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Linking the environmental factors and heterotrophic bacteria to the variability of carbon isotope ($\delta^{13}C$) across crustacean zooplankton in shallow freshwater ecosystems

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Abstract

Planktonic crustaceans are important part of carbon cycling in in shallow, temperate freshwater habitats. The objective of the study was to examine the link between selected environmental variables, densities of various groups of bacteria and $\delta^{13}C$ of calanoid copepods, cyclopoid copepods and *Daphnia* sp. Generalized Linear Models (GLMs) with stepwise-selected environmental predictors showed that $\delta^{13}C$ of calanoid copepods was positively correlated with the Day of the year and Other bacteria density and negatively correlated with NH_4^+ and NO_3^- concentrations. Cyclopoid copepods' $\delta^{13}C$ was correlated exclusively with water temperature, which showed a positive effect on the stable carbon isotopes ratio. GLM for *Daphnia* sp. was the most complex and retained the Day of the year, Other bacteria density and PO_4^{3-} as positively correlated predictors, while β-proteobacteria NH_4^+ and NO_3^- were included in the model as negatively correlated predictors for $\delta^{13}C$. Our study highlights the complex impact of environmental conditions, microbial processes and functional trait-driven responses on $\delta^{13}C$ values in planktonic crustaceans. Thanks to integration of plankton ecology, microbiology and the analysis of stable isotopes the study improves the understanding of carbon flow in shallow freshwater ecosystems

1. Introduction

Functioning of freshwater ecosystems is strongly reliant on pelagic food webs with planktonic crustaceans (copepods and cladocerans; members of zooplankton community) interconnecting primary producers (bacteria, algae) and higher trophic levels (invertebrates, fish). Filter-feeding cladocerans of genus *Daphnia* are widely accepted keystone taxon, constituting phytoplankton-herbivorous zooplankton link of energy transfer (Ger et al., 2016; Sommer et al., 1986; Straile, 2015). *Daphnia* is also recognized as an efficient bacteria grazer which obviously is an advantage, under shortage of algal food (Martin-Creuzburg et al., 2011). However, currently observed global climatic changes, forcing alterations in freshwater food webs, may lead to loss of significance of cladocerans of genus *Daphnia*, as conduits of energy flow and to destabilization of freshwater food webs (revised by Wojtal-Frankiewicz, 2012). Findings of de Senerpont Domis et al. (2007) suggest that far-reaching climate warming (+6°C) may trigger phenological mismatches between *Daphnia* and its prey. Thus, the 'center of gravity' in functioning of freshwater food webs may be switched to copepods which frequently are an abundant component of zooplankton community and beside ability of suppressing biomass of phytoplankton, extensively forage on protozoans (De Meester et al., 2023; Krztoń et al., 2022; Sommer et al., 2003).

Planktonic crustaceans are important part of "fast carbon cycle", which refers to carbon cycling in biosphere. These animals rely on three distinct carbon sources: carbon dioxide (CO_2), dissolved organic carbon (DOC), and methane (CH_4), mobilized by phytoplankton, heterotrophic bacteria and methanotrophic bacteria (methane-oxidizing bacteria; MOB), respectively (Jones and Grey, 2011). Substantial part of the carbon flowing to crustaceans may originate from bacteria (Wylie and Currie, 1991), which strongly contribute to biogeochemical cycle of carbon (Baltar et al., 2015). This is a reason why bacteria are recognized as an important part of in the energy flow through the food web. Some authors showed that the bacteria's significance vary depending on temporary environmental conditions (Hanashiro et al., 2023; Louati et al., 2023). Study of Hall and Cotner (2007) demonstrates strong dependence of bacteria-mediated carbon cycling on temperature and nutrients availability, which is a significant observation given the increase in temperature caused by climate change.

