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Interpreting Seasonal Patterns and Long-Term Changes of Zooplankton in a Deep Subalpine Lake Using Stable Isotope Analysis

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Abstract: The purpose of this study was to elucidate the patterns and mechanisms driving seasonal and interannual variations of carbon and nitrogen stable isotopes in the zooplankton crustacean community of Lake Maggiore (Italy), during the period 2009–2020. Different zooplankton taxa and groups showed different ranges of $\delta^{13}C$ signatures, giving an insight into food sources and niche partition. In particular, cyclopoids had a restricted range with more negative $\delta^{13}C$‰ values and an increase in $\delta^{13}C$ fractionation with the establishment of water thermal vertical stratification, highlighting the importance of vertical distribution as a key factor for taxa coexistence in a vertically heterogenous environment. The $\delta^{13}C$ values of the zooplankton community and of *Daphnia* were positively related to water temperature ($R^2 = 0.58$ $p < 0.0001$ and $R^2 = 0.68$ $p < 0.0001$, respectively), and the $\delta^{13}C$ *Daphnia* signature was positively related to chlorophyll *a* ($R^2 = 0.32$, $p < 0.0001$). Decomposition of the time-series data for zooplankton carbon and nitrogen signatures and environmental parameters identified increasing trends in water temperature, chlorophyll *a* and water conductivity and a decrease in nitrate that matched changes in carbon isotopic signature trends in some zooplankton taxa (*Bosmina*, *Daphnia* and Diaptomids). Overall, the observed patterns in zooplankton isotopic signatures were interpreted as integrations of the effects of climate warming in Lake Maggiore, affecting both the availability of food sources and environmental conditions.

Keywords: freshwater zooplankton; stable isotope analysis; lake; climate change; food web

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1. Introduction

Lake ecosystems are very sensitive to climatic changes and are characterised by strong seasonal variability, determined by physical constraints in the temperature and light regime, controlling vertical mixing, nutrient cycling and, ultimately, primary and secondary production within pelagic waters [1]. Lakes are also subject to long-term changes determined by global climatic forcing, external inputs from catchments and by internal dynamics [2–6]. The effects of global climatic changes on terrestrial and aquatic ecosystems have become increasingly evident during the last two decades [7–9].

Worldwide climatic change has the potential to affect many fundamental properties of lakes, with the increase in temperature modifying numerous chemical and physical parameters of aquatic environments and, ultimately, their biotic communities [10,11]. Great efforts are required to understand the effects on aquatic communities, especially on population structure (e.g., biodiversity, density, biomass; [12–14]), ecosystem functionality (trophic relationships, redundancy, resilience; [15–18]) and on variation in the trophic position of organisms. The ecological role of an organism in an aquatic ecosystem is subject to seasonal fluctuations, depending on ecological variables such as variations of the population structure, food source availability, and chemical and physical environmental variables [19–22]. It is clear that each taxon is not a fixed entity within an ecosystem, but its ecological "role" and its relative relationships with other taxa vary over time. Consequently, trophic webs are not rigid schemes of organism trophic positions but should be considered as networks of dynamic interactions among different organisms.

The use of carbon and nitrogen stable isotope analysis (SIA) represents a useful way to identify dynamic interactions among aquatic organisms in trophic networks, since it makes it possible to determine their relative trophic positions and variations over time. Stable isotope analysis has been increasingly used in ecological studies since it provides a functional approach to the investigation of trophic webs and ecosystem functioning [21–25]. Providing an integrated measure over time of C and N signatures in the different trophic levels, stable isotope analysis allows the tracing of the path of environmental variability through the trophic web. Analysing stable isotopes over plurennial timeframes along with environmental variables provides an insight into the paths and mechanisms through which global changes, such as warming, are processed through the food web.

In this study, carbon and nitrogen isotope signatures (δ^{13}C‰ and δ^{15}N‰) of different zooplankton species (*Daphnia* sp., *Bosmina*, *Diaphanosoma brachyurum*, *Bythotrephes longimanus*, *Leptodora kindtii*) and groups (diaptomids, cyclopoids) were examined along with environmental variables during 12 annual production cycles (2009–2020) in Lake Maggiore, Italy. We aimed at detecting seasonal and inter-annual variability, as well as determining factors that might affect the long-term trend evolution of zooplankton δ^{13}C and δ^{15}N. To our knowledge, there are no other studies on carbon and nitrogen isotopes of freshwater zooplankton communities over such a long time period. Through the use of isotopic signatures, we also aimed to trace habitat preferences, food sources and the niche partition of zooplankton taxa.

Zooplankton is crucial for the transfer of matter and energy through the pelagic food web. The signature of δ^{13}C in zooplankton primary consumers reflect changes in their diet, therefore following seasonal baseline variations, which mainly depend on particulate organic matter in winter and early spring and on phytoplankton in late spring and summer [26–28]. A detailed seasonal study of plankton δ^{13}C and related variables in a dynamic lake system can provide important information for understanding the impact of environmental factors on the pelagic food web.

We therefore aimed at exploring the potential effects of climatic and environmental variables that could have driven inter-annual differences and long-term trends in stable isotopic zooplankton signatures. Among the environmental variables, we focused special attention on water temperature, which is expected to influence zooplankton isotopic signature [22,29] as it controls the solubility of carbon dioxide and, in turn, affects the fractionation of ^{13}C during phytoplankton uptake [30]. We also explored water column thermal stratification, known to influence cell activity; assimilation and competition for nutrients; light competition; the rate of CO_2 and HCO_3^- diffusion; and phytoplankton species succession, abundance and community structure. As water thermal stratification has such a strong effect on the phytoplankton isotopic carbon fingerprint [31], it follows that it should also influence zooplankton isotopic signatures. We also explored chlorophyll *a*, which has been found to have increased in recent years in Lake Maggiore due to an "eutrophication-like" effect of climate warming [32,33], as primary productivity and CO_2 concentration are known to affect carbon isotopic signatures. We investigated also changes in nitrogen concentration, as the nutrient cycling, particularly of nitrogen, affects isotope ratios [34].

There are two main objectives of this paper:

(1) to examine the seasonal and inter-annual variability of the isotopic signals of δ^{13}C and δ^{15}N in the different zooplankton groups and taxa.
(2) to investigate the long-term trends of zooplankton isotopic signals of δ^{13}C and δ^{15}N and their relationships with environmental variables.

This study represents a detailed seasonal analysis, along a decadal period (2009–2020) of freshwater zooplankton δ^{13}C and δ^{15}N signatures, in relation to patterns of environmental variables in Lake Maggiore (Italy). Because freshwater zooplankton represents a crucial link within lake food webs, their study can provide important information for understanding the impact of changes of environmental and climatic factors on pelagic food webs.

2. Study Site

Lake Maggiore is a deep oligomictic lake situated in the subalpine area in Northern Italy. It is an ideal environment to study the relationships between carbon and nitrogen stable isotopic zooplankton signatures and environmental variables because its limnology has been studied over the long term. Lake Maggiore recovered from eutrophication in the 1970s and 1980s through the abatement of external phosphorus loads and reached oligotrophic status by the end of the 1990s [35,36]. Since about 2008, the total phosphorous (TP) concentration in the epilimnion has remained stable, with low concentrations of 6 μg L^{-1}. Long-term studies have revealed a trend of decreasing concentrations in nitrate (NO_3^-) and total nitrogen (TN) in the lake since 2009, when the depletion of NO_3^- in the epilimnion started to be particularly evident, especially in the summer period, with concentrations below 500 μg L^{-1}.

Chlorophyll *a* concentration and phytoplankton biomass have confirmed the oligotrophication process of the lake from the mid-1980s to about 2005 (reduction of annual mean chlorophyll *a* concentration from 4.5–5.5 μg L^{-1} to 2.0–3.0 μg L^{-1}). However, since 2007, strong fluctuations of chlorophyll *a* values and annual mean values around 4.0 μg L^{-1} started to reoccur. Analysis of the phytoplankton community has shown marked interannual changes in biovolume in the last decade, with increases in some years (2011 and 2016) due to algal blooms during the summer months [37,38].

After reaching a stable trophic status, climatic drivers became more important in Lake Maggiore in moulding the dynamics of phytoplankton species fluctuations and the pelagic food web as a whole [5,12,39,40]. Long-term studies have shown how water temperature has increased significantly in Lake Maggiore [5,41,42]. The annual average values of the Schmidt stability have increased over time (MKT $p < 0.001$), confirming the increasing resistance of the water column to mixing. The mixing depth and mixed volume of water at spring vertical mixing in Lake Maggiore have been constantly declining in recent years due to the increasing thermal stability of the water column, an effect of climate change on deep lakes, and it is likely to continue in the near future [5,41,42].

The lake undergoes thermal stratification and a clear separation of epilimnetic and hypolimnetic waters (e.g., [43]). Water temperature vertical profiles (Figure A1) of the period investigated show between-year differences, with gradual warming of surface temperatures and a long-lasting thermal stratification, spanning from spring to full autumn [44]. A clear seasonality characterises the zooplankton population, with cyclopoid copepods prevailing in the winter months and cladocerans becoming increasingly more relevant during spring and summer until autumn (Figure A2).

3. Materials and Methods

3.1. Zooplankton Sampling and Isotope Analyses

Zooplankton sampling was performed in Lake Maggiore at Ghiffa station (45°58′30″ N; 8°39′09″ E, in correspondence with the maximum depth (370 m).

