

Article

Zooplankton as Mercury Repository in Lake Maggiore (Northern Italy): Biomass Composition and Stable Isotope Analysis

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Abstract: Total mercury (THg) and methylmercury (MeHg) concentrations were analyzed in zooplankton (≥ 450 and ≥ 850 μm size fractions) collected seasonally over 6 years in Lake Maggiore (Northern Italy), characterized by a legacy mercury contamination. Analysis of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ stable isotopes was carried out to trace how taxa with different trophic levels and carbon sources contributed to mercury concentrations and trends. THg ranged between 44–213 $\mu\text{g kg}^{-1}$ d.w. and MeHg 15–93 $\mu\text{g kg}^{-1}$ d.w., representing 24–61% of THg. Values showed strong seasonal variations, with peaks in winter, due to the high biomass of predator taxa (*Bythotrephes longimanus*, *Leptodora kindtii*) and of *Daphnia longispina-galeata* gr. A positive correlation between THg and MeHg and $\delta^{15}\text{N}$ signature was observed. *D. longispina-galeata* gr. prevailed in both size fractions, substantially contributing to THg and MeHg concentrations. $\Delta^{13}\text{C}$ signature was strictly bound to lake thermal circulation dynamics. Mercury stock in the zooplankton compartment ranged between 19–140 ng THg m^{-2} and 6–44 ng MeHg m^{-2} for the ≥ 450 μm size fraction and between 2–66 ng THg m^{-2} and 1–7 ng MeHg m^{-2} for the ≥ 850 μm fraction, with the highest values in spring when zooplanktivorous fish actively prey in the pelagic zone. The results highlighted the crucial role of zooplankton as a repository of mercury, easily available to higher trophic levels.

Keywords: invertebrates; methylmercury; bioaccumulation; *Daphnia*; freshwater zooplankton



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1. Introduction

Mercury is a contaminant of concern in freshwater environments because it is very toxic and persistent and effectively accumulates in food chains [1]. The historical contamination of Minamata Bay in Japan showed how exposure to mercury may result in detrimental effects on animals and humans, affecting mainly the central nervous system [2]. Thanks to environmental policy and governance, the Minamata Convention agreement and changes in use, mercury release in the environment has decreased consistently in Europe over the last 30 years [2,3]. However, due to high persistence and to long-distance transport through the atmosphere, mercury remains a problem of global concern [4]. Even if concentrations in the water may be very low, i.e., in the order of a few ng L^{-1} [5–8], values in biota may reach high levels due to bioaccumulation and biomagnification along the trophic chains [9–11]. This is frequently observed in many fish species, even where point sources of mercury are not present [12,13], reaching levels considered a threat to wildlife and human health through fish consumption [2,14,15].

In fish, the prevalent mercury form is methylmercury (MeHg) [15]. MeHg is the most toxic and bioavailable species of mercury, and it is responsible for biomagnification [16]. It is formed by sulphate-reducing bacteria in anoxic conditions, mostly in bottom sediments,

and it is then released in porewater [17]. The main route of mercury transfer to higher trophic levels in freshwater is thus generally considered to be linked to benthic organisms as vectors [18,19]. However, recent studies report that in deep lakes methylation can occur also in suspended particles present in the oxic layer of the water column, enhancing the mercury uptake by planktonic organisms such as zooplankton [20].

In fact, pelagic zooplanktivorous fish proved to reach higher Hg concentrations than littoral species feeding on benthic invertebrates [10,13,21]. Moreover, mercury concentration in pelagic fish were closely related to concentrations in zooplankton [10,22]. Zooplankton is therefore a key vector of Hg to higher trophic levels in lakes [5], accounting for the most significant standing stock biomass in the pelagic zone during the productive seasons [23].

Since a significant amount of zooplankton biomass is needed for mercury analysis, most studies are focused on total mercury (THg) concentrations in the whole zooplankton community or in different size fractions (e.g., [11,24]). Only a few report data on single taxa (e.g., [25]). For the same reason, data on MeHg concentrations in field-collected zooplankton are still scarce (e.g., [9,10]). Mercury concentrations in zooplankton are highly variable across lakes, seasons and years, depending on different lake characteristics (e.g., productivity) and zooplankton taxa assemblages [22,26]. Therefore, site-specific studies on Hg incorporation at the base of the food web are crucial for understanding the subsequent transfer to higher trophic levels.

Seasonality is one of the most important factors driving zooplankton standing stock biomass, density and taxa assemblages in temperate lakes [27,28]. As a consequence, as already observed for other contaminants such as DDTs and PCBs [29], mercury concentrations in zooplankton are also expected to show strong variations across seasons. Changes in system productivity, in the form of algal blooms, may reduce the uptake of mercury by higher-trophic-level organisms such as zooplankton, promoting strong seasonal variability in zooplankton mercury concentrations [30]. In fact, bio-dilution of Hg enhanced by a larger amount of biomass in productive seasons may result in lower concentrations in the lake biota [13,24,30].

Different taxa assemblages may also explain variations in mercury concentrations in bulk zooplankton. Predator taxa such as *Bythotrephes*, *Leptodora* and cyclopoids are expected to reach higher mercury concentrations than other taxa due to biomagnification [11,28]. However, *Daphnia* species as well, even if primary consumers, were reported to accumulate higher Hg body burdens in comparison to other zooplankton taxa, and in some cases even to predators [25,31]. *Daphnia* is a common taxon in temperate lakes and shows large size and high densities, as well as high filtration rates [9,10]. For these reasons, *Daphnia* biomass dynamics may significantly account for Hg variations in the whole zooplankton community (e.g., [5,9]). In turn, since planktivorous fish tend to select larger and more visible prey [28,32], Hg concentrations in macrozooplankton (comprising both *Daphnia* and predator species) may be significant predictors of Hg in fish [5,8,10,33].

The goal of this paper is to analyze mercury concentrations in pelagic zooplankton of Lake Maggiore, the second largest and deepest lake in Italy, where fisheries account for important commercial activity [34]. Due to the legacy of mercury contamination, THg levels in zooplanktivorous fish species were proved to exceed the European Environmental Quality Standard for biota, posing potential risks of secondary poisoning for top predators [35]. Therefore, this paper addresses zooplankton as a mercury source for fish. To consider between-year variability, the investigation was carried out over a period of 6 years, i.e., from 2014 to 2018. The final aim was to analyze seasonal trends of THg and MeHg concentrations in zooplankton and how they related to changes in zooplankton standing stock biomass and taxa composition.