The detailed assessment of carbon sources in the diet of planktonic crustaceans and studies over the pathways of carbon flow in pelagic freshwater food webs remain important topics in the field hydrobiological researches. Therefore, we measured stable carbon isotope ratio ($\delta^{13}C$) which is a powerful indicator used for tracing carbon flow, and its values vary in tissues of diverse organisms depending on the pathway that carbon is incorporated into the food web (Hobson, 2023). One of the pathways can be constituted by bacteria (microbial loop) that might provide nutritional benefits for crustacean grazers, feeding both directly (cladocerans) and indirectly via transitional consumers (copepods and cladocerans) (Wenzel et al., 2021).

Previous studies showed that isotopic niches of planktonic crustaceans increase in area and overlap during cyanobacterial bloom compared to pre-bloom conditions (Krztoń et al., 2025). Here, we follow this background and aim to explore variability of $\delta^{13}C$ in planktonic crustacean communities.

The objective of the study was to examine the link between selected environmental variables, densities of various groups of bacteria and $\delta^{13}C$ of planktonic crustaceans (calanoid copepods, cyclopoid copepods and *Daphnia* sp.) in shallow, temperate freshwater habitats. Based on the fact that copepods are not likely to feed directly on bacteria, we hypothesized that among studied crustaceans, only *Daphnia*'s $\delta^{13}C$ is driven by densities of various bacteria groups. In turn, we expected $\delta^{13}C$ of copepod to be dependent on physical and/or chemical parameters of water. Addressing these research questions we aimed to explore the role of planktonic crustaceans in biogeochemical cycles in shallow freshwater systems.

2. Materials and methods

2.1 Study area

The study was conducted in five shallow, eutrophic water bodies: three oxbow lakes of the Vistula River: Tyniec 1 (50° 01'47" N, 19° 49' 39.8" E), Tyniec 2 (50°01'28.1" N, 19°48'47.7" E), Wołowice 1 (49°59'18.0"N 19°45'01.3"E) and two artificial ponds: Podkamycze 1 (50° 05'11" N, 19° 50' 01.6" E) and

Podkamycze 2 (50° 04′ 59.6″ N, 19° 50′05.4″ E) located in the area of Cracow and nearby (Southern Poland).

2.2 *Sampling and environmental factors analyses*

Samplings were carried out bi-weekly in the vegetation season (April - October) in 2019. Samplings were performed at the central point of each waterbody with the use of a 5 L sampler. Integrated water samples covered the whole column of the water (sampling interval: each 0.5 m starting from surface to the bottom). Each sample included: physico-chemical parameters of water (temperature, pH, conductivity, PO_4^{3-}, NH_4^+, NO_3^- concentration), bacterioplankton composition and density and samples for analyses of stable isotopes of carbon ($\delta^{13}C$) in tissues of planktonic crustaceans (see below). In addition to collecting samples, maximum depts and transparency were measured in situ, each time, in each reservoir. In total 75 environmental samples were collected.

Water transparency was measured using Secchi disc. Water temperature, pH and conductivity were measured using YSI ProDSS sonde. Samples for ions concentrations (PO_4^{3-}, NH_4^+, NO_3^-) were cold-stored and immediately transported to laboratory of the Institute of Nature Conservation, Polish Academy of Sciences. The samples were determined in the laboratory using Dionex ion chromatograph (DIONEX, IC25 Ion Chromatograph; ICS-1000, Sunnyvale, CA, USA).