Samples were collected using a 58 cm diameter zooplankton net (450 μm mesh size) hauled vertically in the water layer 0–50 m (where at least 90% of total organisms are distributed [45]) several times, in order to obtain a sufficient amount of organisms needed to perform the analyses (min. = 2, max = 15), filtering a total volume of at least 26 m^3 per sample. The large net mesh sizes enabled high filtration efficiency, excluding clogging by large phytoplankton colonies, while ensuring collection of the most important taxa representative of the lake net zooplankton (i.e., the Cladocerans *Daphnia longispina-galeata* gr., *Bosmina*, *Diaphanosoma brachyurum*, *Leptodora kindtii*, *Bythotrephes longimanus*, as well as adults and the last developmental stages of Diaptomids and Cyclopoids). A large mesh size net was chosen also to avoid the smaller fraction of zooplankton population (i.e., nauplii, copepodites and rotifers) because of the difficulty to obtain algae-free samples in the amount required by the analysis. Samples were collected seasonally (January, May, August, November) in duplicate from 2009 to 2020. In vivo specimens from one sample were concentrated in laboratory into ca. 1 L of lake water, frozen at −20 °C and subsequently used for carbon (^{13}C) and nitrogen (^{15}N) Stable Isotope Analysis (SIA). The other sample was fixed and preserved in 96% ethanol for subsequent microscopic analysis and counting at 6.3x SIA was performed on pooled zooplankton samples of the size fraction $\geq$450 µm and on the taxa/groups present. We pooled individuals of each taxon under a dissecting microscope in order to reach a minimum dry weight (DW) of ca. 2 mg per sample (ca. 70–700 individuals depending on individual dry weight). Samples were oven-dried for 24 h at 60 °C before homogenizing and transferal into tin capsules of 5 mm × 9 mm in size. The isotopic composition of organic carbon and nitrogen was determined by Ján Veizer Stable Isotope Laboratory (Ottawa University, ON, Canada) following a prescribed method [46].

Since *Daphnia* sp. feeding behaviour has appeared to be non-selective, both in different seasons and in different lakes, many authors (e.g., [47]) have suggested using the isotopic composition of *Daphnia* sp. as representative of the pelagic baseline in lakes. Isotope signatures of crustacean taxa were examined separately as they may exhibit differing feeding behaviours and preferences [48]. Potential modifications in their food sources and in their relative trophic positions along time were investigated by comparing their stable isotopic compositions to that of *Daphnia* sp.

As suggested by the authors of [47], if a taxon depends exclusively on the pelagic baseline, its $\delta^{13}C$ value is comparable to *Daphnia*'s, with a residual variance lower or equal to that expected for the $\delta^{13}C$ enrichment per trophic level (S.D. = 1.3‰; [49]). A common carbon source is attributed when fractionation $F = \delta^{13}C_{\text{predator}} - \delta^{13}C_{\text{prey}} \leq 0.8‰$ ($\pm$1.1‰ S.D. [50]). Consequently, if an organism relies on one food type only, it will exhibit isotopic values similar to that of its food (adjusted for trophic fractionation), while organisms feeding on and incorporating different food items with different isotopic values will be characterised by a mean isotopic value, which reflects the different isotopic contributions in their diet (including fractionation).

In the case of comparing zooplankton taxa with *Daphnia* as the pelagic baseline, the fractionation is $F = \delta^{13}C_{\text{taxon}} - \delta^{13}C_{Daphnia}$.

Water temperature was measured at discrete depths with mercury-filled thermometers until 2010 [51]; later on, thermal profiles were recorded with a multi-parameter probe (Idronaut CTD 304, IDRONAUT S.r.l, Brugherio, Italy), with the two sets of measures being used to calibrate the two series of measurements. Lake water chemical analyses were performed by the chemistry laboratory of the CNR Water Research Institute (CNR IRSA Verbania Pallanza), using standard methods for freshwater samples [52,53]; more details can be found at http://www.idrolab.irsa.cnr.it/ (accessed on 27 August 2023). Integrated samples for chlorophyll *a* analysis were collected through the 0–20 m layer [39]. Chlorophyll *a* was measured by spectrophotometric technique in the period 1984–2009 [53], while, from 2010, measurements were taken using a vertical profiling instrument (FluoroProbe, bbe Moldaenke GmbH, Schwentinental, Germany) after a careful check of the comparability of the two methods.

3.2. Statistical Analyses

Statistical analysis and data visualization were performed with R software [54,55]. The decomposition of time-series data in R [55] used a classical seasonal decomposition through a moving average. Analyses were performed on both $\delta^{13}C$‰ and $\delta^{15}N$‰ data (2010–2020) of zooplankton taxa and groups. The analyses were also extended to the environmental parameters related to isotopic signatures. We carried out an ANOVA with Bonferroni post hoc tests (Datadesk Version 6.1) to determine the statistical significance of differences among the nitrogen and carbon isotopic signatures of zooplankton taxa and groups [56].

4. Results

4.1. Variations in δ^{13}C‰ and δ^{15}N‰ in Zooplankton Taxa

During the decadal period (2009–2020), the three filter-feeder cladocerans *Daphnia longispina-galeata* gr., *Bosmina* and *Diaphanosoma brachyurum* had comparable values of δ^{15}N‰ (mean values of 5.9 δ^{15}N‰, 4.8 δ^{15}N‰ and 4.2 δ^{15}N‰, respectively) and were not significantly different from each other in Bonferroni post hoc tests ($p \geq 0.68$) (Figure 1a). On some occasions, *Bosmina* δ^{15}N‰ was very low, with values approaching zero. The other zooplankton components were placed at higher δ^{15}N‰ values. The two predatory cladocerans (*Bythotrephes longimanus* and *Leptodora kindtii*) had comparable mean and median values of δ^{15}N‰ (although statistically significantly different, $p = 0.01$), with *Bythotrephes* having a wider range than *Leptodora* (range of δ^{15}N‰ = 17.3 against a range of δ^{15}N‰ = 8.3; median value of 8.6 δ^{15}N‰ and 7.2 δ^{15}N‰, respectively). The smallest range of variation for nitrogen isotopic signatures was found for Cyclopoids (range of δ^{15}N‰ = 5.2), which had similarly high/enriched values as Diaptomids (median was 9.1 δ^{15}N‰ for Diaptomids and 9.8 δ^{15}N‰ for Cyclopoids, not significantly different ($p = 1$)).

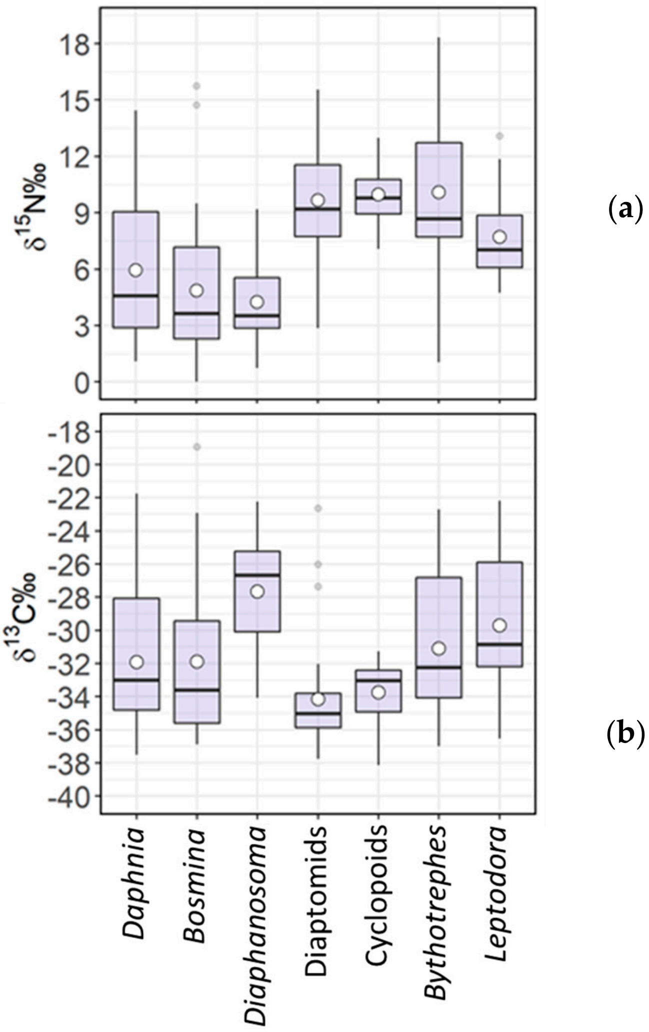

Figure 1. Boxplot of values (circles refer to means, horizontal bars to medians, boxes to upper and lower quartiles, vertical lines to ranges and dots indicate outliers) of δ^{15}N‰ (**a**) and δ^{13}C‰ (**b**) of crustacean zooplankton taxa and groups during the decadal period.