Carbon and nitrogen isotopic signatures provided a basis for understanding how succession of taxa with different trophic position composing the zooplankton community might explain variations in mercury concentration along with season. Stable isotope analysis is an efficient method for tracing how taxa with different trophic levels contribute to concentration of persistent organic pollutants [28] and of mercury [1,36]. In particular,

investigating taxa composition and taxa-specific contribution to zooplankton biomass along with stable isotope signatures allows for tracing the path through which matter, energy and pollutants flow through the food web [13,37]. The basic assumption of this approach is that pollutants are in a steady state condition.

Zooplankton was regarded also as a mercury repository for higher trophic levels. Therefore, in addition to measuring mercury concentrations, estimation of the total mass of THg and MeHg stocked in the zooplankton compartment is also provided by considering seasonal changes in zooplankton biomass.

2. Materials and Methods

2.1. Site Description and Brief History of Its Mercury Pollution

Lake Maggiore is a large (surface area = 213 km², volume = 38 km³), deep (maximum depth = 370 m), subalpine lake, located close to the Central Alps, shared between Italy (about 80%) and Switzerland (20%). The lake is classified as holo-oligomictic, as it rarely undergoes complete vertical mixing [38]. The mean theoretical lake water renewal time was estimated as more than 4 years [38]. The lake has a relatively large (6599 km²) watershed, mostly formed by mountain areas, inhabited by more than 500,000 people. Moreover, seasonal tourism accounts for more than 300,000 additional equivalent inhabitants. Lake Maggiore has 33 tributaries and one outlet, the Ticino River. Oligotrophic by nature, the lake suffered from anthropogenic eutrophication during the second half of the 20th century [39]. Concern for water quality resulted in a specific monitoring program for the lake, its drainage basin and tributaries, with funds “ad hoc” provided by the International Commission for the Protection of the Italian–Swiss Waters (CIPAIS; www.cipais.org, accessed on 14 January 2022). In the following decades, the building of sewage treatment plants and the progressive ban of phosphorus from detergents caused a decrease of P load, until arriving at values close to pre-industrial concentrations [40].

The monitoring program, initially focused on the lake trophic status, was later extended to the investigation of toxic substances in sediments of the lake and its tributaries and in biota. Thanks to these monitoring activities, long-term data on present and past concentrations of different toxic substances in the main lake compartments (sediments, benthic invertebrates, zooplankton and fish) along with basic limnological data are available for Lake Maggiore (www.cipais.org accessed on 14 January 2022).

Regarding mercury, concentrations in sediments and biota are constantly monitored. In fact, as a result, Lake Maggiore was shown to be affected by a number of anthropogenic sources [35,41]. Gold mining is documented between AD 1700 and AD 1900 [42], and it acted as a source of mercury for the aquatic environment. Moreover, since 1800, industrial production led to the discharge of several pollutants in the lake and in its tributaries, among them Hg used in the hat-making industry and, in the 1900s, in a mercury-cell chlor-alkali plant located along the Toce River, one of the main tributaries of the lake. Further details can be found in Marziali et al. [41].

2.2. Sampling

The sampling station was located in the central part of Lake Maggiore (45°58′30″ N; 8°39′09″ E), corresponding to the zone of maximum depth (370 m) (Figure 1).

Zooplankton samples belonging to two size fractions were collected in the four seasons (i.e., in January, May, August and November) of 2014 and 2016–2020. The lake’s basic features (productivity and hydrochemistry) did not vary in this time lapse. The research program did not include the analysis of mercury in zooplankton in 2015, therefore some data are missing.

Plankton samplers with a large-opening mouth (diameter of 58 cm), equipped with nylon nets of 450 µm and 850 µm of light, were towed vertically from a depth of 50 m to the surface. The sampled water layer is representative of the net zooplankton population, as the occurrence of zooplankton in deeper layers is very rare and restricted to complete winter vertical mixing events [43]. Quantitative samples were collected in duplicate for microscope

and stable isotope analysis ($\geq 450 \mu\text{m}$ and $\geq 850 \mu\text{m}$ zooplankton “pool” samples). In addition, an appropriate number of specimens of different taxa composing the pool samples were extracted for the analysis of taxon-specific isotopic signatures on each sampling date. Individual biomass was obtained by applying taxon-specific length–weight regression equations [44], and the zooplankton standing stock biomass was estimated by combining single taxon weights with abundance in each sample (individuals m^{-3}).

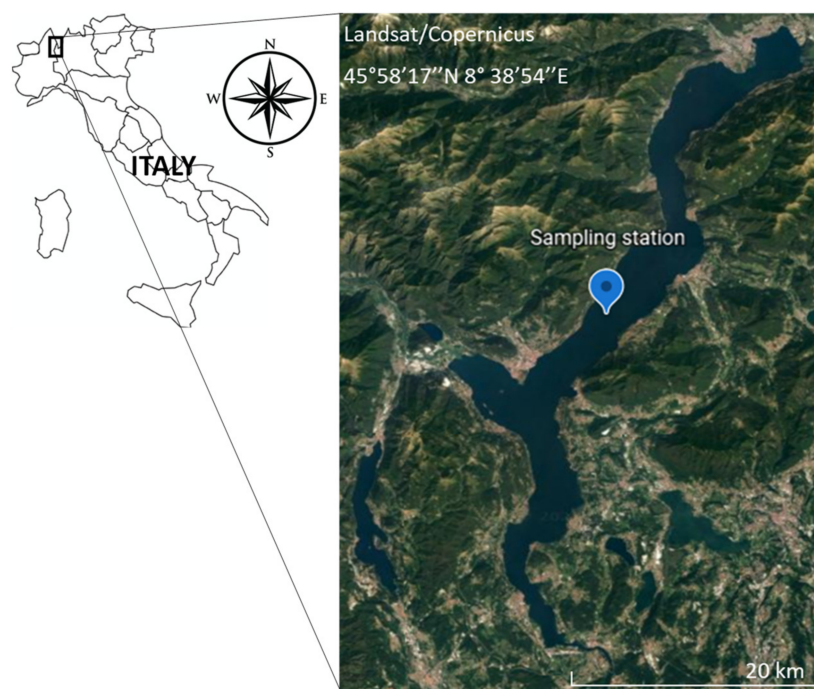


Figure 1. Map of Lake Maggiore (Northern Italy) with the sampling station. Modified from Google Earth (accessed on 14 January 2022).