2.3 *Bacterial communities analysis*

The water samples (vol. 10mL) for bacteria community analysis were fixed on filters (0.2 µm pore size; type GTTP; Millipore). Bacterial community composition was determined using fluorescent in situ hybridization (FISH) with Cy3-labelled oligonucleotide probes, following the protocols described by Glöckner et al. (1996) and Pernthaler et al. (2001). The probe set targeted the major phylogenetic groups of Proteobacteria, including Alpha- (α-), Beta- (β-), Gamma- (γ-), and Delta- (δ-) subclasses. Probe target sites and hybridization conditions followed the recommendations of Amann et al. (1990), Daims et al. (1999), Alm et al. (1996), Manz et al. (1992) and (Lücker et al., 2007). To assess autofluorescence and non-specific probe binding, all samples were simultaneously hybridized with the NON338 probe, complementary to EUB338. The proportion of non-specifically stained cells did not exceed 5% in any sample. Filter sections were examined using an epifluorescence microscope (Olympus BX61) equipped with a ×100 oil-immersion objective, UV illumination, and a CCD camera with DAPI and Cy3 filter sets (Olympus, Poland). Images were processed using Cell F software (Olympus, Poland). The abundance of FISH-positive cells was quantified in triplicate by counting at least 1,000 DAPI-stained cells per sample.

2.4 *Analysis of stable isotopes*

The samples for analysis of stable isotopes of carbon ($\delta^{13}C$) in tissues of planktonic crustaceans were collected using vertical hauls performed with plankton net of mesh size 50 µm. In the laboratory, using a light microscope and micromanipulator, samples were segregated to the following fractions: 1) Calanoid copepods (N = 36), 2) cyclopoid copepods (N =29), 3) and *Daphnia* sp. (N = 12). Single samples consisted of 30 - 100 adult specimens. In total, $\delta^{13}C$ were determined in 77 samples of planktonic crustaceans.

The stable carbon isotope analyses were conducted at the Stable Isotope Laboratory of the Institute of Geological Sciences, Polish Academy of Sciences (Warsaw, Poland), using a Flash 1112 HT elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA) operating in continuous-flow mode with helium as the carrier gas. Carbon isotope ratios are expressed in δ notation as per mil (‰) relative to the Vienna Pee Dee Belemnite (VPDB) standard. Duplicate measurements were calibrated against three international reference materials: USGS 41a (L-glutamic acid, $\delta^{13}C$ = + 36.55 ± 0.08 ‰), USGS 40 (L-glutamic acid, $\delta^{13}C$ = -26.389 ± 0.042 ‰) and IAEA 600 (caffeine, $\delta^{13}C$ = -27.771 ± 0.043 ‰). Instrument performance and drift were monitored using USGS 65 (glycine, $\delta^{13}C$ = –20.29 ± 0.04 ‰) as an internal laboratory standard. Analytical precision and reproducibility, based on repeated measurements of the laboratory standard during each analytical run, were better than ±0.1‰ (1σ) for $\delta^{13}C$. Carbon-to-nitrogen (C:N) ratios were determined from the same analyses with an accuracy of ±0.1. Because lipids are depleted in ^{13}C and may bias $\delta^{13}C$ measurements in planktonic crustaceans, carbon isotope values were corrected for lipid content using the elemental C:N ratio following the equation proposed by (Syväranta and Rautio, 2010).

2.5 Statistical analyses

All statistical analyses and data visualizations were performed with R V. 4.2.2 and RStudio statistical software (R Core Team, 2022). Values of $\delta^{13}C$ were tested for differences among studied groups of planktonic crustaceans using ANOVA (‘stats’ package; (R Core Team, 2022). Tukey's Honest Significant Difference test (HSD; ‘stats’ package; R Core Team, 2022) was applied in order to calculate confidence intervals between means of $\delta^{13}C$ among particular crustacean groups.

To identify environmental and microbial drivers of carbon isotopic variation ($\delta^{13}C$) in zooplankton, generalized linear models (GLMs) with Gaussian error distribution and identity link function were fitted separately for Calanoid copepods, Cyclopoid copepods, and *Daphnia* sp. For each group, the initial (full) model included sampling day, bacterial abundance variables (total Proteobacteria, α-proteobacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria, and the group Other bacteria), water temperature, and nutrient concentrations (NH_4^+, NO_3^-, PO_4^{3-}) as explanatory variables. Prior to model selection, multicollinearity among predictors was assessed using variance inflation factors (VIFs; ‘car’ package, Fox et al., 2019), which remained below commonly accepted thresholds. Model selection was performed using stepwise Akaike Information Criterion (AIC) optimization with both forward and backward selection (*stepAIC*, Venables and Ripley, 2013) to identify the most parsimonious models explaining variation in $\delta^{13}C$. The results of stepwise regression were visualized with use of 'visreg' package (Breheny and Burchett, 2017).

