional groups, the primary consumers, *Daphnia longispina galeata* gr., *Diaphanosoma brachyurum*, and *Eubosmina longispina*, and the secondary consumers, *Bythotrephes longimanus*, *Leptodora kindtii*, and the copepods. The secondary consumers were more ^{15}N -enriched than primary consumers. Cyclopoid copepods were feeding on the highest trophic level, which can be estimated to be 2–3 trophic levels higher than the primary producers, depending on whether a fractionation factor of 2.55 [29] or 3.4‰ [14] is used.

In this study, three or four trophic levels were identified in crustacean zooplankton of Lake Maggiore. In bio-magnification and energy and matter transfer in a pelagic food web, zooplankton is considered a crucial link between the primary producers and fish. In bio-energetic models, zooplankton are categorized as a "source" [56], or pooled into a singular grouping [57] of the trophic level of 2 [58,59], without taking into account species-specific traits or differences in life stage. Because the relative biomass of zooplankton taxa can significantly change during a calendar year [60], and because trophic positions of taxa and their relationships are dynamic during the year, we can conclude that freshwater zooplankton cannot be clustered together in the same ecological compartment. We propose the inclusion of seasonality and the dynamic of species trophic roles of zooplankton in the construction of models predicting bio-magnification capability.

In our study, we have demonstrated that the use of $\delta^{13}C$ and $\delta^{15}N$ stable isotope analysis represents an effective way to investigate the relationships present in the zooplankton community of a lake. In fact, the preferred habitat, food selectivity, and trophic position of each species could be defined. Through the use of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes as a proxy of zooplankton functional traits, we can gain a better understanding of the ecological roles of the zooplankton species in the lake and thus define the functional diversity of the ecosystem. Moreover, combining the use of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes with dietary analysis, we provided evidence that this could be an effective approach to infer functional groups, helping us understand the impact of functional differences in resource use. In particular, we have demonstrated that seasonal variations of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes indicated a dynamic process of change in the relationships among zooplankton taxa, according to the different

availability of food sources and of potential bottom-up and top-down (in particular fish predation) mechanisms present in the lake.

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References

1. Moss, B.R. *Ecology of Fresh Waters: Man and Medium, Past to Future*; John Wiley & Sons: Hoboken, NJ, USA, 2009.
