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ANALYSIS OF INDIVIDUAL CLADOCERA AND COPEPODS FROM MESOTROPHIC LAKE USING THE PHENOM PROX-SEM/EDS

The stoichiometric analysis of Cladocera and Copepods from four different habitats of Obsterno mesotrophic lake in Belarus have been conducted in September 2016 using a method based on X-ray microanalysis, Phenom Prox Scanning Electron Microscope (SEM) with an elemental detection system (EDS), for measurement of atomic weight fractions of carbon, nitrogen and phosphorus elements in a designated area of zooplankton tissues. Phenom Prox SEM/EDS provided atomic weight percent of C:N, C:P, N:P ratios for Cladocera in pelagial, rush beds and nymphaea and for Copepods in pelagial, bare littoral, rush beds and nymphaea respectively. For Cladocera, the content of carbon in tissues was significantly higher in pelagial than in rush beds and nymphaea habitats ($p < 0.01$), opposite to nitrogen and phosphorous which contents were significantly lower in the pelagial ($p < 0.01$) comparing with other habitats. In the case of Copepods contents of carbon and nitrogen in their tissues did not differ between habitats. Significant among habitats differences were found for phosphorus – its content was significantly higher in Copepods from pelagial than from nymphaea habitat ($p < 0.01$).The purpose of this research is to determine the main biochemical elements content in zooplankton samples for different biotopes and to assess the elemental composition.

Keywords: elemental analysis, Cladocera, Copepods, pelagial, littoral.

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АНАЛИЗ ОТДЕЛЬНЫХ ВИДОВ CLADOCERA И COPEPODA МЕЗОТРОФНОГО ОЗЕРА С ИСПОЛЬЗОВАНИЕМ PHENOM PROX-SEM/EDS

Цель данного исследования – определение содержания основных биохимических элементов в отдельных видах зоопланктона из разных биотопов мезотрофного озера и оценка стехиометрического состава зоопланктона. Стехиометрический анализ Cladocera и Copepoda из четырех различных мест обитания в литоральной и пелагической зонах озера Обстерно (Беларусь) проведен в сентябре 2016 г. с использованием метода, основанного на рентгеновском микроанализе, Phenom Prox Scanning Electron Microscope (SEM) с элементной системой обнаружения (EDS). С помощью Phenom Prox SEM/EDS определен атомный массовый процент углерода, азота и фосфора в поверхностной области тканей карапакса зоопланктона и рассчитаны соотношения C:N, C:P, N:P для Cladocera из зарослей камыша и кубышки и для Copepoda из пелагиали, чистой литорали, камыша и кубышки. Для Cladocera содержание углерода в тканях было значительно выше в пелагиали ($p < 0{,}01$), а содержание азота и фосфора, напротив, было ниже в пелагиали ($p < 0{,}01$) по сравнению с другими средами обитания. В случае с Copepoda содержание углерода и азота в их тканях не различалось в зависимости от среды обитания. Значительные различия концентраций в зависимости от места обитания обнаружены для фосфора – его содержание было значительно выше у Copepoda из пелагиали по сравнению с копеподами из кубышки ($p < 0{,}01$).

Ключевые слова: элементный анализ, Cladocera, Copepoda, пелагиаль, литораль.

Introduction. Since Redfield [1, 2] delineated the linkage between the plankton composition and chemistry in waters, the elemental analysis of plankton has become a focal mutable for understanding oceans and freshwaters dynamics, and a key parameter in marine biogeochemistry, limnology, phytoplankton physiology, model formulation of ecological stoichiometry and global climate change [3–7]. The Redfield ratio elicited from the gain of nutrient concentration with depth represents a mean value of plankton elemental composition, and can alter accordingly with changes in the planktonic community [1]. A review of the current literature illustrates that this ratio spans at least one order of magnitude [4] and alternates at two levels: differences between species and larger taxonomic groups, and phenotypic variation between populations that are habituated to different physical or chemical parameters [5, 8].

It conforms that phytoplankton elemental stoichiometry is a good ecophysiological indicator for modeling and detecting variations in water biogeochemistry and plankton dynamics, but precise measurements of this parameter in the natural environment is the main requirement. It conforms that phytoplankton elemental stoichiometry is a good ecophysiological character for modeling and detecting variations in water biogeochemistry and plankton dynamics but precise measurements of this parameter in the natural environment is the main requirement.