3. Results

3.1 Environmental variables

Water temperature in studied lakes ranged from 10.8 to 25.6 °C (mean: 18.2°C), reaching highest values in July and August. Concentrations of NO_3^- ranged between <0.01 and 18.8 mg/L (mean: 4.6 mg/L). Highest mean concentrations of NO_3^- were detected in the period of beginning of April to end of May, while the lowest concentrations were noted between beginning of June to mid-August. PO_4^{3-} concentrations measured in collected samples ranged between <0.01 and 0.44 mg/L (mean: 0.42 mg/L). The highest mean concentrations of PO_4^{3-} were detected in the period of mid-May to end of the August. Concentrations of NH_4^+ ranged from <0.01 to 0.99 mg/L (mean: 0.18 mg/L). Highest mean concentrations of NH_4^+ were detected from beginning of May to end of June, and then from beginning of September to end of October. Lake specific ranges, mean values, standard deviations and coefficients of variance of studied parameters are given in Table 1.

Table 1. Lake specific ranges, mean values, standard deviations (SD) and coefficients of variance (CV) of water temperature and concentrations of NO_3^-, PO_4^{3-} and NH_4^+.

		Waterbody				
		Tyniec 1	Tyniec 2	Wołowice 1	Podkamycze 1	Podkamycze 2
Temperature [°C]	Mean	18.89	19.73	14.85	17.27	19.86
	Range (min-max)	11.8 – 25.5	11.2 – 25.5	10.8 – 18.3	10.9 – 23.7	12.1 – 25.6
	SD	4.62	5.03	2.16	4.01	5.19
	CV	24.43	25.5	14.56	23.24	26.13
	Mean	1.44	1.39	8.99	11.43	1.99

NO_3^- [mg/L]	Range (min-max)	0.003 – 4.91	0.02 – 6.17	0.05 – 18.83	9.43 – 15.03	0.19 – 6.13
	SD	1.5	1.59	6.44	1.47	2.21
	CV	103.95	114.59	71.76	12.88	110.31
PO_4^{3-} [mg/L]	Mean	0.02	0.05	0.077	0.08	0.003
	Range (min-max)	0 – 0.1	0 – 0.11	0 – 0.44	0 – 0.18	0 – 0.02
	SD	0.033	0.04	0.12	0.07	0.003
	CV	205.31	80.72	154.89	83.52	126.87
NH_4^+ [mg/L]	Mean	0.1	0.2	0.45	0.03	0.02
	Range (min-max)	0.01 – 0.2	0.001 – 0.99	0.06 – 0.97	0.003 – 0.09	0 – 0.09
	SD	0.06	0.29	0.32	0.03	0.02
	CV	59.96	145.29	71.24	104.62	98.81

3.2 Bacterial communities

Analyses of bacteria revealed presence of following groups of bacteria: Proteobacteria, α-proteobacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria and Other bacteria. Total number of planktonic bacteria cells ranged from 5.55×10^6 to 8.58×10^7 cells per mililiter. The largest mean densities were observed for Proteobacteria and Other bacteria, while the lowest for α-proteobacteria group. The largest variability of densities (based on CV) was found for the group of Other bacteria while the lowest for β-proteobacteria (Tab. 2).