For mercury analysis, aliquots of the samples were freeze-dried (72 h at 0.1 atm and -45°C ; Telstar LyoQuest) and preserved in dark glass bottles until analysis.

2.3. Mercury Analysis

Concentrations of total mercury (THg) in zooplankton were determined by thermal decomposition, amalgamation and atomic absorption spectrometry according to the US-EPA 7473 method [45] using an Automated Mercury Analyzer (AMA-254, FKV srl, Bergamo, Italy). The instrument sensitivity is 0.01 absolute ng of Hg. Considering a sample weight of 0.025 g, the LOD is $0.4 \mu\text{g kg}^{-1}$. The Limit of Quantification (LOQ), calculated as mean plus ten times the standard deviation of the blank and considering a sample mass of 0.025 g, is 0.009 mg kg^{-1} . The absolute instrument working range is 0.05 to 600 ng Hg. For quality assurance, the certified reference material BCR-CRM414 plankton powder (Institute for Reference Materials and Measurements—IRMM, European Commission, Geel, Belgium; reference value = $276 \pm 18 \mu\text{g THg kg}^{-1}$) was analyzed. Mean recovery was $92 \pm 3\%$ ($n = 6$). Analyses were run in duplicate or triplicate, according to availability of biomass. Coefficient of variation was $\leq 5\%$.

Methylmercury (MeHg) was analyzed in the $\geq 450 \mu\text{m}$ size fraction samples collected from autumn 2019 onwards, and in the corresponding $\geq 850 \mu\text{m}$ size fraction when biomass was sufficient. A minimum of 0.25 g dry weight (d.w.) per sample was necessary. The analytical method is published in Valsecchi et al. [46]. The analysis involved a microwave-assisted digestion in controlled pressure and temperature and subsequent instrumental analysis by static headspace and GC-MS, while quantification was performed by isotope dilution technique. Briefly, ^{201}Hg -enriched internal standard (96.5%, ISC Science, Oviedo, Spain)

and 3 mL of 1M HCl were added to the sample, then microwave digested at 70 °C for 3 min. After filtration, the sample was brought to pH 5 with the addition of an acetate buffer and a KOH solution. MeHg ethylation was achieved by adding a 1% sodium tetraethylborate solution to the sample and incubating it for 12 min at 90 °C using the static headspace TriPlus RSH™ autosampler (Thermo Fisher Scientific, Rodano, Milan, Italy). GC analysis was carried out with a single quadrupole GC-MS system acquiring the signal in SIM mode and monitoring the ions (m/z) 215 + 244 and 216 + 245 for native MeHg and $^{201}\text{MeHg}$, respectively. The cross contribution due to the Hg isotope pattern was corrected by software (Xcalibur 1.4, Thermo Fisher Scientific, Rodano, Milan, Italy). The LOD, based on the signal-to-noise ratio, was $0.7 \mu\text{g kg}^{-1}$. For quality assurance, the certified reference material BCR-CRM414 plankton powder (reference value = $200 \pm 10 \mu\text{g MeHg kg}^{-1}$ according to GeoReM database, <http://georem.mpch-mainz.gwdg.de/>, accessed on 14 January 2022) was analysed. Mean recovery was $97 \pm 3\%$ ($n = 6$).

2.4. Stable Isotope Analysis (SIA)

An adequate amount of sample (1 mg d.w.) was required for stable isotope analysis of both pool and taxa-specific samples. Depending on the season, not all taxa detected under the microscope could be processed for SIA, since for small-sized taxa very high numbers are needed to reach the minimum weight necessary (e.g., up to 800 individuals per sample for *Eubosmina longispina*). Samples were prepared in duplicate for each sampling date. A total of 96 samples for the two size fractions and of 250 for taxa-specific samples were sealed in 5×9 mm tin capsules and sent to Ján Veizer Stable Isotope Laboratory at University of Ottawa for SIA.

Samples and standards were weighed into tin capsules and loaded into an elemental analyser interfaced to an Isotope Ratio Mass Spectrometer (IRMS). Sample/Standard were flash combusted at about 1800 °C (Dumas combustion) and the resultant gas products were carried by helium through columns of oxidizing/reducing chemicals optimised for CO_2 and N_2 . The gases were separated by a “purge and trap” adsorption column and sent to IRMS interface and then to IRMS. Laboratory internal standards were ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$ in ‰): C-51 Nicotiamide (0.07, −22.95), C-52 mix of ammonium sulphate + sucrose (16.58, −11.94), C-54 caffeine (−16.61, −34.46), blind standard C-55: glutamic acid (−3.98, −28.53). Data are reported in Delta notation (δ), the units are per mil (‰) and defined as $\delta = ((R_x - R_{\text{std}})/R_{\text{std}}) \times 1000$, where R is the ratio of the abundance of the heavy to the light isotope, x denotes sample and std is an abbreviation for standard. All $\delta^{15}\text{N}$ is reported as ‰ vs. AIR and normalized to internal standards calibrated to the International Standards IAEA-N1 (+0.4‰), IAEA-N2 (+20.3‰), USGS-40 (−4.52‰) and USGS-41 (47.57‰). All $\delta^{13}\text{C}$ is reported as ‰ vs. V-PDB and normalized to internal standards calibrated to the International Standards IAEA-CH-6 (−10.4‰), NBS-22 (−29.91‰), USGS-40 (−26.24‰) and USGS-41 (37.76‰). Please note that the PDB and V-PDB scales are identical and interchangeable. Analytical precision was based on an internal standard (C-55) which is not used for calibration, and it was generally better than 0.2‰.

3. Results and Discussion

Total mercury concentrations ranged from 44 to $213 \mu\text{g kg}^{-1}$ d.w. and, by considering all datasets, showed no significant differences between the two size fractions ($98 \pm 45 \mu\text{g kg}^{-1}$ d.w. in the $\geq 850 \mu\text{m}$ fraction and $95 \pm 40 \mu\text{g kg}^{-1}$ d.w. in the $\geq 450 \mu\text{m}$ fraction) (t -test, $p > 0.05$). MeHg concentrations ranged between 15 and $93 \mu\text{g kg}^{-1}$ d.w., and, again, values were comparable between size fractions ($35 \pm 17 \text{ ng g}^{-1}$ d.w. in the $\geq 850 \mu\text{m}$ size fraction and $26 \pm 10 \text{ ng g}^{-1}$ d.w. in the corresponding $\geq 450 \mu\text{m}$ fraction samples) (t -test, $p > 0.05$). Percent MeHg on THg ranged between 24 and 61%.