Zooplankton grazing on phytoplankton can transfer more than 50 % of carbon fixed by primary production to higher trophic levels [9]. Zooplankton excretion strongly influences trophic dynamics in freshwater ecosystems by contributing inorganic N and P for primary and bacterial production [10–12]. Estimates of the fraction of N and P regenerated by zooplankton and then utilized by phytoplankton range from 14 to 50 % [13–15]. The main factors controlling this fraction include temperature, zooplankton and phytoplankton biomass and species composition. Because these factors interact dynamically, it has been difficult to quantify the role of zooplankton in nutrient cycling. Ecological stoichiometry deals with the patterns and processes associated with the chemical content of species. The aim of this study was to determine the differences in the stoichiometry (C, N, and P content as well as C:N, C:P, N:P ratios) of Cladocera and Copepods, as the dominant systematic groups of Obsterno lake, inhabited four different habitats of this ecosystem: pelagial, littoral, rush beds and nymphaea. Our analysis may provide insight into topics like nutritional and energetic status of different types of lake's habitats, as well as planktonic trophic interactions.

Material and methods. The study area was the mesotrophic Lake Obsterno, which is located in the south of Miory district of Vitebsk region (north-west of Belarus). Its maximum and mean depths are 12 and 5 m. The zooplankton samples were collected in September 2016, from three littoral zones (approximate depth of 1.2–1.5 m) and one pelagic station (depth of 5 meters) using GPS coordinates. During sampling there were no wind and no wave in lake and transparency amounted 4 meters. In littoral 3 stations were chosen: one from the open water including open sandy intertidal zone covering *Chara sp.*(clean littoral) and two among the macrophytes – one of them with common rush beds (*Schoenoplectus lacustris*), and the second one among nymphaea (*Nuphar lutea*), extending from the bottom to the surface (in pelagic zone – once and other three stations two/three times) inorder to filter water. This volume for pelagial, clean littoral, rush beds and nymphaea were respectively 206.06, 235.5, 105.13 and 168.9 liters. Also collecting zooplankton samples was carried out via taw net with 25 cm diameter and 100 μm mesh size and fixed in ethanol 95 % to measure elemental analysis. Previously, we checked fresh zooplanktons with fixing ones and measured them via EDS, after seeing no difference between samples, ethanol was chosen as a fixative.

All biotope sample collections were taken once a day in 3 replicates at 10 – 12 a. m., then fixed by 4 % formalin solution transformed to the lab in order to define the species composition. Taxonomic identification was performed under a stereo microscope MBS-10. Simultaneously the samples for the analysis of carbon, nitrogen and phosphorus content in zooplankton tissues have been prepared. EDS analyzer uses an energy-dispersive spectrometer detector that simultaneously identifies and quantifies not only the most important elements in marine and freshwater ecology such as C, N and P, but also O, Mg, Na, Al, Si, S, Cl, K, and Ca that are present on single natural cells or tissues. This technique has been available for more than 20 years for determining elemental composition in marine and freshwater phytoplankton [16–18] and bacterioplankton [19, 20] used successfully to analyze the complete elemental composition of single marine bacteria and cyanobacteria [21, 22], [18] too. Elemental analysis were performed using a Phenom ProX desktop scanning electron microscope (LOT-QuantumDesign) equipped with a thermionic CeB6 source and a high sensitivity multi-mode backscatter electron (BSE) detector, 15 Kv EHT (primary-beam energy) for animals with ≤ 1.5 mm length. Finally 26 Cladocera and Copepods were used for analysis. For each animal five homogeneous zones of the same area was determined for the detection of three major elements: atomic carbon (% C), atomic nitrogen (% N) and atomic phosphorus (% P). For some under detection phosphorus atomic ratios, an amount of < 0.2 % has been considered for atomic weight fractions. Moreover, fixation and dehydration, critical point drying, coating with gold and using sputter coater image processing (software Scandium) was done in order to make a SEM pictures of the zooplankton individuals. Analysis using Phenom Prox Scanning Elektron

Microscope (SEM) with an elemental detection system (EDS), were conducted at Faculty of Biology and Environmental Protection University of Lodz, Department of Applied Ecology in December 2016.