Table 2. Range (Min-Max), Mean, standard deviation (SD) and coefficient of variation (CV) of densities of studied bacteria groups in samples collected through the sampling season

	Range (Min-Max) [Cells/mL]	Mean [Cells/mL]	SD [Cells/mL]	CV
Proteobacteria	2.94×10^6 - 5.98×10^7	1.49×10^7	9.65×10^6	0.65

α-proteobacteria	1×10^5 - 3.01×10^6	1.03×10^6	7.43×10^5	0.72
β-proteobacteria	4.34×10^5 - 5.45×10^6	2.15×10^6	1.02×10^6	0.47
γ-proteobacteria	1.34×10^5 - 5.77×10^6	1.51×10^6	1.18×10^6	0.78
δ-proteobacteria	2.11×10^5 - 5.70×10^6	2.45×10^6	1.49×10^6	0.61
Other bacteria	2.37×10^5 - 4.80×10^7	9.57×10^6	1.01×10^7	1.06
Total bacteria	5.55×10^6 - 8.58×10^7	3.16×10^7	1.68×10^7	0.53

3.3 Crustacean zooplankton

Composition and distribution of studied planktonic crustaceans varied throughout the sampling period (Figure 1). Calanoid copepods were found in all of the samples collected throughout the year (beginning of April - end of October). Cyclopoid copepods and Daphnia sp. showed similar frequency, with exception for the beginning of April (when *Daphnia* sp. was absent) and mid-July (when cyclopoid copepods were absent).

3.4 Stable isotopes

Mean values of $\delta^{13}C$ were found in calanoid copepods accounted for -31.8‰ with range between -41.5‰ to -23.7. Mean $\delta^{13}C$ values of cyclopoid copepods was -30.6‰ and range: -40‰ to -22.2‰). $\delta^{13}C$ measurements of *Daphnia* sp. showed mean value of -31.2‰ and range between -42.5‰ and –24.4‰)

No statistically significant differences among crustacean groups in mean $\delta^{13}C$ values were found among Calanoid copepods, Cyclopoid copepods ($p<0.01$) and *Daphnia* sp. (Supplementary material, Tables S1 and S2).

For calanoid copepods, the full GLM model explained a substantial proportion of deviance relative to the null model. Stepwise AIC selection retained a reduced model including day of the year, β-proteobacteria, Other bacteria, and concentrations of NH_4^+, NO_3^-, PO_4^{3-}. $\delta^{13}C$ values in calanoid copepods increased significantly over time, as indicated by a positive effect of day of the year. NH_4^+ and NO_3^- concentrations were strong predictors, showing significant negative relationships with $\delta^{13}C$, whereas PO_4^{3-} exhibited a weak positive effect. Among bacterial predictors, β-proteobacteria showed a marginal negative association with $\delta^{13}C$, while Other bacteria were positively associated. Remaining tested bacterial groups as well as water temperature did not significantly improve model fit and were excluded during model selection (Tab. 3.; Fig. 2.).

Tab. 3. Results of Generalized Linear Model with stepwise-selected environmental predictors for Calanoid copepods, Cyclopoid copepods and *Daphnia* sp. $\delta^{13}C$ values (dependent variable)