2. Barnett, A.J.; Finlay, K.; Beisner, B.E. Functional diversity of crustacean zooplankton communities: Towards a trait-based classification. *Freshw. Biol.* **2007**, *52*, 796–813. [CrossRef]
3. Díaz, S.; Cabido, M. Vive la difference: Plant functional diversity matters to ecosystem processes. *Trends Ecol. Evol.* **2001**, *16*, 646–655. [CrossRef]
4. Tilman, D.; Knops, J.; Wedin, D.; Reich, P.; Richie, P.; Siemann, E. The influence of functional diversity and composition on ecosystem processes. *Science* **1997**, *277*, 1300–1302. [CrossRef]
5. Walker, B.; Kinzig, A.; Langridge, J. Plant attribute diversity, resilience, and ecosystem function: The nature and significance of dominant and minor species. *Ecosystems* **1999**, *2*, 95–113. [CrossRef]
6. Elton, C.S. *Animal Ecology*; University of Chicago Press: Chicago, IL, USA, 1927; p. 260.
7. Leibold, M.A. The niche concept revisited: Mechanistic models and community context. *Ecology* **1995**, *76*, 1371–1382. [CrossRef]
8. Petchey, O.L.; Gaston, K.J. Functional diversity: Back to basics and looking forward. *Ecol. Lett.* **2006**, *9*, 741–758. [CrossRef] [PubMed]
9. Mouchet, M.A.; Villeger, S.; Mason, N.W.; Mouillot, D. Functional diversity measures: An overview of their redundancy and their ability to discriminate community assembly rules. *Funct. Ecol.* **2010**, *24*, 867–876. [CrossRef]
10. Loseto, L.L.; Stern, G.A.; Ferguson, S.H. Size and biomagnification: How habitat selection explains beluga mercury levels. *Environ. Sci. Technol.* **2008**, *42*, 3982–3988. [CrossRef] [PubMed]
11. Forest, A.; Sampei, M.; Makabe, R.; Sasaki, H.; Barber, D.G.; Gratton, Y.; Fortier, L. The annual cycle of particulate organic carbon export in Franklin Bay (Canadian Artica): Environmental control and food web implications. *J. Geophys. Res. Oceans* **2008**, *113*, C03S05.
12. Petchey, O.L.; Gaston, K.J. Functional diversity (FD), species richness and community composition. *Ecol. Lett.* **2002**, *5*, 402–411. [CrossRef]
13. Peterson, B.J.; Fry, B. Stable isotopes in ecosystem studies. *Annu. Rev. Ecol. Syst.* **1987**, *18*, 293–320. [CrossRef]
14. Post, D.M. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. *Ecology* **2002**, *83*, 703–718. [CrossRef]
15. Minagawa, M.; Wada, E. Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between δ^{15}N and animal age. *Geochim. Cosmochim. Acta* **1984**, *48*, 1135–1140. [CrossRef]
16. Visconti, A.; Manca, M. Seasonal changes in the δ^{13}C and δ^{15}N signatures of the Lago Maggiore pelagic food web. *J. Limnol.* **2011**, *70*, 263–271. [CrossRef]
17. Szepanski, M.M.; Ben-David, M.; Van Ballenberghe, V. Assessment of anadromous salmon resources in the diet of the Alexander Archipelago wolf using stable isotope analysis. *Oecologia* **1999**, *120*, 327–335. [CrossRef] [PubMed]
18. Phillips, D.L. Mixing models in analyses of diet using multiple stable isotopes: A critique. *Oecologia* **2001**, *127*, 166–170. [CrossRef] [PubMed]