The comparison with other case studies may be hampered by different lake characteristics (e.g., lake morphometry, trophic state, etc.), which influence mercury cycling mechanisms and bioavailability, by different sampling seasons and by zooplankton taxa assemblages and size-fractionation [22]. Notwithstanding these limitations, concentra-

tions in Lake Maggiore seem to be generally similar to or higher than those observed in other oligotrophic lakes without direct sources of mercury. For example, concentrations in bulk zooplankton ($> 153 \mu\text{m}$ size) collected in Lake Superior reached $130 \mu\text{g kg}^{-1}$ d.w. of THg and $50 \mu\text{g kg}^{-1}$ d.w. of MeHg, representing 30–77% of THg [24]. Average mercury level in zooplankton ($>300 \mu\text{m}$ size) of Lake Balaton was $31.0 \pm 6.8 \mu\text{g kg}^{-1}$ d.w. for THg and MeHg represented 4–30% of THg [6]. Concentrations in some lakes in New Hampshire and Vermont (USA) were comparable to those of Lake Maggiore: for example, for the $>202 \mu\text{m}$ size-fraction in Echo Pond, a range of $54\text{--}430 \mu\text{g kg}^{-1}$ d.w. for THg and of $40\text{--}100 \mu\text{g kg}^{-1}$ d.w. for MeHg were reported [22]; in Horseshoe Pond, $91\text{--}310 \mu\text{g kg}^{-1}$ d.w. of THg and $36\text{--}170 \mu\text{g kg}^{-1}$ d.w. of MeHg were found. Ward et al. [22], as well as Chen et al. [5], reported higher concentrations for other US lakes, which may be indicative of significant contamination: up to $7500 \mu\text{g kg}^{-1}$ d.w. for THg and to $430 \mu\text{g kg}^{-1}$ d.w. for MeHg.

Lake Maggiore can be regarded as mildly polluted with respect to other investigated lakes. Due to the activity of the chlor-alkali plant, peak Hg concentrations in the lake sediments were reached in the 1940s, with values up to 26mg kg^{-1} d.w. for THg [35,38]. After 1996, thanks to the drastic reduction of industrial activities, treatment of effluents and stabilization of contaminated soils, the contaminant inputs into the aquatic ecosystem dropped significantly. However, at the time of this writing, mercury concentrations in zooplanktivorous fish such as whitefish (*Coregonus lavaretus*) and landlocked shad (*Alosa fallax lacustris*) still largely exceed the European Environmental Quality Standard for mercury in biota ($20 \mu\text{g kg}^{-1}$ wet weight as THg), reaching concentrations up to 10 times higher [35]. The risk for human health may be limited, since concentrations in fish fillets are significantly lower than thresholds for human consumption (e.g., 0.5mg kg^{-1} wet weight as THg according to the European Commission Regulation No. 78/2005). However, the environmental risk of secondary poisoning for the top predators of food chains still arises. Zooplankton is considered to play an important role in mercury transfer to higher trophic levels and might be used as an early warning tool for new contamination pulses [29,35].

A marked seasonality characterized mercury concentration in both zooplankton size fractions. Generally, for THg, peak values were observed in winter (mean of $179 \pm 27 \mu\text{g kg}^{-1}$ d.w. in $\geq 850 \mu\text{m}$ size fraction and $141 \pm 22 \mu\text{g kg}^{-1}$ d.w. in $\geq 450 \mu\text{m}$ size fraction) and the lowest ones in summer (mean of $71 \pm 20 \mu\text{g kg}^{-1}$ d.w. and $66 \pm 17 \mu\text{g kg}^{-1}$ d.w. in the two size fractions, respectively), as for other contaminants [28,47] (Figure 2). The same trend was observed for MeHg (Figure 2).

Lower summer values in comparison to other seasons were also observed in other case studies and were related to bio-dilution (i.e., decreasing concentrations with increasing trophic level) and/or different taxa compositions [21,48]. For example, Back et al. [24] speculated a summer increase of phytoplankton and/or zooplankton biomass without a concomitant Hg pulse. In those conditions, lower mercury concentrations are expected in zooplankton due to the increased biomass of phytoplankton and, consequently, of zooplankton. In Lake Maggiore, the main inputs of mercury derive from the Toce River, where the mercury-cell chlor-alkali plant was active until the end of 2018, and by two tributaries, the Bardello and Boesio rivers, whose watersheds suffer intense urban and industrial pressures [41]. However, contamination is mostly linked to the transport of Hg-rich sediments, while Hg release in the water column is generally rather limited [49,50]. Moreover, past Hg pulses into the lake were associated with intense hydrological events, which were not observed during our study period [41]. Therefore, mercury bio-dilution may have occurred in productive seasons.

In contrast, during lake destratification periods, higher mercury values in zooplankton were observed in Lake Maggiore, as well as in other case studies, such as in Post Pond (Vermont, USA) [10]. In Lake Maggiore, winter values of the size fraction $\geq 850 \mu\text{m}$, which was dominated by *Daphnia longispina-galeata* gr., were generally higher than those of the fraction $\geq 450 \mu\text{m}$, composed mostly by Cyclopoids and Diaptomids (Figure S1). Notably, in winter 2018 and 2019, peak concentrations matched the increased contribution of

D. longispina-galeata gr. to the total zooplankton biomass (85.7% and 92.3%, respectively). The key role of Cladocera in driving Hg concentrations in bulk zooplankton was highlighted also by Stewart et al. [21], who reported peak MeHg concentrations in periods characterized by the dominance of this group, and by Karimi et al. [10], who found THg peaks matching with highest *Daphnia* biomass.