		Estimate	Std. Error	T value	P value
Calanoid copepods	Intercept	-34.60	2.332	-14.84	**<0.001**
	Day of the year	2.97×10^{-2}	9.45×10^{-3}	3.14	**<0.01**
	β-proteobacteria density	-1.05×10^{-6}	5.83×10^{-7}	-1.81	0.079
	Other bacteria density	1.29×10^{-7}	6.34×10^{-8}	2.03	**<0.05**
	NH_4^+	-7.86	2.54	-3.10	**<0.01**
	NO_3^-	-3.17×10^{-1}	1.18×10^{-1}	-2.70	**<0.05**
	PO_4^{3-}	1.58×10^{1}	8.51	1.86	0.071
Cyclopoid copepods	Intercept	-38.83	2.40	-16.14	**<0.001**
	Day of the year	0.015	0.008	1.90	0.064
	Water temperature	0.30	0.115	2.61	**<0.05**
Daphnia sp.	Intercept	-41.36	6.49	-6.37	**<0.001**
	Day of the year	4.59×10^{-2}	1.80×10^{-2}	2.55	**<0.05**
	Proteobacteria	7.22×10^{-8}	6.71×10^{-8}	1.08	0.300
	α-proteobacteria	2.31×10^{-6}	1.17×10^{-6}	1.97	0.069
	β-proteobacteria	-3.21×10^{-6}	1.23×10^{-6}	-2.61	**<0.05**
	γ-proteobacteria	-1.68×10^{-6}	9.15×10^{-7}	-1.83	0.088
	δ-proteobacteria	1.17×10^{-6}	6.56×10^{-7}	1.79	0.095
	Other bacteria density	2.90×10^{-7}	8.15×10^{-8}	3.56	**<0.01**
	Water temperature	2.41×10^{-1}	2.03×10^{-1}	1.19	0.253
	NH_4^+	-1.32×10^{1}	4.33	-3.06	**<0.01**
	NO_3^-	-4.66×10^{-1}	2.05×10^{-1}	-2.28	**<0.05**
	PO_4^{3-}	4.68×10^{1}	1.74×10^{1}	2.69	**<0.05**

In contrast, $\delta^{13}C$ variation in cyclopoid copepods was weakly explained by the full model, and stepwise AIC selection resulted in a highly simplified final model including only day of the year and water temperature. Cyclopoid $\delta^{13}C$ showed a significant positive relationship with water temperature,

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whereas the effect of day of the year was marginal. Neither bacterial groups abundances nor nutrient concentrations contributed significantly to explaining variation in cyclopoid δ^{13}C, and all were removed during model selection (Tab. 3.; Fig. 3.).

For *Daphnia* sp., the full model was retained as the best-supported model according to AIC, indicating strong explanatory power without further simplification. *Daphnia* sp. δ^{13}C increased significantly with the day of the year. β-proteobacteria abundance was negatively associated with δ^{13}C, whereas the “Other bacteria” showed a strong positive effect. Positive α-, and δ-proteobacteria effects and negative γ-proteobacteria effect remained marginal in final model.

Nutrient concentrations were also significant predictors, with negative effects of NH_4^+ and NO_3^- concentrations and a strong positive effect of PO_4^{3-} concentration. Proteobacteria abundance and water temperature did not significantly explain additional variation in *Daphnia* sp. δ^{13}C (Tab. 3.; Fig. 4.).

4. Discussion

During the last few decades great progress has been made in understanding carbon cycling in freshwaters (Tranvik et al., 2018), but it is still not entirely understood. Our study is another step forward, as we provide an evidence for environmental factors – bacteria – zooplankton link, which significantly contributes to the understanding of carbon flux within planktonic networks. Values of δ^{13}C in grazers community (zooplankton) is strongly influenced by δ^{13}C values of their food sources (Tiselius and Fransson, 2016). However, conducted analyses showed that the variability of δ^{13}C values in planktonic crustaceans was driven by distinct environmental and microbial factors and the set of the factors was ultimately linked to specific crustacean group. Planktonic crustaceans bear diverse functional traits (e.g. the method of food acquisition) and therefore their interaction with the environment and response for its’ changes is different (De Meester et al., 2023).

Day of the year was a significant predictor, showing positive effects on δ^{13}C values of calanoid copepods and *Daphnia* sp. In case of δ^{13}C values of cyclopoid copepods, day of the year was retained as a predictor (with positive effect) within the final model, however its’ effect remained marginal. This finding corroborates with similar studies addressing the seasonality of δ^{13}C in freshwater phytoplankton (Lammers et al., 2017), herbivorous zooplankter *Daphnia middendorfiana* (Gu et al., 1999) and particulate organic matter (POM; Moschen et al., 2009). To the certain extent this can be an effect of water temperature but also the seasonal succession in source of food, e.g. phytoplankton community since algal δ^{13}C values greatly influence grazers’ isotope signal.