The smallest ranges of δ^{13}C (Figure 1b) were found for Diaptomids and Cyclopoids, both characterised by more negative values (mean of −34.2 δ^{13}C‰ for Diaptomids and mean of −33.8 δ^{13}C‰ for Cyclopoids) than the other zooplankton taxa. *Diaphanosoma* had the least negative δ^{13}C signature (mean of −27.5 δ^{13}C‰, median of −26.7 δ^{13}C‰, significantly different from all other taxa ($p \leq 0.03$), likely due to its limited presence during the year, preferring warm months, and its tendency to occupy superficial waters. *Daphnia* and *Bosmina* had similar δ^{13}C ranges and mean values (−31.8 δ^{13}C‰ and −31.9 δ^{13}C‰, respectively) and were not significantly different ($p = 1$) (Figure 1b).

The seasonality of carbon and nitrogen isotopic signatures of pooled zooplankton samples (≥450 µm), detected over the decadal time scale, is shown in Figure 2. Winter signatures are positioned in the top-left of the graph and are opposed to summer signatures, which lie in the lower right. Spring signature values extend into winter signals the left side of the graph but are characterised by lower δ^{15}N‰ values. Summer signature values had similar δ^{15}N‰ values as spring but differed substantially for the least depleted carbon signatures (δ^{13}C‰ between −29 and −22 for summer and δ^{13}C‰ −37 to −32 for spring). Autumn values, lying in the central part of the graph, have the largest between year variability (δ^{13}C‰ between −35 and −25) and tend to extend into the spring and summer areas.

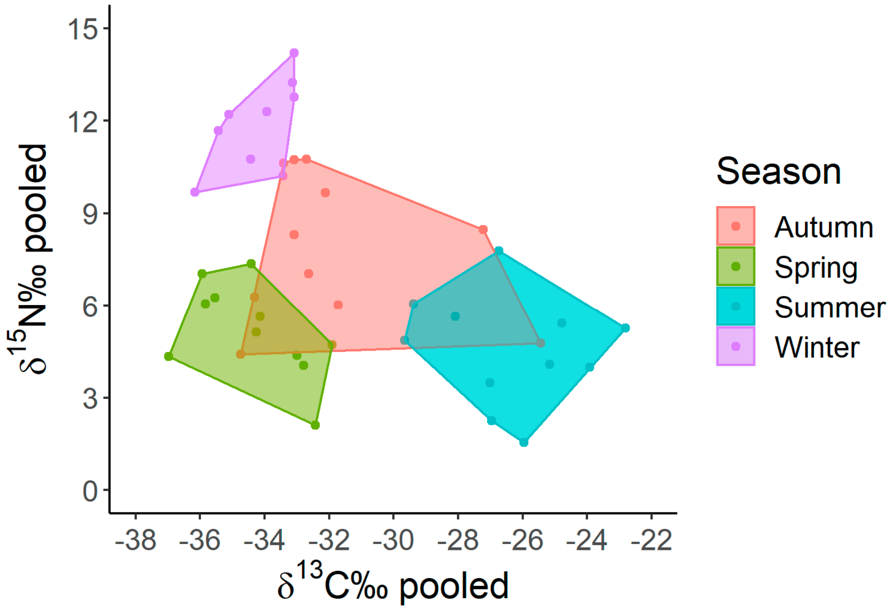

Figure 2. Trophochemical graph of carbon and nitrogen stable isotope signatures of pooled zooplankton samples (≥450 µm) measured in four seasons of the decadal period.

The large variation in the autumnal δ^{13}C signature resulted from less ^{13}C-depleted signatures of *Diaphanosoma*, *Bosmina* and the Diaptomids in October 2009 and of *Bythotrephes* and *Leptodora* in November 2010. Warm temperatures characterised the two above-mentioned months, and water temperature stratification was still present in autumn 2009 and 2010, with warmer temperatures around 16 °C down to 15 m depth.

The four areas characterising the decadal seasonality of isotopic signatures are better separated when only *Daphnia* is considered (Figure 3). Between-year variability is still larger for autumn than for the other seasons for both carbon and nitrogen isotopic signatures (δ^{13}C‰ between −35 and −28; δ^{15}N‰ between 3 and 10). The close correspondence between the isotopic values of pooled zooplankton and *Daphnia* reflects the major contribution of *Daphnia* to total zooplankton biomass throughout the year [57].

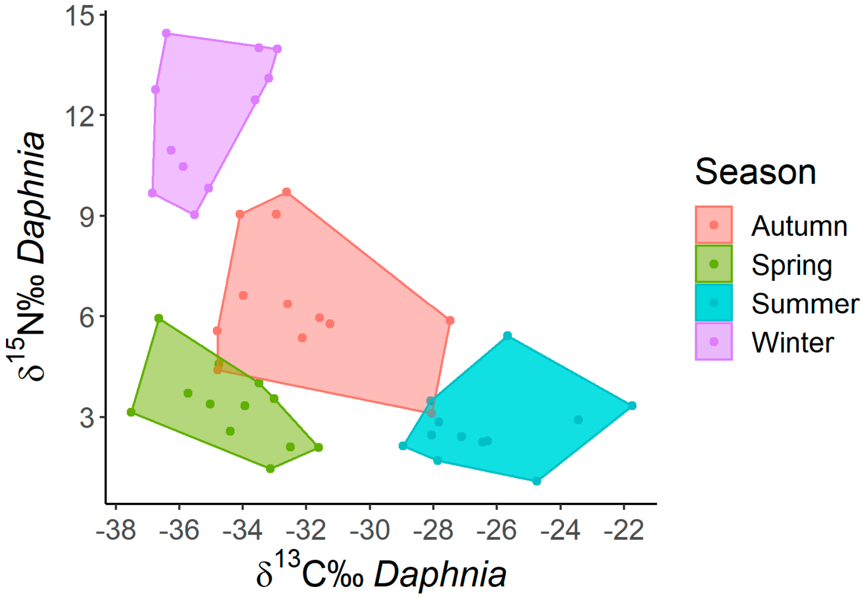

Figure 3. Trophochemical graph of *Daphnia* stable isotope signatures measured seasonally during the decadal period.

4.2. $\delta^{13}C$‰ and Environmental Variables

4.2.1. Temperature and $\delta^{13}C$‰ Fractionation

Carbon isotopic signatures of both pooled zooplankton and *Daphnia* were related to water temperature measured in the 0–50 m layer ($\delta^{13}C$ pooled vs. temperature 50 m: R^2 = 51% $p \leq 0.0001$ $n = 42$ and $\delta^{13}C$ *Daphnia* vs. temperature 50 m: R^2 = 61%, $p \leq 0.0001$; $n = 45$). This relationship improved when the temperature in the 0–20 m layer was used ($\delta^{13}C$ pooled vs. temperature 20 m: R^2 = 58%, $p \leq 0.0001$; $n = 42$ and $\delta^{13}C$ *Daphnia* vs. temperature 20 m: R^2 = 68.7%, $p \leq 0.0001$; $n = 45$).

Warmer temperatures corresponded to less ^{13}C-depleted zooplankton values, while colder temperatures were associated with the most ^{13}C-depleted signatures.

Between-year variability was large, and it reflected between-year variations in the temperature of the sampled layer, as well as differences among temperature vertical profiles. For example, in autumn, relatively higher surface temperatures (15.8 °C) down to 15 m depth and stronger stratification (temperature range of 9 °C in the layer 0–50 m) recorded in 2009 and, to a lesser extent, in 2019 (Figures A1 and A3) corresponded to the least negative values of *Daphnia* $\delta^{13}C$ (-28 to -27 $\delta^{13}C$‰), as shown in Figure 3. In contrast, lower autumn temperatures (11 °C) and lighter thermal vertical stratification (temperature range of 3 to 4 °C in the layer 0–50 m) in 2014 and 2015 corresponded to the most negative autumn values (-34 $\delta^{13}C$‰) on the left side of the graph (Figure 3).

Cyclopoids shared the same carbon signature as *Daphnia* during the winter, having close to zero fractionation ($F_{Cyclopoids} = \delta^{13}C‰_{Cyclopoids} - \delta^{13}C‰_{Daphnia}$; Figure 4). Fractionation gradually increased with warmer temperatures toward the summer and the progression of water thermal stratification.

The fractionation of cyclopoids increased substantially (from -4 to -10) at mean water temperatures $\geq$15 °C. The changes were quantitative, and a regression between $\delta^{13}C_{Daphnia}$ and $F_{Cyclopoids}$ was statistically significant (R^2 = 78%, $p < 0.0001$; $n = 44$).

The decomposition analysis for the carbon isotopic signatures detected marked seasonality for the zooplankton taxa and groups considered. Variation of the carbon isotopic signature in the seasonally decomposed trends allowed the observation of patterns and changes over the decadal period.