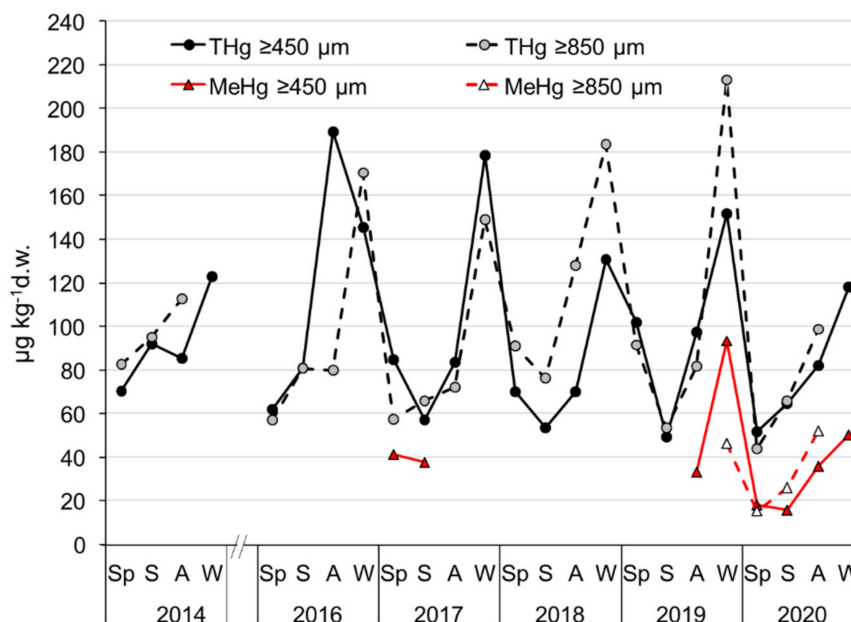


Figure 2. Seasonal changes in mercury concentrations (total mercury and methylmercury) in two zooplankton size fractions of Lake Maggiore during the years 2014 and 2016–2020. Sp = spring; S = summer; A = autumn; W = winter. For further explanations, see text.

Moreover, Hg concentrations in Cladocera with a substantial contribution of *Daphnia* biomass were proved to be linked to Hg concentrations in zooplanktivorous fish. For example, Karimi et al. [10] found that Hg concentrations are higher in fish consuming cladoceran-dominated zooplankton, due to a higher Hg concentration in Cladocera in comparison to Copepoda. Fish predation on zooplankton is strictly size specific: since the early 1980s, it was well defined that zooplanktivorous fish preferably prey on the $>850\ \mu\text{m}$ size fraction. Fish also select by visibility, preferring large, ovigerous *Daphnia* over smaller and more transparent preys [51–53]. Differently from the large predatory Cladocera, the range of body size of *Daphnia* species spans from ca. $450\ \mu\text{m}$ for newborn *Daphnia cucullata* up to 4–5 mm in large *Ctenodaphnia* sp. adults (e.g., [54,55]). Due to this large range of sizes, *Daphnia* pertains to both the ≥ 450 and ≥ 850 size fractions. However, the contribution of ovigerous females will be undoubtedly larger in the largest fraction. The literature, in fact, suggests that the minimum size of the primiparae, depending on temperature and food, ranges from $850\ \mu\text{m}$ to 1.4 mm in species of the *Hyalodaphnia* group, to which the *D. longispina-galeata* gr. of Lake Maggiore belongs [52]. Because of this size overlapping, it was largely expected that mercury did not differ substantially between the two fractions analysed, except for those samples where *D. longispina-galeata* gr. was largely dominant. However, analysing the amount of mercury stocked in the largest fraction may be relevant for tracing the trophic transfer to zooplanktivorous fish.

Seasonal trend of $\delta^{15}\text{N}\text{‰}$ signature supported higher Hg concentrations in winter. In fact, $\delta^{15}\text{N}$ showed higher values in the winter period and lower ones during summer, while intermediate values characterized both spring and autumn. As expected, nitrogen isotopic signature ($\delta^{15}\text{N}\text{‰}$ range = 1.55‰ – 16.84‰) was positively correlated with mercury concentrations (Pearson's correlation; for THg, $r = 0.79$, $N = 44$, $p < 0.001$; for MeHg,

$r = 0.93$, $N = 10$, $p < 0.001$), since mercury levels are linked to trophic position [1,21] (Figure 3).

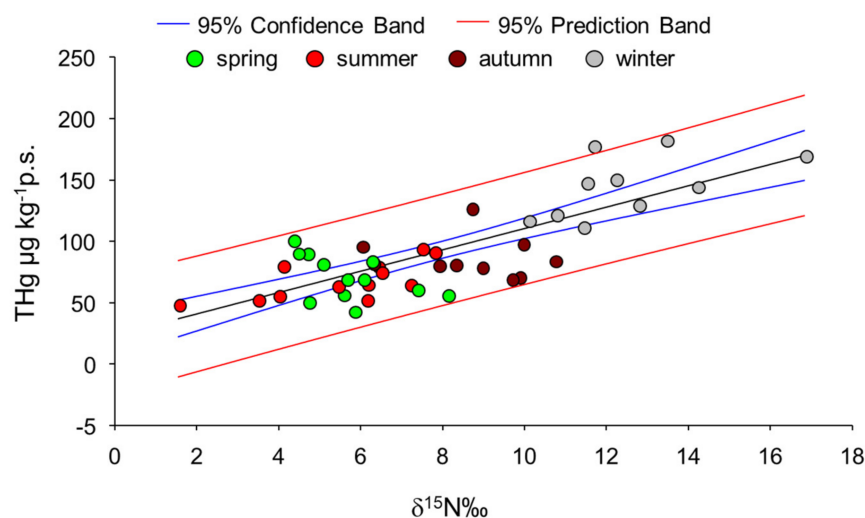


Figure 3. Relationship between total mercury concentrations and $\delta^{15}\text{N}$ isotopic signature for zooplankton ($\geq 450\ \mu\text{m}$ and $\geq 850\ \mu\text{m}$ size fractions) in Lake Maggiore during the four seasons of the investigated years (2014 and 2016–2020).

In fact, the seasonal trend of $\delta^{15}\text{N}$ observed was related to different taxa composition of zooplankton, characterized in winter by higher biomass of predator species (Figures S1 and S2) and by larger-sized *D. longispina-galeata* gr. individuals compared to the summer period. Season is a key factor driving zooplankton taxa composition [28]. Spring warming and thermal water stratification generally promote a spring peak in phytoplankton biomass, followed by a peak in zooplankton biomass, mainly due to primary consumers. This peak is followed by an increased contribution of zooplankton secondary consumers to the total zooplankton biomass in late summer, with a prevalence of large-sized organisms.