19. Phillips, D.L.; Gregg, J.W. Uncertainty in source partitioning using stable isotopes. *Oecologia* **2001**, *127*, 171–179. [CrossRef] [PubMed]
20. De Bernardi, R.; Giussani, G.; Manca, M.; Ruggiu, D. Trophic status and the pelagic system in Lago Maggiore. *Hydrobiologia* **1990**, *191*, 1–8. [CrossRef]
21. Guilizzoni, P.; Marchetto, A.; Lami, A.; Gerli, S.; Musazzi, S. Use of sedimentary pigments to infer past phosphorus concentration in lakes. *J. Paleolimnol.* **2011**, *45*, 433–445. [CrossRef]
22. Manca, M.; Ruggiu, L. Consequences of pelagic food-web changes during a long-term lake oligotrophication process. *Limnol. Oceanogr.* **1998**, *43*, 1368–1373. [CrossRef]
23. McCutchan, J.H.; Lewis, W.M.; Kendall, C.; McGrath, C.C. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos* **2003**, *102*, 378–390. [CrossRef]
24. DeNiro, M.J.; Epstein, S. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta* **1981**, *45*, 341–351. [CrossRef]
25. Matthews, B.; Mazumder, A. Temporal variation in body composition (C: N) helps explain seasonal patterns of zooplankton δ^{13}C. *Freshw. Biol.* **2005**, *50*, 502–515. [CrossRef]
26. McConnaughey, T.; McRoy, C.P. Food-web structure and the fractionation of carbon isotopes in the Bering Sea. *Mar. Biol.* **1979**, *53*, 257–262. [CrossRef]
27. Matthews, B.; Mazumder, A. Compositional and interlake variability of zooplankton effect baseline stable isotope signatures. *Limnol. Oceanogr.* **2003**, *48*, 1977–1987. [CrossRef]
28. Kiljunen, M.; Grey, J.; Sinisalo, T.; Harrod, C.; Immonen, H.; Jones, R.I. A revised model for lipid-normalizing δ^{13}C values from aquatic organisms, with implications for isotope mixing models. *J. Appl. Ecol.* **2006**, *43*, 1213–1222. [CrossRef]
29. Petchey, O.L.; Gaston, K.J. Dendrograms and measuring functional diversity. *Oikos* **2007**, *116*, 1422–1426. [CrossRef]
30. Petchey, O.L.; Hector, A.; Gaston, K.J. How do different measures of functional diversity perform? *Ecology* **2004**, *85*, 847–857. [CrossRef]
31. McCann, K.S. The diversity–stability debate. *Nature* **2000**, *405*, 228–233. [CrossRef] [PubMed]
32. Woodward, G.; Papantoniou, G.; Edwards, F.; Lauridsen, R.B. Trophic trickles and cascades in a complex food web: Impacts of a keystone predator on stream community structure and ecosystem processes. *Oikos* **2008**, *117*, 683–692. [CrossRef]
33. Petchey, O.L.; O'Gorman, E.J.; Flynn, D.F. A functional guide to functional diversity measures. In *Biodiversity, Ecosystem Functioning, and Human Wellbeing. An Ecological and Economic Perspective*; Oxford University Press: Oxford, UK, 2009; pp. 49–60. ISBN 9780199547951.
34. Visconti, A.; Volta, P.; Fadda, A.; Di Guardo, A.; Manca, M. Seasonality, littoral versus pelagic carbon sources, and stepwise 15N-enrichment of pelagic food web in a deep subalpine lake: the role of planktivorous fish. *Can. J. Fish. Aquat. Sci.* **2013**, *71*, 436–446. [CrossRef]
35. Poma, G.; Volta, P.; Roscioli, C.; Bettinetti, R.; Guzzella, L. Concentrations and trophic interactions of novel brominated flame retardants, HBCD, and PBDEs in zooplankton and fish from Lake Maggiore (Northern Italy). *Sci. Total Environ.* **2014**, *481*, 401–408. [CrossRef] [PubMed]
36. Lehmann, M.F.; Bernasconi, S.M.; Barbieri, A.; Simona, M.; McKenzie, J.A. Interannual variation of the isotopic composition of sedimenting organic carbon and nitrogen in Lake Lugano: A long-term sediment trap study. *Limnol. Oceanogr.* **2004**, *49*, 839–849. [CrossRef]
37. Caroni, R.; Free, G.; Visconti, A.; Manca, M. Phytoplankton functional traits and seston stable isotopes signature: A functional-based approach in a deep, subalpine lake, Lake Maggiore (N. Italy). *J. Limnol.* **2012**, *71*, 84–94. [CrossRef]
38. Grey, J.; Jones, R.I.; Sleep, D. Seasonal changes in the importance of the source of organic matter to the diet of zooplankton in Loch Ness, as indicated by stable isotope analysis. *Limnol. Oceanogr.* **2001**, *46*, 505–513. [CrossRef]
39. Vuorio, K.; Meili, M.; Sarvala, J. Taxon-specific variation in the stable isotopic signatures (δ^{13}C and δ^{15}N) of lake phytoplankton. *Freshw. Biol.* **2006**, *51*, 807–822. [CrossRef]