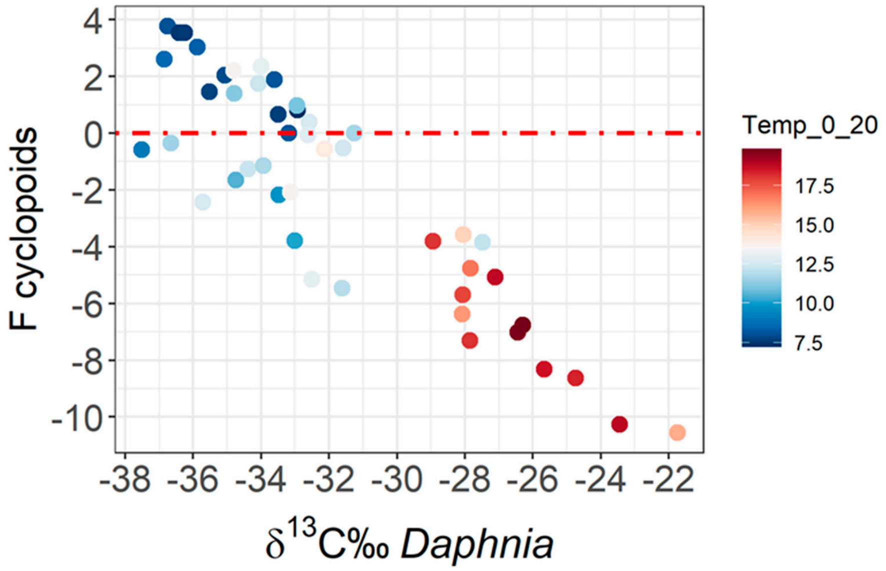

Figure 4. *Daphnia*'s carbon isotopic values vs. Cyclopoids' carbon fractionation (F).

4.2.2. Chlorophyll *a* and Nitrates

Chlorophyll *a* was related to the pool zooplankton δ^{13}C signature, indicating the influence of food availability (Figures 5 and A3). The linear relationship between chlorophyll *a* concentrations and *Daphnia* δ^{13}C signatures was statistically highly significant ($R^2 = 31.9\%$, $p < 0.0001$; $n = 41$).

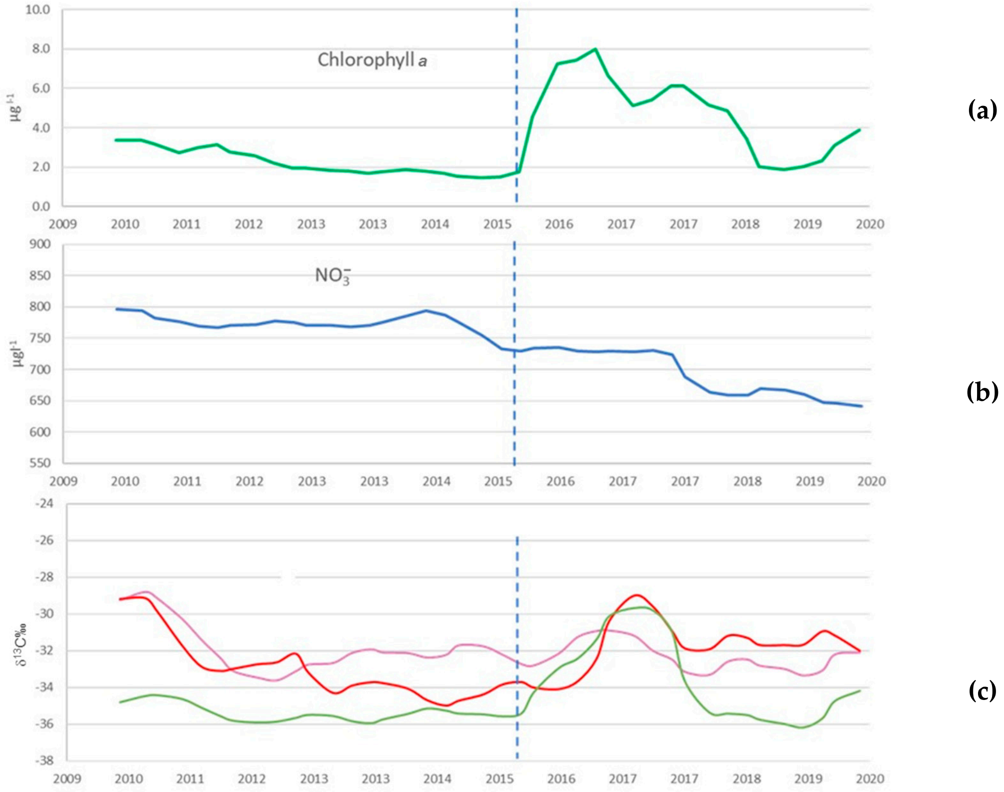

Figure 5. Comparison of seasonally decomposed trends of chlorophyll *a* ((**a**); dark green line) and nitrates ((**b**); blue line) with carbon isotopic signatures of selected zooplankton taxa (**c**): red line = *Bosmina*; green line = diaptomids; purple line = *Daphnia*), during the period 2009–2020; blue dotted line indicates the beginning of changes in environmental variables (see text for further explanations).

Seasonally decomposed trends clearly identified phases of changes in environmental variables (Figure A3); water temperature increased in the studied period, with a marked acceleration of the warming after 2015–2016. A gradual decreasing trend in nitrates (Figures 5 and A3) was detected up to 2015, followed by a sharper decline, with values falling below 600 μg L^{-1} measured in the epilimnion during the summer periods. The trend in chlorophyll *a* concentration initially showed a gradual decline until 2015, followed by a surge in concentration in 2016–2018 (Figure 5a), matching a phase of increase in the trend of water conductivity in the same period. Seasonally decomposed trends clearly identified phases of increased water temperature and a sharp acceleration of warming after 2015/2016 (Figure A3).

The carbon signature trend of *Daphnia* varied little in the decadal period, with the exception of a decline from less negative values during the first two years (2010–2012) of the studied period (Figure 5c). The trend in the carbon signature of *Bosmina* showed a gradual decrease from the beginning of the time period until 2015, followed by a steeper increase in 2016. The carbon signatures trend of diaptomids were quite stable at relatively depleted values (about −34‰) until 2016, when the signal started to increase toward less negative values (around −30‰ with extreme values up to −25‰) for a period of two years (2016–2018). Almost regular fluctuations in the carbon isotopic signature of the cyclopoids were observed (Figure A5), with carbon signatures varying in a relatively very small range, estimated between −38 and −32‰.

Our results for the δ^{15}N‰ *Daphnia* and δ^{15}N‰ *Bosmina* time-series decomposition (Figure A5) showed a common decreasing trend after 2014–2015, corresponding to the time of decrease in concentration in the epilimnion of the lake (while no decreasing or increasing trend in δ^{15}N‰ was detected for the other taxa and groups).

5. Discussion

In lake ecosystems, δ^{13}C% and δ^{15}N% values of suspended particulate matter (SPM) vary over time [31,58]. External loadings, phytoplankton species composition and primary productivity, as well as sources and concentrations of dissolved inorganic C and N, affect this variability [59,60], which is transmitted along the food chain to higher trophic levels, like zooplankton organisms, which are crucial in transferring matter and energy in pelagic food webs [47]. To date, most studies on the relationships between δ^{13}C variability and environmental variables are incomplete and have primarily focused on marine systems [29,30,61–65]. An analysis of the literature data revealed that carbon-stable isotopes of POM are related to lake latitudinal location, water temperature and CO_2 concentration, with no relationship with trophic state indices [66]. In contrast, an analysis of δ^{13}C and δ^{15}N of zooplankton in 18 reservoirs found correlations with Secchi depth, water temperature, nutrients and chlorophyll *a* [67].

The results of this study showed that carbon and nitrogen zooplankton signatures are ruled by a strong seasonality, which was maintained across the 12-year period considered. Trophochemical graphs (Figures 1 and 2) showed a similar pattern of seasonal partition when considering the pooled zooplankton community and *Daphnia*. The close correspondence between the isotopic values of pooled zooplankton and *Daphnia* reflects the major contribution of *Daphnia* to the total zooplankton biomass throughout the year. As proved in previous studies, the pooled zooplankton signature can be obtained from the sum of taxa specific isotope signatures weighted by taxa contribution to the pooled zooplankton biomass obtained on the same date [57].

5.1. δ^{13}C and Temperature

Carbon isotopic signatures of both pooled zooplankton and *Daphnia* were significantly correlated with water temperature for both water layers of 0–50 m depth and 0–20 m depth. The choice of these two water layers was determined by the vertical distribution of zooplankton in Lake Maggiore. Previous studies have shown that zooplankton only occasionally (e.g., water column mixing) inhabit water layers deeper than 50 m during daytime because of the unsuitable temperatures (too low) and food unavailability [45,68–70].

Warm temperatures corresponded to less ^{13}C-depleted values, while colder temperatures were associated with the most ^{13}C-depleted signatures. Temperature is an important determinant of seasonal changes in zooplankton $\delta^{13}C$‰ values [30,71,72]. Seasonal fluctuations of zooplankton $\delta^{13}C$‰ values generally reflect the phytoplankton ^{13}C fingerprint in lake ecosystems [60,73,74]. In monomictic lakes, a positive correlation between $\delta^{13}C$‰ values and temperature is often observed [28,31,46,60].

The solubility of CO_2 is controlled by water temperature and, in turn, influences the fractionation of carbon isotopes during phytoplankton uptake [30,59,72]. Particularly in late spring and summer, the carbon baseline signature reflects the contribution by phytoplankton [27,28]. Changes in phytoplankton community structure and functional groups are also related to the carbon isotopic signature [60], as temperature is also related to intrinsic changes of $\delta^{13}C$‰ values for zooplankters, due to the balance between carbon assimilation and respiration. In our study, values of $\delta^{13}C$‰ in zooplankters from −37 to −33.5 in winter corresponded to mean water temperatures (within the layer 0–50 m) below 8.5 °C; in summer, $\delta^{13}C$‰ values ranged between −28 and −24 with mean water temperatures above 12.5 °C.