Results and discussion. All statistical analyses were conducted using Statistica 12.0 (StatSoft, Inc; license of Faculty BEP UL: JPZP605E651727AR-T). Elemental atomic ratios were estimated as the average of individual ratios in a given population. To test for the effect of habitat type on the animals' stoichiometry, a one-way ANOVA was used with Tukey post hoc test.The dominant species in the respective habitats were determined by the mentioned abundance percentage of total zooplankton community, as follow: a) in bare littoral: *Eudiaptomus graciloides* 38.83 %, *Diaphanosoma brachyurum* 12.62 %, *Thermocyclops oithonoides* 10.67 % and *Mesocyclops leuckarti* 10.67 %; b) in pelagial: *Eudiaptomus graciloides*. 56.39 % and *D. brachyurum* 19.30 %; c) in rushbeds: *Allonella sp.* 24.48 %, *Ceriodaphnia pulchella* 20.91 %, *Thermocyclops oithonoides* 16.32 % and *Mesocyclops leuckarti* 9.18 %; d) in nymphae: *Bosmina* sp. 25.19 %, *C. pulchella* 18.89 %, *Allonella sp.* 16.19 %, *Thermocyclops oithonoides* 10.79 % and *M. leuckarti* 6.29 %. According to the statistical analysis atomic weight fractions of carbon and nitrogen in tissues of Copepods did not differ between habitats (Fig. 1, 2). Significant differences among habitats were found in the case of phosphorus ($F_{3,61} = 4.56$; $p < 0.01$). Its content was significantly higher in tissues of Copepods from pelagial than from nymphae habitat (Fig. 3).

Table 1. **Atomic weight ratios for individuals of Cladocera in different habitats**

Cladocera	C:N	N:P	C:P
Pelagial	2.41:1	106.24:1	245.46:1
Rush beds	1.53:1	76.47:1	109.61:1
Nymphae	1.34:1	123.36:1	158.9:1

Table 2. **Atomic weight ratios for individuals of Copepods in different habitats**

Copepods	C:N	N:P	C:P
Pelagial	1.50:1	54.94:1	80.97:1
Bare littoral	1.27:1	87.30:1	110.75:1
Rush beds	1.08:1	123.92:1	123.92:1
Nymphae	1.36:1	115.5:1	144.4:1

In the case of Cladocera contents of all three elements differed significantly among habitats ($F_{2,26} = 11.62$, $p < 0.001$; $F_{2,26} = 12.54$, $p < 0.001$ and $F_{2,28} = 7.46$, $p < 0.01$ for carbon, nitrogen and phosphorus, respectively). Content of carbon in tissues of Cladocera from pelagial was significantly higher than those from other two habitats. In turn carbon contents did not differ between Cladocera from nymphaea and rush beds habitats (Fig. 1).

Analysis of atomic nitrogen in tissues of Cladocera revealed that content of nitrogen as significantly lower in pelagial than those in other two habitats. Similarly as in the case of carbon nitrogen contents did not differ between nymphaea and rush beds habitats (Fig. 2).

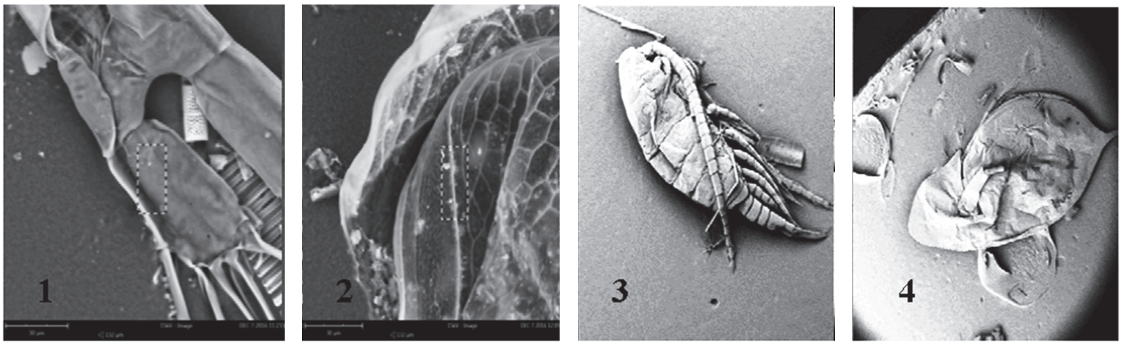

Fig. 1. The zones of elements' detection in Copepod and Cladocer (1 and 2). SEM pictures of individual Copepod and Cladocera (3 and 4)