$\Delta^{15}\text{N}$ signatures were basically the same in both size fractions (Figure S2), and this may explain why mercury values were generally similar between size fractions. Only in some cases, mercury values were higher in the $\geq 850\ \mu\text{m}$ size fraction than in the corresponding $\geq 450\ \mu\text{m}$ fraction (e.g., in winter 2018 and 2019 for THg, Figure 2). However, $\delta^{15}\text{N}$ signatures did not emphasize a higher trophic level for the former group (Figure S2). Therefore, macrozooplankton was not proven to derive Hg from the ingestion of smaller zooplankton (i.e., from biomagnification), as reported in other case studies [5,22]. In those sampling dates, *D. longispina-galeata* gr. biomass dominated the $\geq 850\ \mu\text{m}$ size fraction, while it was less relevant in the $\geq 450\ \mu\text{m}$ size fraction. This may explain the observed differences in Hg concentrations. In fact, *Daphnia* species, even if primary consumers, proved to accumulate mercury more efficiently than other species and to be often dominant in the zooplankton community [10,25,31]. In some cases, mercury concentrations in zooplankton proved to be correlated with *Daphnia* biomass, which may drive mercury trends in the whole zooplankton community [9,10]. Additionally, the seasonal changes in $\delta^{15}\text{N}$ ‰ signature in bulk zooplankton may result not only from different abundances of predator taxa, but also from different nitrogen resources available for *Daphnia* in different seasons. In summer, a prevalence of phytoplankton in particulate organic matter may lead *Daphnia* to lower $\delta^{15}\text{N}$ ‰ values, while in winter trophic resources could also be composed of bacteria, protozoa and detrital particles such as exuviae, which are more enriched in ^{15}N isotope [28,36]. Moreover, Todorova et al. [9] showed that MeHg concentration in *Daphnia* increases with body size. In the case of Lake Maggiore, the maximum body length (and the maximum clutch size) of *D. longispina-galeata* gr. is reached in winter–early spring, when the highest mercury concentrations are observed. Figure 4 shows the relationship between the percent of *D. longispina-galeata* gr. biomass relative to the total zooplankton biomass

and THg concentrations in zooplankton normalized to $\delta^{15}\text{N}$. As a result, *Daphnia* biomass can be used as a good predictor of mercury concentrations in zooplankton ($R^2 = 0.45$, $p < 0.01$) by excluding summer values. In summer, the relationship was not clear and often inverse, and other mechanisms seem to drive mercury concentrations in zooplankton, such as bio-dilution, as discussed above.

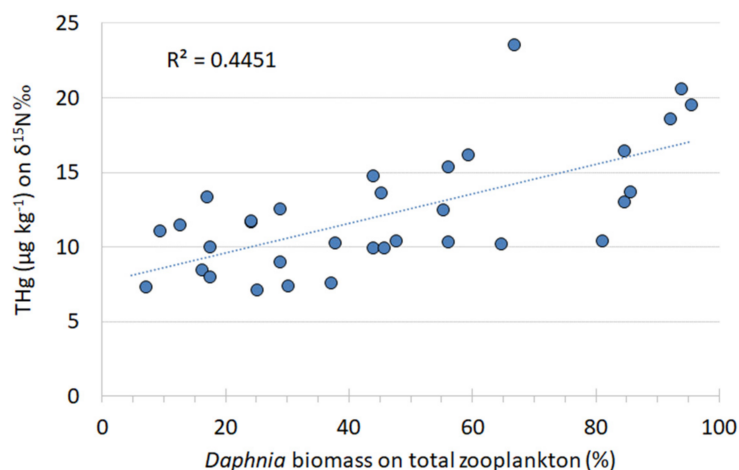


Figure 4. Relationship between the percentage of *D. longispina-galeata* gr. biomass relative to the total zooplankton biomass and the total mercury concentrations in zooplankton of Lake Maggiore (≥ 450 and ≥ 850 μm size fractions) normalized to $\delta^{15}\text{N}$ ‰. Summer values were excluded (see text for explanations).

Therefore, the $\delta^{15}\text{N}$ ‰ signatures of zooplankton samples may result from both seasonal changes in isotopic signatures of single taxa composing the sample and the relative contribution of single taxa to total zooplankton biomass attained in each season [28]. Applying the formula by Piscia et al. [28], we compared the measured vs. estimated (weighting taxa-specific signatures on each date on the relative contribution to the total zooplankton biomass on the same date) $\delta^{15}\text{N}$ ‰ signatures of the pool zooplankton samples on each sampling date. The correlation was very strong (Spearman rank correlation, $r = 0.94$, $N = 45$, $p < 0.001$), pointing out a substantial steady state condition for mercury pollution in the lake, as observed also for POPs [28].

As regards $\delta^{13}\text{C}$ ‰, values estimated by applying the same formula cited above [28] closely corresponded to the $\delta^{13}\text{C}$ ‰ measured of each zooplankton sample (Spearman rank correlation, $r = 0.94$, $N = 45$, $p < 0.001$). Less carbon-depleted values were observed during summer and the most depleted ones were observed in winter (Figure S3). Intermediate values characterized the spring and autumn periods. The inverse relationship between $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ is typical in lakes [37], and it was also observed in Lake Maggiore. Carbon stable isotope analysis can be used to identify carbon sources. Less-depleted values in zooplankton may be related to allochthonous carbon sources such as inorganic particles detritus and bacteria, which are generally prevalent during thermal stratification in comparison to destratification periods, where higher contribution from autochthonous resources (phytoplankton) may determine higher ^{13}C depletion [21]. Phytoplankton cell size and taxa composition are also important factors [56]. Changes in carbon isotopic signatures are closely related to mean water temperatures measured in the sampled water layer (Spearman rank correlation, $r = 0.82$, $N = 48$, $p < 0.001$). Values of $\delta^{13}\text{C}$ of -37 ‰ to 33.5 ‰ in winter corresponded to mean water temperature values of 7.5 – 8.5 °C (Figure 5). Summer $\delta^{13}\text{C}$ values ranged between -28 ‰ and -24 ‰ for temperatures between 11.75 °C and 13.2 °C. Spring temperatures appeared more closely related to winter ones, while autumn temperatures were closer to summer ones. This pattern reflects the dynamics of vertical water temperature profiles in the lake, highlighting how stratification erosion in fall is slower than spring stratification onset (Figure 5). Between-year variations in $\delta^{13}\text{C}$ ‰ signature

were well explained by differences in thermal stratification regime and water temperatures in the sampled layer. Spring 2019 in particular was the coldest period, with spring 2020 being the warmest and well thermally stratified (Figure 5). No thermal stratification and the lowest winter-like water temperatures characterized spring 2019. Stratification and warm temperatures to 18 m depth characterized summer 2017, when the highest surface temperature of the analysed period was reached (25.2 °C).