5.2. Carbon Isotopic Signature in Different Taxa/Groups as Niche Indicators

Carbon isotopic signature is related to habitat and food sources. Food selectivity also can play a role, particularly as phytoplankton size and shape seem to be important in the seasonality of carbon isotopic signature in general [60]. The relationship between temperature and carbon isotopic signature is also indicative of the vertical distribution of the zooplankton taxa.

Several studies have supported the concept that carbon isotopic signature is affected by water depth [31,46,58,75]. In deep lakes, depth strongly influences the carbon isotopic signature of organisms living in deeper layers, which are characterised by more negative values than those living closer to the surface [76–78]. During the production year of a deep lake, SPM and zooplankton ^{13}C-signature increases with water warming and the onset of thermal stratification [28,46,60].

In our study, we observed differences in the ^{13}C signature between *Daphnia* and adult Cyclopoids, likely due to the differences in food sources and habitat preferences. The changes were quantitative, and the correlation between $\delta^{13}C$ *Daphnia* and the delta fractionation of cyclopoids ($F_{Cyclopoids}$) was statistically significant (Figure 4). Cyclopoids increasingly disengage from the water layer in which *Daphnia* feeds and lives when warming and vertical thermal stratification increase. However, the question remains whether food differentiation or habitat changes are more important in the resulting $\delta^{13}C$ signal of the two zooplankton taxa during the warmer months characterised by thermal stratification. However, it seems evident that there is a distinct separation of the cyclopoids from the predatory cladocerans, which closely align with *Daphnia*'s isotopic carbon signature, likely inhabiting and moving within more superficial and warmer zones of the pelagic. Another study in a deep lake of the same Italian lake district [79] drew similar conclusions, with cyclopoids likely occupying a different and deeper habitat (>20 m depth) than *Daphnia*. The authors of [80] conducted an experimental study in an oligotrophic lake in Japan and found that the population of a cyclopoid copepods (*Cyclops* sp.) inhabited the deep waters during the summer stratification but was scattered throughout the whole water column in the winter circulation period. Other studies [81,82] have found spatial segregation during summer stratification, with daphnids residing above the thermocline, whereas copepods were found at the lower edge of the thermocline.

Another explanation of the increasing variations between *Daphnia* and the Cyclopoids could result from their different life history strategies and metabolism. Some authors [83] have suggested that the $\delta^{13}C$‰ values of adult Cyclops respond more slowly to changes in the isotopic baseline because they have a lower carbon turnover rate compared with

Daphnia that quickly respond to variations in the $\delta^{13}C‰$ values of its phytoplankton food source. Such changes in the isotopic turnover between the herbivorous daphnids and predatory copepods has been confirmed in experimental studies [77] and is attributed to distinct life history strategies [84].

The least negative $\delta^{13}C$ signature of the filter-feeding cladoceran *Diaphanosoma brachyurum* was likely due to its limited seasonal presence during the warmest months of the year (i.e., usually absent in winter and spring samples) and its tendency to occupy superficial water. This habitat preference of *D. brachyurum* has been demonstrated in previous studies in Lake Maggiore [70], with species biomass increasing during the establishment of thermal stratification in the warmer, upper 10 m of the lake.

Littoral carbon sources have less ^{13}C-depleted signatures [46,85–88]. Seasonality has also been demonstrated in the littoral carbon signature, with the same pattern observed for the pelagic carbon signature, i.e., a transition from more (in winter) to less (in summer) negative $\delta^{13}C‰$ values. Previous studies on Lake Maggiore [57,60,87] have demonstrated that ranges of isotopic signatures measured in the same season were quite distinct for littoral and pelagic habitats, allowing a correct interpretation of data when speculating on the migration of zooplankters between different lake zones.

The two-particle feeder cladocerans *Daphnia* and *Bosmina* shared the same $\delta^{13}C‰$ range and similar values, indicating they share the same food sources and have similar mobility across lake habitats. The two taxa have, however, distinct feeding modes: *Daphnia* is a strict filter feeder, processing food particles through the filtering combs of the third and fourth thoracic limbs [89]. Whereas the feeding mode of *Bosmina* diverges significantly from filter feeding, permitting a high efficiency in the removal of edible flagellates, particularly when these items occur at a low density [90]. In our 12-year study, on some occasions, *Bosmina*'s $\delta^{13}C$ signature was less negative than *Daphnia*'s, likely reflecting a mobility through the water column of this small cladoceran and a tendency to feed in more superficial layers than *Daphnia*. The relatively low $\delta^{15}N$ signature of *Bosmina*, with values approaching zero during the summer in a few years, confirms the capability of this zooplankter to exploit nitrogen fixing cyanobacteria [79,87,90,91]. Cyanobacteria are usually lower in nitrogen isotopic signature than other phytoplankton groups, in particular nitrogen-fixing taxa [92–94] found in the phytoplankton community of Lake Maggiore [60]. The feeding mode of *Bosmina* avoids clogging during the growth of filamentous phytoplankton colonies, including those of cyanobacteria.

The range of variation of carbon isotopic signature (Figure 1b) was small for Cyclopoids and Diaptomids, indicating consistency of food sources throughout the studied period and that Cyclopoids and Diaptomids tend to be conservative in their habitat during the year. For Diaptomids, however, a more skewed distribution was observed toward less negative/depleted $\delta^{13}C‰$ values, suggesting an opportunistic feeding strategy with a discrete mobility across different zones or habitats of the lake. In particular, because less depleted values of $\delta^{13}C‰$ occurred during the summer periods, the migration of Diaptomids toward more superficial waters was considered [46].

Within the zooplankton community of Lake Maggiore, a narrower range of variation for nitrogen isotopic signatures (Figure 1a) was found for Cyclopoids, which, combined with their small range of carbon isotopic signatures, suggests an overall niche specificity of this zooplankton group. Enrichment with respect to the pelagic baseline, however, cannot be calculated, being linked with a carbon isotopic signature restricted to the unstratified thermal phase.

The conceptual diagram represents a summary of all the above-described variations in food sources, habitat preferences and seasonal variations of the zooplankton taxa considered in our study in Lake Maggiore (Figure 6). The diagram clearly illustrates how the relative $\delta^{13}C‰$ values of zooplankton taxa are collocated with respect to each other and to vertical lake depth in the different seasons. In particular, it illustrates the shift of $\delta^{13}C‰$ of different zooplankton taxa during two very different times of the year: late winter/springtime, when water column circulation generally occurs in the lake, and summertime, when stratification takes place (Figure A1).

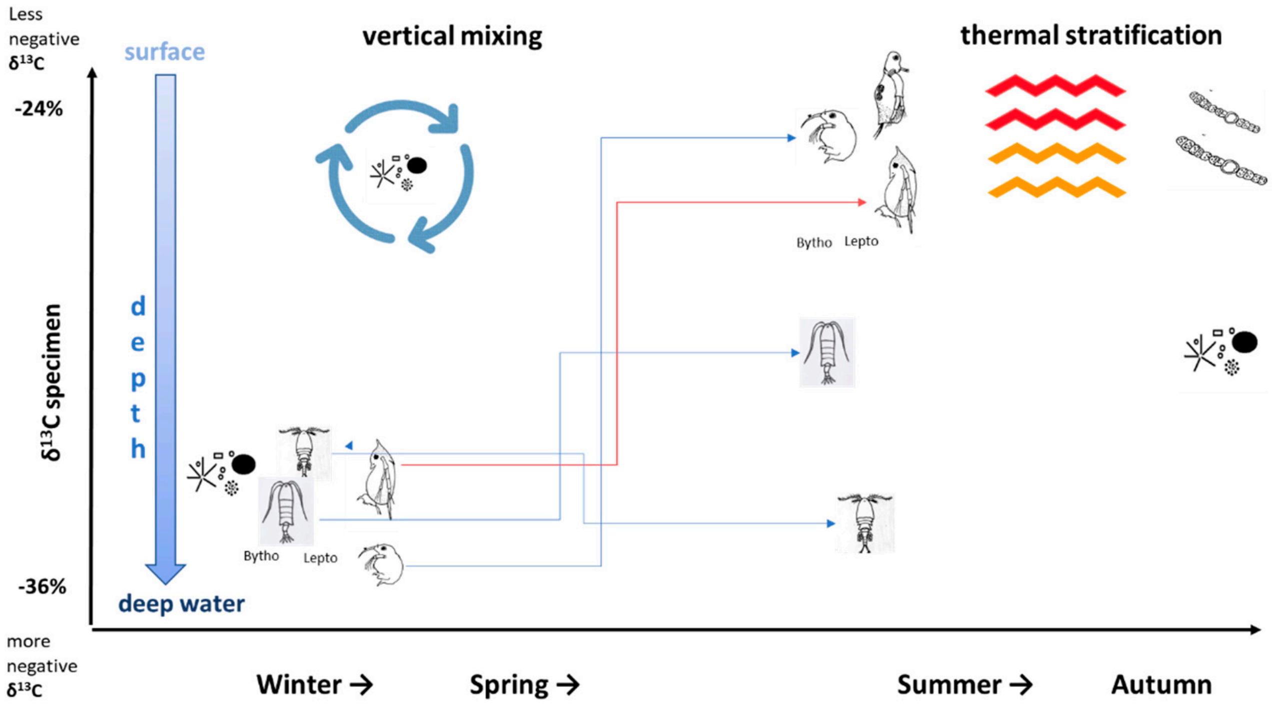

Figure 6. Conceptual diagram summarizing variations in the food sources and habitat preferences of crustacean zooplankton species and groups of Lake Maggiore during the period 2009–2020.