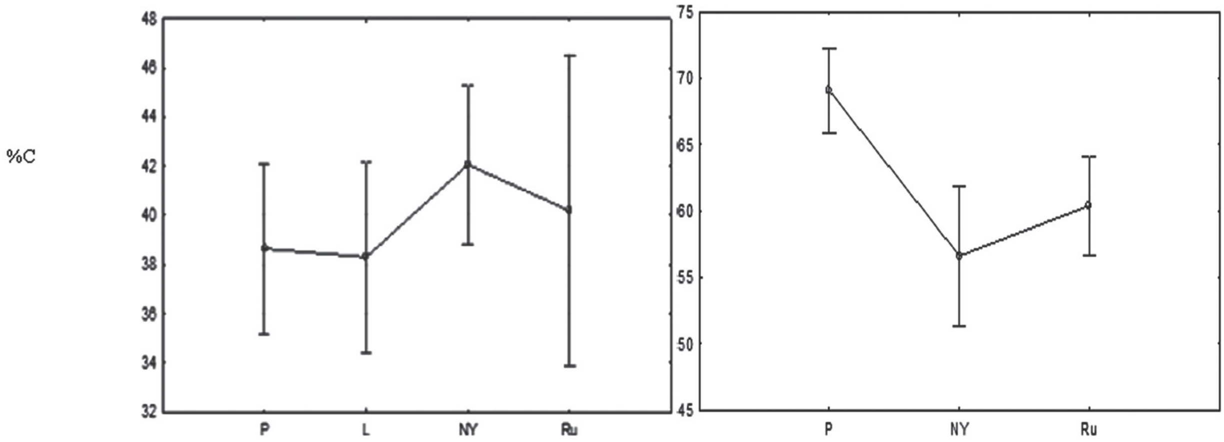

Fig. 2. Atomic carbon percent: A) in tissues of Copepod from pelagial, littoral, nymphae, rushbeds and B) in tissues of Cladocera from pelagial, nymphaea and rushbeds

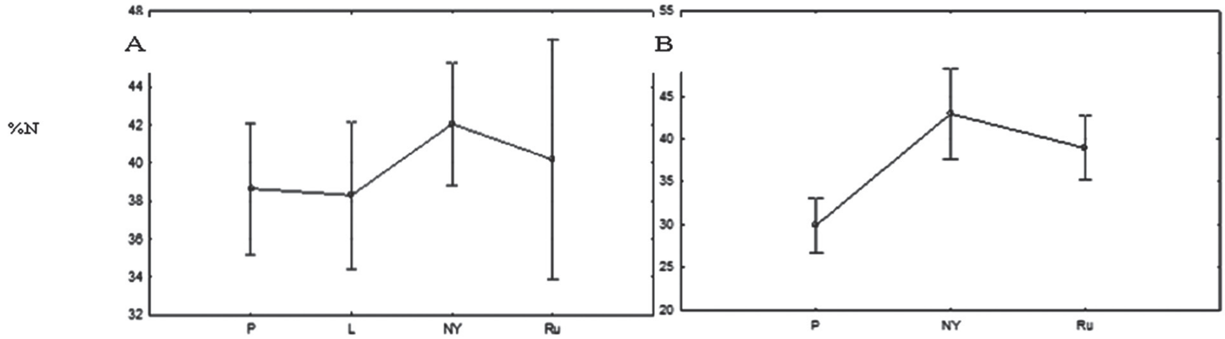

Fig. 3. Atomic nitrogen percent: A) in tissues of Copepod from pelagial, littoral, nymphaea, rushbeds and B) in tissues of Cladocera from pelagial, nymphaea and rushbeds

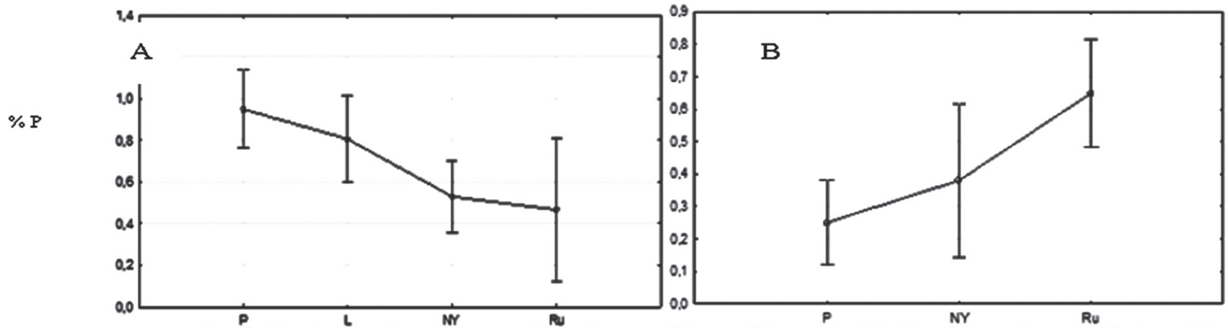

Fig. 4. Atomic phosphorus percent: A) in tissues of Copepod from pelagial, littoral, nymphaea, rushbeds and B) in tissues of Cladocera from pelagial, nymphaea and rushbeds

Statictical analysis of phosphorus in tissues of Cladocera showed that its content was significantly higher in rush beds than in pelagial (Fig. 3). Other differences were not significant. The phosphorus content was more variable than the nitrogen content in our Copepods (Fig. 2, 3). However no significant difference among Copepods has been found.