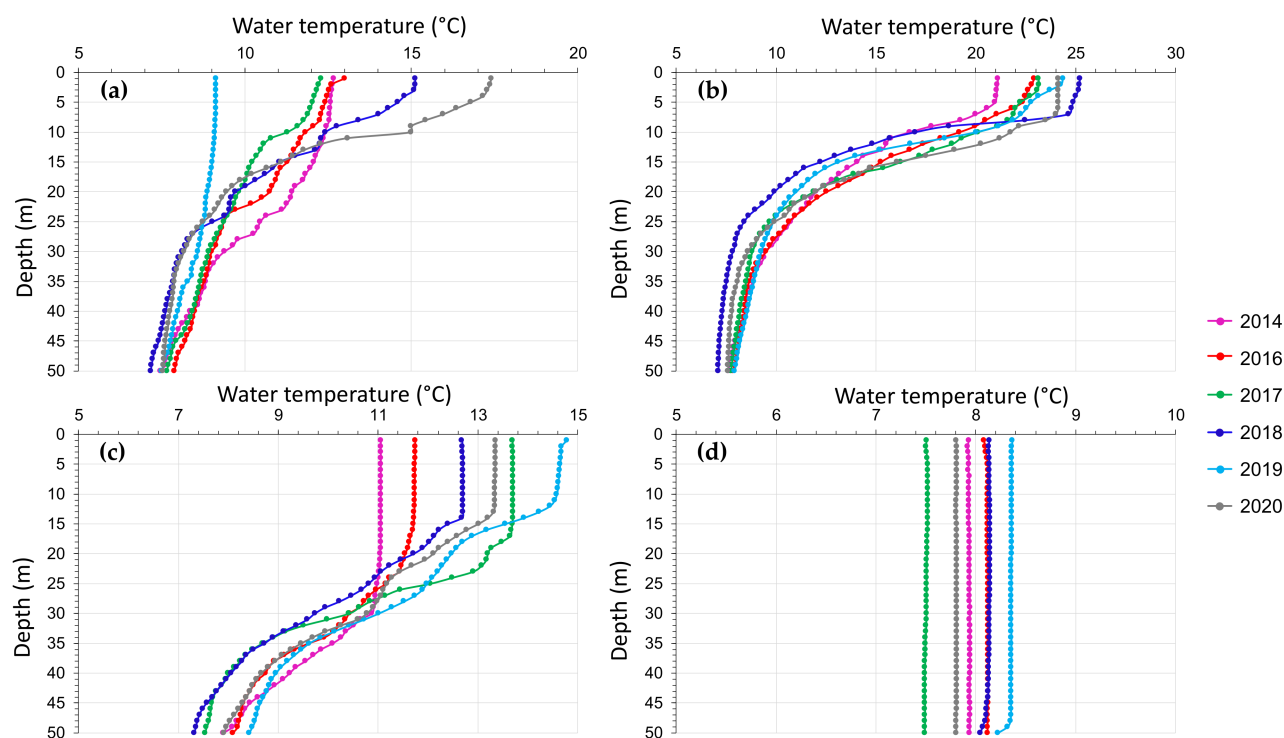


Figure 5. Vertical profiles of water temperature of the upper 50 m in Lake Maggiore during different seasons of the investigated years (2014 and 2016–2020): (a) spring; (b) summer; (c) autumn; (d) winter.

The total zooplankton biomass in Lake Maggiore (of the $\geq 450 \mu\text{m}$ size fraction) ranged between 3 and $33 \mu\text{g L}^{-1}$, with a mean of $13 \mu\text{g L}^{-1}$. These values are in line with the most oligotrophic sectors of Lake Champlain (USA), which showed biomass comprised between about 9 and $21 \mu\text{g L}^{-1}$ [8].

Miller et al. [8] calculated for Lake Champlain a Hg pool stocked in bulk zooplankton in the productive season (considering both $45\text{--}202 \mu\text{m}$ and $>202 \mu\text{m}$ size fractions) between 0.50 and 1.54 pg L^{-1} for THg and between 0.12 and 0.51 pg L^{-1} for MeHg. Most mercury was carried by the large-sized fraction of organisms, which included omnivores. From data reported by Stewart et al. [21] for a mining-impacted reservoir in California, the MeHg pool in bulk zooplankton can be calculated, with values up to 9960 pg L^{-1} in the most productive periods and down to 5.5 pg L^{-1} in winter. For Lake Maggiore, the results of this estimation demonstrated a THg stock between 0.38 and 2.80 pg L^{-1} (mean = 1.14 pg L^{-1}) for the $\geq 450 \mu\text{m}$ size fraction and between 0.03 and 1.32 pg L^{-1} (mean = 0.30 pg L^{-1}) for the $\geq 850 \mu\text{m}$ fraction. The lowest numbers occurred in the winter period and the highest in spring, following the zooplankton biomass dynamics. The same calculation for MeHg gave a stock between 0.13 and 0.88 pg L^{-1} (mean = 0.40 pg L^{-1}) for the $\geq 450 \mu\text{m}$ size fraction and between 0.02 and 0.14 pg L^{-1} (mean = 0.09 pg L^{-1}) for the $\geq 850 \mu\text{m}$ fraction.

The mercury pool in zooplankton was calculated also in relation to the lake surface (ng m^{-2} , Figure 6). To our knowledge, this estimation is not reported in other publications, but provides a simple and reliable method for the comparison between case studies. In fact, different sampling strategy, comprising both vertical tows at different depths and, with minor extent, horizontal tows, may hinder the comparison of Hg pools based on

filtered m^3 of water. In comparison, this estimation, based on lake surface (per m^{-2} instead of per m^{-3}), may help in understanding the significance of zooplankton as a repository for mercury readily available for higher trophic levels. In Lake Maggiore, THg stock was comprised of between 19 and 140 ng m^{-2} (mean = 57 ng m^{-2}) for the $\geq 450 \mu\text{m}$ size fraction and between 2 and 66 ng m^{-2} (mean = 15 ng m^{-2}) for the $\geq 850 \mu\text{m}$ fraction (Figure 6). For MeHg, a stock between 6 and 44 ng m^{-2} (mean = 20 ng m^{-2}) for the $\geq 450 \mu\text{m}$ size fraction and between 1 and 7 ng m^{-2} (mean = 4 ng m^{-2}) for the $\geq 850 \mu\text{m}$ fraction were calculated (Figure 6).