In large, deep lakes at middle latitudes during late winter, significant water mixing typically occurs, caused by a change in the air temperature and helped by windy conditions, thus increasing homogeneity in water conditions and environmental parameters along the water column (temperature, dissolved oxygen, pH, etc.). Phytoplankton are consequently distributed in the water column, and in Lake Maggiore, the phytoplankton community is mainly represented by diatoms which require a high degree of turbulence to maintain in suspension [46] and have ^{13}C-depleted values [31,95]. At this time of the year, we registered values of δ^{13}C‰ for zooplankton taxa within in a narrow range, likely as a result of the homogeneity of phytoplankton type and of habitat.

With the advancing of warmer temperatures, water conditions change, establishing thermal stratification and creating a vertical gradient of the main environmental parameters. These conditions create heterogeneity in the lake water column, reflected also in more diverse plankton communities and vertical distribution of groups and biomass. Filamentous green algae, smaller diatoms, cyanobacteria and other species capable of depth regulation are favoured with intensified stratification [96]. In Lake Maggiore during the summer, cyanobacteria, green algae and diatoms are all relevant groups contributing to the phytoplankton community. A detailed study on Lake Maggiore [60] revealed a vertical gradient of phytoplankton density and biomass in three different lake stations during thermal stratification. Recent phytoplankton monitoring with a fluorescent probe (FluoroProbe, bbe Moldaenke GmbH, Schwentinental, Germany) and with high-frequency chlorophyll *a* in situ sensors (Turner Design, San Jose, USA) confirmed different chlorophyll *a* concentrations and phytoplankton groups with water depth (pers. com., data unpublished). In this decadal study, δ^{13}C‰ values for different zooplankton taxa in summer consistently underwent a clear separation from those of springtime when taxa δ^{13}C‰ values were similar.

We hypothesize that it is likely because the conditions of thermal stratification during the summer create vertical heterogeneity in both food sources and habitats, allowing partial niche partitions for the different zooplankton taxa.

5.3. Zooplankton (Daphnia) δ^{13}C and Chlorophyll a: Indication of Food Quality and Availability

In our decadal study of Lake Maggiore, chlorophyll *a* concentration was related to zooplankton δ^{13}C signatures, indicating the importance of phytoplankton standing crop as food availability and utilization for zooplankton. Previous studies on Lake Maggiore have reported significant correlations between chlorophyll *a* and sestonic carbon concentration over the long term [97], suggesting that POM in the lake is mainly of autochthonous origin and likely comprised of algal cells, with the seston δ^{13}C signature mainly related to phytoplankton. The correlation found in this study between zooplankton δ^{13}C signatures and chlorophyll *a* concentration suggests that the phytoplankton biomass is important in determining zooplankton carbon signatures, likely representing the main food source for zooplankton in Lake Maggiore. In other freshwater studies [59], it has been shown that zooplankton tend to utilize mainly algal-derived autochthonous carbon during the more productive months. In addition, the authors of [74] found a correlation between the herbivorous zooplankter *Daphnia* and chlorophyll *a* in their study of four annual production cycles in a subarctic lake.

Because chlorophyll *a* variability is often a function of primary productivity in freshwaters [98,99], the δ^{13}C seasonality can be interpreted as resulting from changing phytoplankton growth rates [74]. This finding is in good agreement with other field investigations [34,100–102] and culture experiments [101,103,104], which have revealed a strong relationship between the growth rate and δ^{13}C‰ values of aquatic primary producers. Several models have predicted a rise in δ^{13}C‰ values with the increasing algal growth rate or carbon demand [29,103–105].

Plankton collected during periods of high primary production tends to exhibit the heaviest (i.e., less negative) δ^{13}C signatures, due to a reduction of the isotopic fractionation at high cell densities or growth rates, or a shift to utilizing HCO_3^- as CO_2 concentration is depleted at high cell densities or high growth rates [106–108]. The relationships are also mediated by the difference in the δ^{13}C signatures of the phytoplankton groups present in a certain season; during the summer months, Cyanobacteria tend to increase in Lake Maggiore [60], and this phytoplankton group is known to possess relatively enriched δ^{13}C values [109].

5.4. δ^{13}C and δ^{15}N Time Series and Environmental Variables

The decomposition of time-series data allowed the detection of seasonality and trends of environmental parameters during the considered period (2010–2020). In particular, the seasonally decomposed trend of chlorophyll *a* concentration increased, while the nitrate trend decreased.

Recent long-term studies on Lake Maggiore [5] have shown that during the oligotrophication phase of the lake between 1988 and 2005, chlorophyll *a* and phytoplankton biovolume significantly decreased to low concentrations of 2.0–3.0 μg L^{-1}. However, in the last decade, despite nutrient concentrations remaining quite stable and low, the chlorophyll *a* concentration has slightly increased, with fluctuations observed especially during summer periods. These data were recently confirmed by satellite observations, detecting a change in the chlorophyll *a* seasonal pattern with less defined spring blooms and a tendency for annual maxima to occur in summer since 2015 [110]. In this study, we detected those increases and fluctuations in chlorophyll *a*, especially between 2015 and 2020, matching an increasing trend of δ^{13}C‰ values of zooplankton taxa such as *Bosmina* and Diaptomids. An explanation might be found in the new water conditions with increasing algal growth rates or carbon demand, leading to an increase in the δ^{13}C signature of phytoplankton as predicted by several models [29,103–105].

In our study, time-series decomposition analysis also revealed that the $\delta^{15}N$ signature of the cladocerans *Daphnia* and *Bosmina* had a decreasing trend during the last decade. A number of studies have demonstrated that the plankton $\delta^{15}N$ fingerprint is a good indicator of nitrogen sources, nitrogen cycling processes and consumer trophic position in aquatic systems [111]. Changes in phytoplankton species composition may influence the use of nitrogen sources (N_2 fixation vs. uptake of dissolved combined nitrogen). When nitrogen availability is limited, cyanobacterial N_2 fixation is stimulated, resulting in depleted $\delta^{15}N$‰ values, which may then be transferred to zooplankton through feeding, resulting in a low zooplankton $\delta^{15}N$ signature [112]. Although daphnids cannot ingest the largest forms of cyanobacteria, they are capable of feeding on the smaller, decomposing pieces [113] and on the associated bacteria, so they will still reflect the $\delta^{13}C$ signature of the baseline during cyanobacterial blooms [83]. The capability of *Bosmina* to exploit cyanobacteria has been already mentioned in this paper. Overall, the decreasing trend in the $\delta^{15}N$ signatures of both *Daphnia* and *Bosmina* during the last decade in our results match the conditions of nitrogen limitation and the decreasing concentrations in nitrate and total nitrogen in the epilimnion of Lake Maggiore, as revealed by recent long-term studies [114].

Our 12-year study of carbon and nitrogen stable isotopes revealed that zooplankton isotope signatures incorporate changes in lake environmental parameters, integrating the trends that have occurred during the last decade, thereby representing candidate sentinels capable of integrating the influence of climate change on the ecosystem of the lake.

Although the nutrient status in Lake Maggiore has reached oligotrophy, climatic change has affected lake thermal dynamics by increasing the water column stability, aggrading resistance of the water column to mixing and diminishing the water mixing depth [5], all factors which affect the primary production of the lake. The increase of fluctuations and of concentrations in chlorophyll *a* experienced in Lake Maggiore during most recent times could be the result of the recent changes in the lake which have modified conditions altering lake primary production. The changes observed in the zooplankton community provide direct evidence of how climate change can drive cascading effects through lake ecosystems. Such impacts are likely to occur at the regional scale and beyond [17,110], though the context is crucially only revealed through long-term monitoring.

Limnologists are realising that climate change is intensifying the symptoms of eutrophication in freshwaters [1,13], and results from direct experiments suggest that warming can increase the symptoms of eutrophication and changes in ecosystem structure. Our results, together with the most recent long-term studies on Lake Maggiore, could be interpreted as signs of eutrophication-like effects on a deep lake. Because increased temperatures and water column stability promoted a prolonged period of water stratification and of epilimnion segregation during the warmest months, these new conditions could resemble those in the better-studied shallow lakes, with similar final consequences in promoting lake primary production.

Figure 5 compares the seasonally decomposed trend of chlorophyll *a* and nitrate with the carbon isotopic signature of selected zooplankton taxa. A clear-cut match is evident between a surge in chlorophyll *a* concentration, the decrease in epilimnion nitrates since 2015 and the trend change in carbon isotopic signature of the considered zooplankters.

These conditions suggest that climate change has represented the main driving factor of the lake dynamics during the last decade and that its effects have been integrated in the stable isotopes signatures of the zooplankton community, candidates to become sentinels of climatic changes in the lake.

Temperature as a stressor can impact freshwater ecosystems at lower trophic levels (e.g., primary producers) and can cause a cascading effect on other species through the food chain, which, in turn, affects ecosystem structure and function [115,116]. For aquatic ecosystems, the interaction between phytoplankton and zooplankton determines the structure of the food web, which represents the energy and materials flowing through the population in the system [117–119].