Stoichiometric analysis of Cladocera and Copepods has been investigated mostly for pelagial and trophogenic layer of the lakes where filtrators (and partially predators) are usually dominant species. In our survey, pelagic Cladoceran species in fall was *D. brachyurum* which is a typical planktonic spesies but for pelagic and littoral Copepods, *Eudiaptomus graciloides* has been developed because of similar conditions in these two biotopes including lack of macrophytes and interconnection of water. In rush beds and nymphaea because of detritus presence and death of macrophytes, planktobentic species have been dominated in Obsterno lake. The atomic carbon of Cladocera and Copepods revealed an accordance with Coles result [23] and Copepod (*Eudiaptomus graciloides*) carbon in pelagial (60.28 ± 5.58) was higher than littoral (58.94 ± 8.21). As for Cladoceran dominant species(*D. brachyurum*), average atomic content of carbon in pelagial (69.07 ± 5.24) was higher than in littoral (59.14 ± 6.21).

Eudiaptomus graciloides as a dominant Copepod in pelagial (38.66 ± 5.50 %) and clean littoral (38.30 ± 9.85 %) showed the lower nitrogen rate than Copepods taken from rush beds (40.18 ± 12.01) and nymphaea beds (42.05 ± 6.36 %), in contrast, the nitrogen content of pelagic Cladocera (29.90 ± 5.15 %) revealed the lower ratio than nymphaea beds (42.96 ± 4.53 %) and rush beds (38.95 ± 6.96 %) which was caused by *C. pulchella* as dominants consuming nitrogen and saving it in their tissues but with less carbon. Cole [23] showed that atomic carbon for Cladocera (up to 30%) substantially was higher than Copepod (from 10–30 %) as well as ours. It could be due to deep feeding by vertically migrating zooplankton and consumption of detritus of algal but for nitrogen, the higher rate was reported for *Eudiaptomus graciloides* sp because of feeding on a higher trophic level than feeding on different basal food sources or both. As for our result, atomic carbon for Cladocera and nitrogen for Copepods were higher that was completely in agreement with Coles result but more sophisticated approach may be needed for future to achieve more precise results for secondary consumers.

Storage of elements especially P is relatively well-studied in some organisms. Less is known about elemental storage in many metazoans.It's known that freshwater zooplankton may acquire or assimilate modest amounts of P in excess of immediate growth demands over short time scales. In our research, P amount of pelagic Copepods in fall was higher than pelagic Cladocera – it could be due to *Eudiaptomus* which is a longliving zooplankton and saves more energy for resting period in comparison with Cladocera. Zooplankton has a relatively constant, species-specific C:N ratio [24]. Our survey revealed that in pelagial zone where owns the highest zooplankton abundance of Copepod species *(Eudiaptomus graciloides)*, has relatively high C:N ratio in comparison with the other habitats where dominant Copepods' species were different from pelagial. Thus, it seems that *Diaptomidae* store more carbon and less nitrogen. This could be due to high energy cost on many physiological processes or as Cole [23] reported for *Diaptomus* sp because of feeding on a higher trophic level than feeding on different basal food sources or both. According to Speas and Duffies [25] research, *Daphnia* is not significant to its C balance because there is an evidence that some zooplankton, especially Cladocera, will ingest numerous kinds of particles but this C balance controlling Cladocera is still unclear in our investigate because different species were dominated in fall in Lake Obsterno. On the other side, Daphnia has an especially low N:P compared to all other zooplankton so far measured. This caused the low N:P ratios of rushbeds Cladocera as well as C:P. We conclude that taxon-specific differences in the N:P ratio in these species are mostly attributable to differences in P content. Despite a known wide variation in the elemental ratios in their food, the Nand P-content of individual zooplankton species show a remarkably stable N:P ratio in Cladocerans from pelagial and littoral. Tamelander [26] by their investigation on Copepod and vertical flux of particulate organic carbon, nitrogen and phosphorus mentioned that sinking of large zooplankton provides an important mechanism for removing phosphorus from the water column. The relative importance of this pathway is that smaller, slower-sinking food is recycled faster in water coloumn. As for our data, mean size of pelagic zooplankton (0.95 mm) is larger than littoral (0.54 mm) and accordingly, removing phosphorus is (or might be) more than littoral. Enhancing in *Daphnia* biomass occurring most strongly in lakes with low particulate C:P as well as our C:P ratio in littoral with *Ceriodaphnia pulchella* and *Thermocyclops. oithonoides* as dominant species [27]. In contrast, [28] found that increasing the food C: P ratio produced greater DOC release by *Daphnia*. In zooplankton, little variability in C:N:P ratios was originally reported within taxonomic groups, and large differences were found among taxa [29, 30]. Interspecific differences in zooplankton elemental composition can be explained at complementary levels that link organismal physiology and evolution [31, 32]. Interspecific variation in zooplankton body C:N:P ratios reflectthe relative contribution of primary cellular biomolecules and different life-history strategies that result from costs associated with maintaining P-rich body stoichiometry [31],[33].