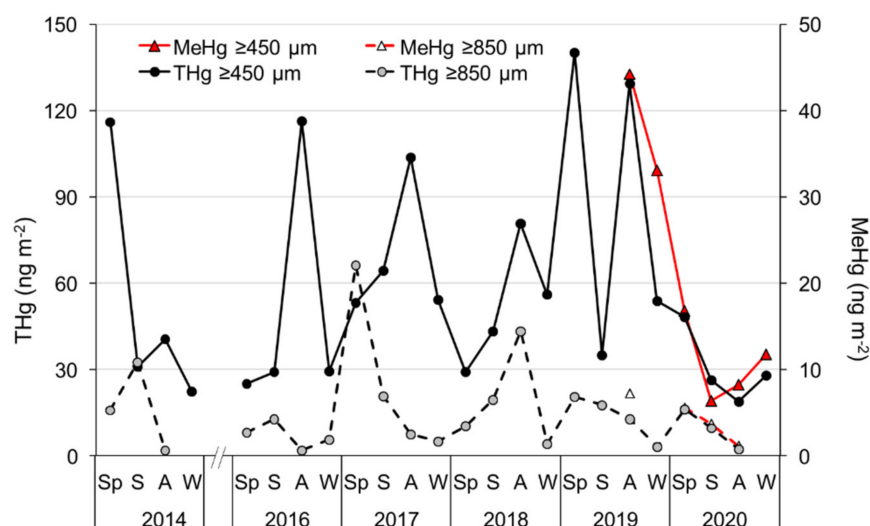


Figure 6. Mercury stock (as total mercury and methylmercury) in Lake Maggiore accumulated in zooplankton for two size fractions ($\geq 450 \mu\text{m}$ and $\geq 850 \mu\text{m}$) in different seasons of the investigated years (2014, 2016–2020). Sp = spring; S = summer; A = autumn; W = winter.

The major portion of mercury was stocked in the $\geq 450 \mu\text{m}$ size fraction, which accounted for the highest biomass, with the exception of spring 2017, when the $\geq 850 \mu\text{m}$ fraction resulted as being slightly higher (Figure 6). Seasonality was marked and similar in the two size fractions. In winter, the amount of mercury stocked in zooplankton was minimal, despite concentrations being highest, because standing stock biomass of zooplankton was the lowest. In the cold season, fish generally migrate toward the littoral zone to find trophic resources, such as benthic invertebrates [23] and to rest for spawning [57,58]. In contrast, in spring and summer, zooplankton may play a key role in the transfer of pollutants to zooplanktivorous fish, which in previous studies have been reported to migrate from the littoral zone (in winter) to the open water starting in spring [23,58]. Even if mercury concentrations in these seasons were lower than in winter, THg mass accumulated in zooplankton standing stock biomass was up to 14 times higher than in winter in the $\geq 850 \mu\text{m}$ fraction, being thus consistent and easily available for predators. The same trend was found for MeHg, the most bioavailable and toxic form of mercury, which reached in spring a total mass up to 5 times higher than in autumn in the $\geq 850 \mu\text{m}$ fraction (data are not available for this fraction for winter, because biomass was not enough for analysis). The percent of MeHg on THg was on average 43% (from 24 to 61%), confirming that zooplankton is an active and relevant vector of mercury to higher trophic levels.

4. Conclusions

This paper highlights the crucial role of zooplankton as repository of mercury pollution in Lake Maggiore. In deep lakes, mercury transfer in the pelagic pathway is generally prevalent over the benthic one, therefore the dynamics of mercury concentrations in zooplankton may help in understanding why the Hg levels of fish in Lake Maggiore still exceed the Environmental Quality Standard for biota, even if the main sources of the contaminant have been reduced and/or removed.

Seasonal dynamics, in particular species composition, biomass and trophic position ($\delta^{15}\text{N}\text{‰}$), proved to be key factors driving mercury levels in zooplankton. Mercury concentrations reached maxima during the cold season. However, the marked seasonality in zooplankton biomass, basically resulting in low biomass during the cold season and high biomass in the warm season, results in a larger mercury stock during the spring and summer, when fish predation is most active, than in winter, when zooplanktivorous fish migrate toward the littoral zone.

Nitrogen isotopic signature was strongly correlated with mercury concentration, as already observed for persistent organic pollutants in this lake and in other temperate lakes all over the world. Such close correlation proved significant for the two size fractions, pointing out the key role of the taxon which contributed most to the total zooplankton biomass, i.e., *Daphnia*. This taxon was largely present and often dominant in both size fractions, being relevant for tracing mercury transfer to zooplanktivorous fish. In fact, fish predation on zooplankton is strictly size specific and focused preferably on large prey, such as the $\geq 850\text{ }\mu\text{m}$ size fraction, which in Lake Maggiore is mainly dominated by *D. longispina-galeata* gr. In fact, mercury variations in terms of concentrations proved to be closely linked to the biomass dynamics of this taxon.

Mercury concentrations in fish of Lake Maggiore suggest a risk of secondary poisoning for top predators of trophic chains, such as piscivorous birds. Therefore, there is a need to better understand the sources and the trophic transfer mechanisms of the contaminant. Future works may better focus on the role of single zooplankton taxa as mercury vectors to fish.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14050680/s1>, Figure S1: Relative contribution of zooplankton taxa to total biomass for two size fractions ($\geq 450\text{ }\mu\text{m}$ and $\geq 850\text{ }\mu\text{m}$) collected seasonally in Lake Maggiore between 2014 and 2020; Figure S2: Nitrogen isotopic signatures for the two size fractions ($\geq 450\text{ }\mu\text{m}$ and $\geq 850\text{ }\mu\text{m}$) of zooplankton collected seasonally in Lake Maggiore in 2014–2020; Figure S3: Carbon isotopic signatures for the two size fractions ($\geq 450\text{ }\mu\text{m}$ and $\geq 850\text{ }\mu\text{m}$) of zooplankton collected seasonally in Lake Maggiore in 2014–2020.

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