6. Conclusions

Tracing ecosystem functioning and the role of ecological variables under changing scenarios is a challenging goal, also in view of predicting the impact of climate change in freshwater environments. We used carbon and nitrogen SIA to identify the seasonal trophic positions of crustacean zooplankton taxa over 12 years in a deep subalpine lake. Across the seasons, water temperature together with the dynamics of thermal stratification and food availability were important factors determining the carbon isotopic signatures of different zooplankton taxa. Vertical heterogeneity of the environment, established during the summer with thermal stratification, promoted vertical spatial segregation of pelagic zooplankton taxa, suggesting that cyclopoid copepods occupy the deepest waters, and cladocerans, such as *Diaphanosoma*, occupy more superficial waters. Zooplankton stable isotopic signatures and their decadal temporal changes were related to temperature, an increase of chlorophyll *a* concentration and a decrease of in the epilimnion, observed during the last decade in Lake Maggiore. Such changes in environmental parameters occurred from the direct and indirect effects of climate change on the lake ecosystem; the isotope composition of zooplankton taxa, reflecting their dietary assimilated carbon and nitrogen and their habitat choice, may therefore integrate these long-term environmental changes. Climate change directly alters lake stratification dynamics, and here we have shown this is of particular significance for zooplankton habitat partition and population dynamics with implications for other trophic levels.

Our study, having considered the seasonal dynamics of carbon and nitrogen isotopes in the zooplankton community, focusing on the main crustacean taxa and extended to a considerable period of 12 years, represents an important insight and context for interpretation of similar investigations restricted to single years.

Overall, we further confirm that stable isotopic studies are a useful tool for tracing the ecological role of organisms in an aquatic ecosystem, subject to seasonal fluctuations and depending on ecological variables such as food sources availability and environmental conditions. Zooplankton can integrate environmental variability in their isotopic signals, and their analysis provides insight for the detection of patterns from a functional perspective.

Author Contributions: Conceptualization, R.C., R.P. and M.M.; formal analysis, R.C.; investigation, R.P. and M.M.; writing—original draft preparation, R.C., R.P. and M.M.; writing—review and editing, G.F.; funding acquisition, M.M. and R.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

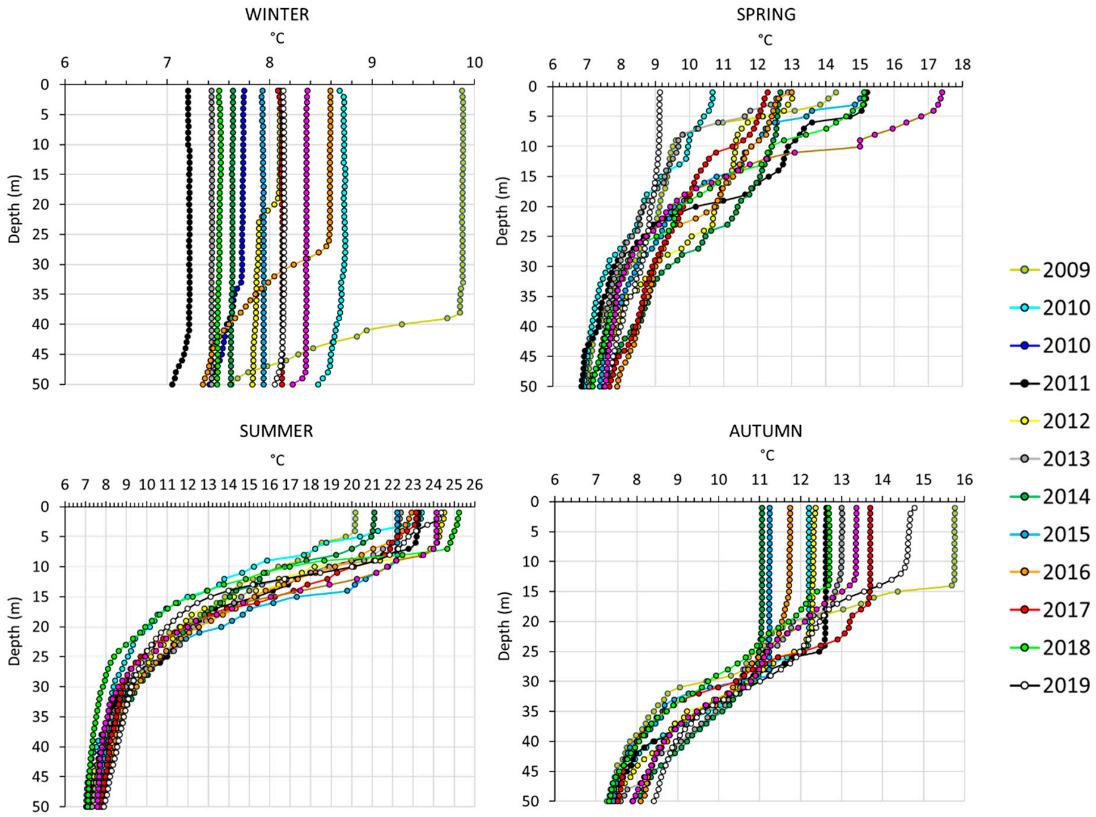

Figure A1. Seasonal vertical profiles of water temperature of the upper 50 m in Lake Maggiore, from 2009 to 2020.

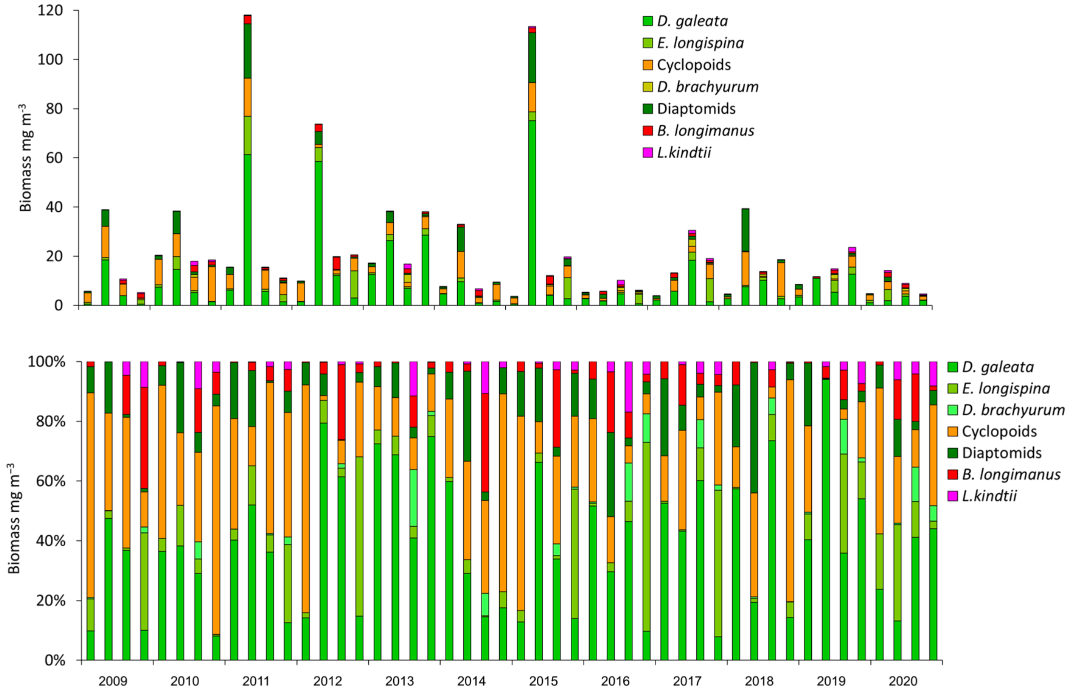

Figure A2. Seasonal variations of zooplankton biomass belonging to the size fraction ≥ 450 μm in Lake Maggiore during the period 2009–2020.