Atomic C:N, C:P and N:P of some marine planktonic Copepods in fall [34] in comparison with our data were respectively 5.7 ± 0.5, 114 ± 34, 20 ± 6 and 1.08–1.50, 80.97–144.4, 54.24–123.92. C:N ratios in Copepods are relatively low. High trophic level species such as *Eudiaptomus grasiloides* experience a variable physical and trophic environment, thus some species accumulate lipids during periods of high food abundance, rest during winter, and invest the stored lipids in reproductive tissue in winter the following year. Less lipid is used for basal metabolism through the winter [35]. This is reflected in high C:N ratios during summer but low C:N ratios during autumn up to the spring bloom [36, 37]. On the other side, the female Copepods as we had in our samples ,can display low, and fairly rigid, C:N ratios indicating that their preadult lipid stores were allocated to moulting and reproduction. Species composition of the zooplankton compartment is important for the N and P allocated in zooplankton biomass. C:N:P ratios in algae only approach Redfield ratios when algae grow at near maximum rates [38, 39]. This affects Copepod food intake, and preferences for fast growing algae [40] may pay off as an increase in fecundity [39]. The composition of the zooplankton community will affect the distribution of nutrients in the food web, as the elemental composition differs among zooplankton taxa. The N:P ratios differ between trophic levels. C:N and C:P ratios in carnivorous zooplanktons such as *Diaptomidae* is low compared to its zooplankton food [41, 42]. This concomitantly higher demand for nitrate- and phosphorus-rich food at higher trophic levels indicates that more N and P compared to C may be retained in organic material, and less is regenerated as inorganic nutrients. Cladocera in marine systems do not have the same unique position as their freshwater relatives, but can be abundant in surface layers. Cladocera analysed in Gismervik s study displayed low C:P ratios of 59 ± 22 and 34 ± 5, respectively which is in contrast with our freshwater Cladocera (C:P from 109 to 245 in pelagial and littoral) [34].

Hall [43] found that zooplankton body composition did not consistently explain the distribution of different species along a P-supply gradient. They concluded that body P content was only a poor predictor of the animal's nutrient demand, as this demand is also affected by the assimilation efficiency for the different elements and respiration rates. However, it was shown that consumer nutrient content correlates strongly with threshold elemental ratios which include respiration losses and assimilation efficiencies to define the ratio of two elements where the limitation switches from one to the other [44]. Thus, the use of body C:N:P ratios may be well applicable to indicate nutrient demand across species.

As a conclusion, the body content of P, N, and C in consumers is one indicator of their demands for these elements. Because the C:N:P ratios of many littoral planktons remain unknown, there is yet little basis to apply recent developments in ecological stoichiometry to studies of ecological processes in littoral systems [45, 46]. Also using a Phenom ProX SEM/EDS method that can be applied to a wide range of ecosystems, we found significant differences in stoichiometry of pelagic zooplankton compare with littoral. The literature reports a range in CNP atomic ratios for zooplankton among habitats and taxa. Zooplankton are selective feeders, some taxa more than others, and phytoplankton is usually a preferred food. As methods improve and more studies are conducted, we expect considerable variation in support of consumers, which should lead to the development of models that explain this variation among ecosystems. Further, improved stable isotopes in food web such as δ13C and δ15N that better incorporate uncertainty in sources and to estimate the relative contribution of diet sources to food web compartments will likely aid in producing better models.

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