40. Morabito, G.; Sili, C.; Panzani, P.; Oggioni, A. Dinamica stagionale e distribuzione orizzontale di fitoplancton, carbonio organico e batterio plankton: Struttura dei popolamenti fitoplanctonici. In *Ricerche Sull'evoluzione del Lago Maggiore. Aspetti Limnologici. Programma Quinquennale 2008–2012*; Commissione Internazionale per la Protezione delle Acque Italo-Svizzere, 2009; pp. 53–66. ISSN 1013-8099. Available online: http://www.cipais.org/pdf/Limno_Maggiore_-_Rapporto_2009.pdf (accessed on 26 January 2018).
41. Grey, J.; Jones, R.I.; Sleep, D. Stable isotope analysis of the origins of zooplankton carbon in lakes of differing trophic state. *Oecologia* **2000**, *123*, 232–240. [CrossRef] [PubMed]
42. Jones, R.I.; Grey, J.; Sleep, D.; Quarmby, C. An assessment, using stable isotopes, of the importance of allochthonous organic carbon sources to the pelagic food web in Loch Ness. *Proc. R. Soc. Lond. B Biol. Sci.* **1998**, *265*, 105–110. [CrossRef]
43. Manca, M.; Vijverberg, J.; Polishchuk, L.; Voronov, D.A. *Daphnia* body size and population dynamics under predation by invertebrate and fish predators in Lago Maggiore: An approach based on contribution analysis. *J. Limnol.* **2008**, *67*, 15–21. [CrossRef]
44. Matveev, V.F. Effect of Competition on the Demography of Planktonic Cladocerans: *Daphnia* and *Diaphanosoma*. *Oecologia* **1987**, *74*, 468–477. [CrossRef] [PubMed]
45. Brandl, Z. Freshwater copepods and rotifers: Predators and their prey. *Hydrobiologia* **2005**, *546*, 475–489. [CrossRef]
46. Williamson, C.E.; Butler, N.M. Predation on rotifers by the suspension-feeding calanoid copepod *Diaptomus pallidus*. *Limnol. Oceanogr.* **1986**, *31*, 393–402. [CrossRef]
47. Kling, G.W.; Fry, B.; O'Brien, W.J. Stable isotopes and planktonic trophic structure in arctic lakes. *Ecology* **1992**, *73*, 561–566. [CrossRef]
48. Luecke, C.; O'Brien, W.J. The effect of *Heterocope* predation on zooplankton communities in arctic ponds. *Limnol. Oceanogr.* **1993**, *28*, 367–377. [CrossRef]
49. Vander Zanden, M.J.; Rasmussen, J.B. Primary consumer δ^{13}C and δ^{15}N and the trophic position of aquatic consumers. *Ecology* **1999**, *80*, 1395–1404. [CrossRef]
50. Vander Zanden, M.J.; Chandra, S.; Park, S.K.; Vadeboncoeur, Y.; Goldman, C.R. Efficiencies of benthic and pelagic trophic pathways in a subalpine lake. *Can. J. Fish. Aquat. Sci.* **2006**, *63*, 2608–2620. [CrossRef]
51. Cattaneo, A.; Manca, M.; Rasmussen, J.B. Peculiarities in the stable isotope composition of organisms from an alpine lake. *Aquat. Sci.* **2004**, *66*, 440–445. [CrossRef]
52. Post, D.M.; Pace, M.L.; Hairston, N.G. Ecosystem size determines food-chain length in lakes. *Nature* **2002**, *405*, 1047–1049. [CrossRef] [PubMed]
53. Quay, P.D.; Emerson, S.R.; Quay, B.M.; Devol, A.H. The carbon cycle for Lake Washington—A stable isotope study. *Limnol. Oceanogr.* **1986**, *31*, 596–611. [CrossRef]
54. Del Giorgio, P.A.; France, R.L. Ecosystem-specific patterns in the relationship between zooplankton and POM or microplankton δ^{13}C. *Limnol. Oceanogr.* **1996**, *41*, 359–365. [CrossRef]
55. Jeppesen, E.; Søndergaard, M.; Christoffersen, K.; Theil-Nielsen, J.; Jürgens, K. Cascading trophic interactions in the littoral zone: An enclosure experiment in shallow Lake Stigsholm, Denmark. *Arch. Hydrobiol.* **2002**, *153*, 533–555. [CrossRef]
56. Barwick, M.; Maher, W. Biotransference and biomagnification of selenium copper, cadmium, zinc, arsenic and lead in a temperate seagrass ecosystem from Lake Macquarie Estuary, NSW, Australia. *Mar. Environ. Res.* **2003**, *56*, 471–502. [CrossRef]
57. Paterson, M.J.; Rudd, J.W.; St. Louis, V. Increases in Total and Methylmercury in Zooplankton following Flooding of a Peatland Reservoir. *Environ. Sci. Technol.* **1998**, *32*, 3868–3874. [CrossRef]
58. Connolly, J.P.; Pedersen, C.J. A thermodynamic-based evaluation of organic chemical accumulation in aquatic organisms. *Environ. Sci. Technol.* **1988**, *22*, 99–103. [CrossRef] [PubMed]
59. Campfens, J.; Mackay, D. Fugacity-based model of PCB bioaccumulation in complex aquatic food webs. *Environ. Sci. Technol.* **1997**, *31*, 577–583. [CrossRef]
60. Morabito, G.; Mazzocchi, M.G.; Salmaso, N.; Zingone, A.; Bergami, C.; Flaim, G.; Accoroni, S.; Basset, A.; Bastianini, M.; Belmonte, G.; et al. Plankton dynamics across the freshwater, transitional and marine research sites of the LTER-Italy Network. Patterns, fluctuations, drivers. *Sci. Total Environ.* **2017**, submitted.