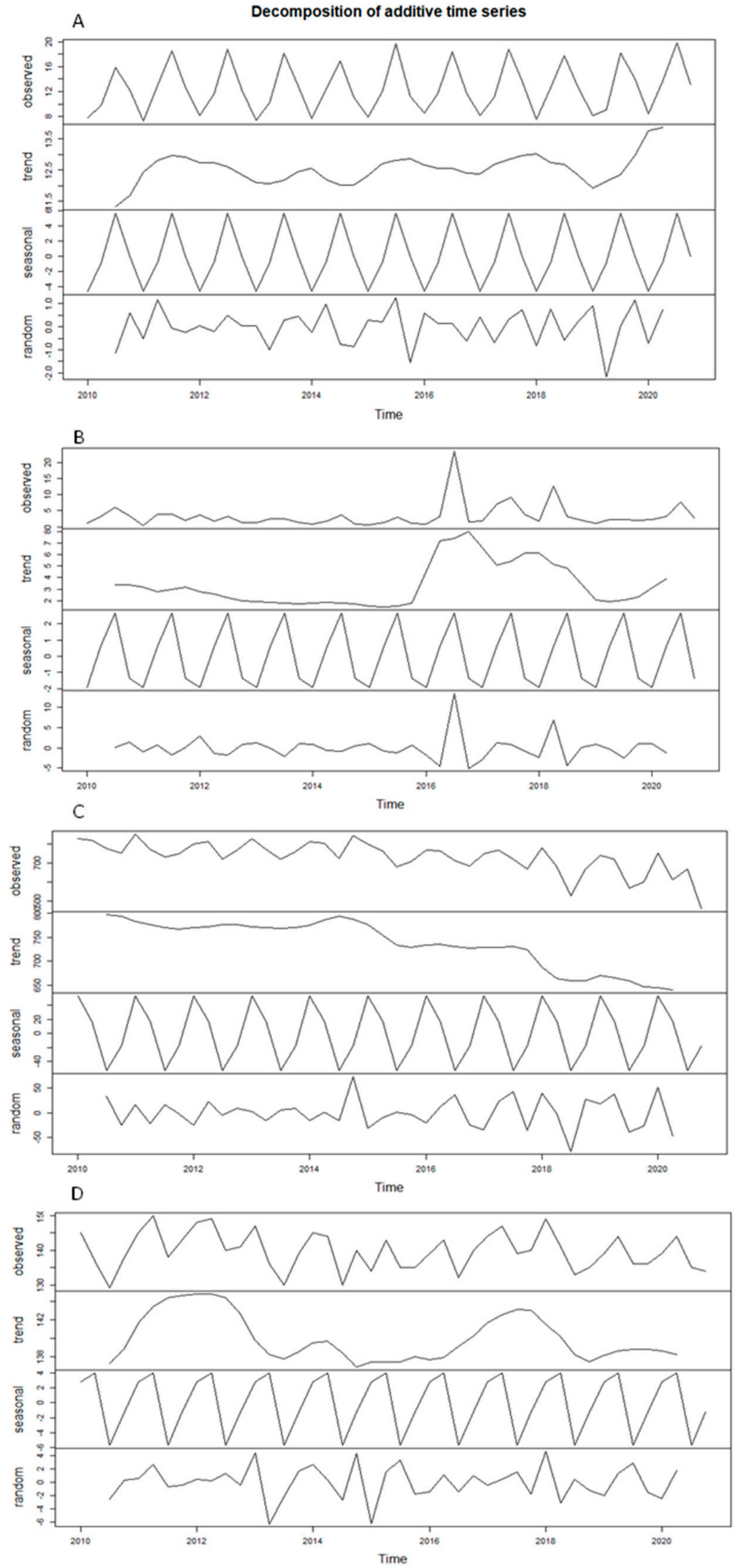

Figure A3. Decomposition of the additive time series of some environmental values: temperature (**A**) mean water temperature of the layer 0–20 m), chlorophyll *a* (**B**), nitrates (**C**) and conductivity (**D**).

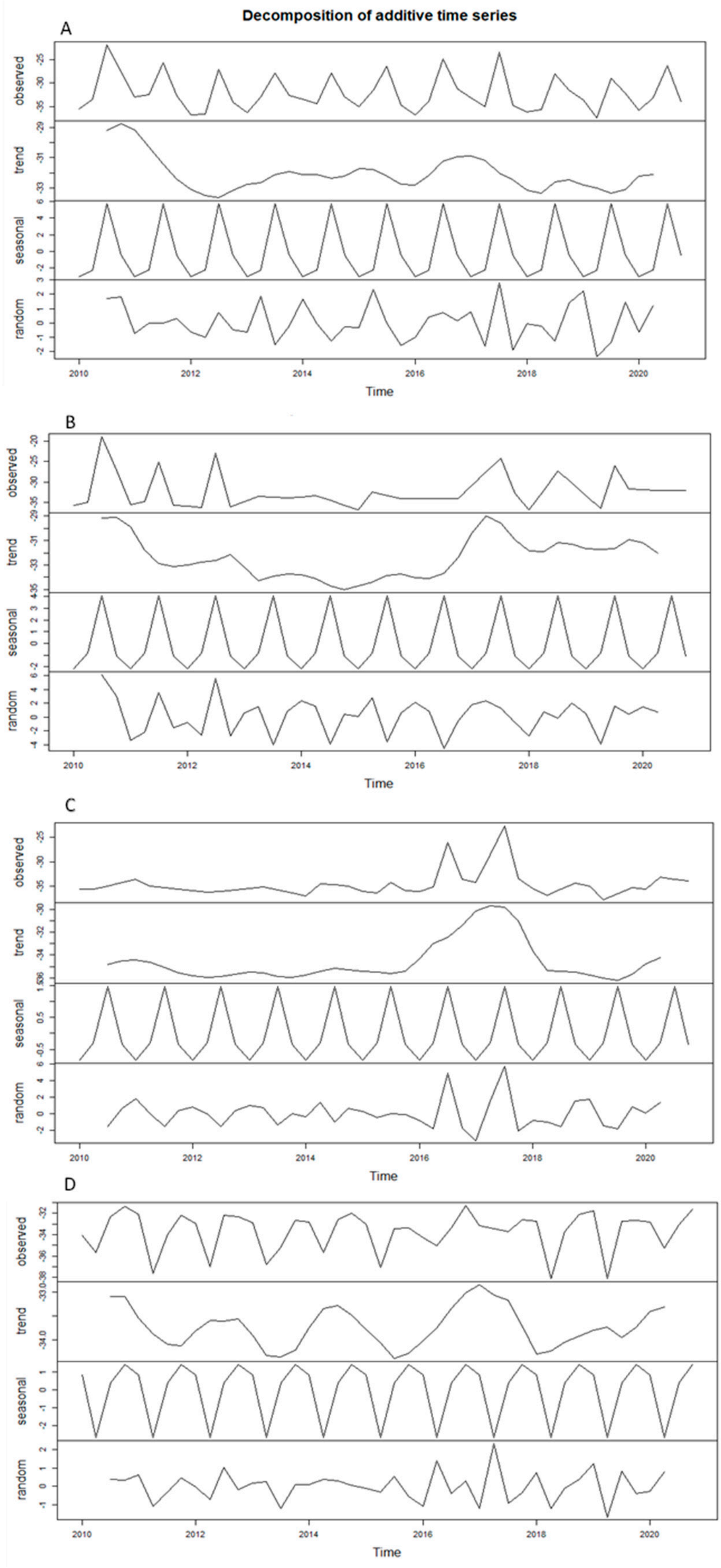

Figure A4. Decomposition of the additive time series of the carbon isotopic values of *Daphnia* (**A**), *Bosmina* (**B**), Diaptomids (**C**) and Cyclopoids (**D**).

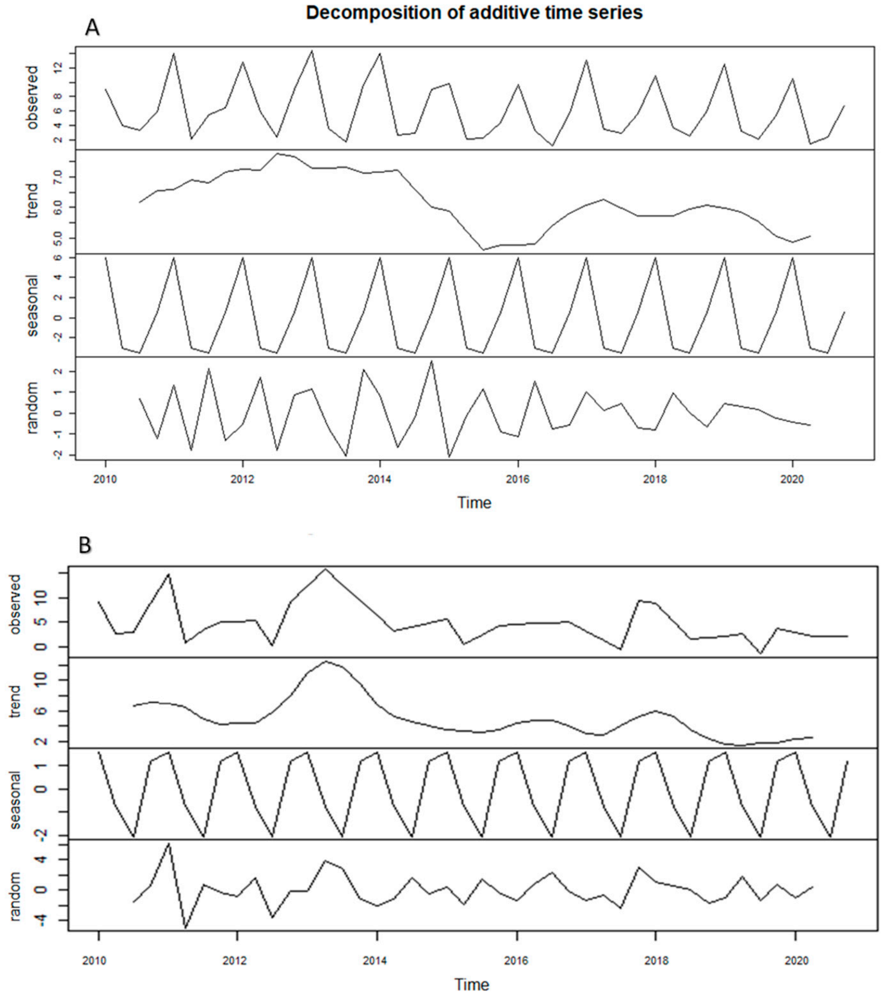

Figure A5. Decomposition of the additive time series of the nitrogen isotopic values of *Daphnia* (**A**) and *Bosmina* (**B**).

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