However, the only factor that positively affected δ^{13}C values of cyclopoid copepods, in the present studies, was water temperature. Copepods tend to decrease their body size with increasing

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temperatures and response of specific taxa to this effect is dependent on food uptake method (Horne et al., 2016). The response of cyclopoids is weaker compared to calanoids (Horne et al., 2016), however any decrease in body size also decreases the optimal food particle size (Hansen et al., 1994) increasing the share of algae in the diet of cyclopoid copepods (Adrian and Frost, 1993), which explains positive effects of temperature on $\delta^{13}C$ values of the animals. In present study, water temperature was in fact retained as the predictor in the final model for $\delta^{13}C$ in *Daphnia* sp. however not as a significant one. This corroborates with the findings of (Schilder et al., 2015) who concluded that temperature is negligible predictor of *Daphnia* $\delta^{13}C$ values.

Availability of nutrients (i.e. NH_4^+, NO_3^- and PO_4^{3-} concentrations) in water also showed significant effects on crustacean $\delta^{13}C$ values. In final models NH_4^+ and NO_3^- concentrations were step-wise selected as significant predictors with negative effect on $\delta^{13}C$ values of *Daphnia* and calanoid copepods. NH_4^+ and NO_3^- are crucial for phytoplankton development and in shallow lakes often become a limiting factor, especially under availability of phosphorous (as in studied lakes; Søndergaard et al., 2017). Some algal groups, such as green algae, cyanobacteria and flagellates show preferences toward NH_4^+ while diatoms are more likely to utilize NO_3^- (Domingues et al., 2011; Glibert et al., 2016). Furthermore, NO_3^-:NH_4^+ ratio is recognized to impact community composition by influencing growth rates of certain taxa (Trommer et al., 2020). Increased growth rates and factors controlling them (such as nutrient availability) are demonstrated to negatively affect fractionation of carbon stable isotopes, and thus leading to more positive $\delta^{13}C$ values in phytoplankton (Burkhardt et al., 1999; Magozzi et al., 2017). Assuming this mechanism to be in force in waterbodies considered in present study, observed negative effect of NH_4^+ and NO_3^- on $\delta^{13}C$ suggest higher concentrations of these nutrients may be associated with slower phytoplankton growth rates, resulting in lower $\delta^{13}C$ values in crustacean consumers. This pattern is opposite to what would be expected if nutrient availability directly stimulated phytoplankton growth, but seasonal dynamics and declining water temperatures effects might be involved. In shallow lakes, increased nitrogen availability is typically observed in early spring and late autumn, when low temperatures constrain primary production despite nutrient abundance (Søndergaard et al., 2017). Furthermore, this result might also involve nutrient recycling by crustacean grazers, which depending on N and P requirements by certain taxa, can differently affect entire food web (Branco et al., 2018). Cladocerans of genus *Daphnia* are demonstrated to excrete substantial amounts of NH_4^+ while grazing, increasing availability of NH_4^+ for phytoplankton (Karjalainen et al., 1997; Wojtal-Frankiewicz and Frankiewicz, 2011). This phenomenon is an additional explanation for effects of NH_4^+ on crustacean $\delta^{13}C$ values throughout the season.

In addition to negative effects of NH_4^+ and NO_3^- we found positive significant effects of PO_4^{3-} on *Daphnia*'s $\delta^{13}C$ values. For optimal performance, *Daphnia* requires high amounts of phosphorus (P) in relation to nitrogen (N) and carbon (C; Hessen, 1992; Hessen et al., 2013), which is a reason why in some systems Daphnia can act as a phosphorus trap (Branco et al., 2018; Karjalainen et al., 1997). The reason can be the fact highlighted by Vrede et al. (1999), who showed that approximately 14% of P in *Daphnia*'s body is allocated within the carapace, that is lost during the molting (which can happen every 2 – 3 days at 20°C;Lynch et al., 1986). This mechanism strongly drains *Daphnia* individuals from P content (Vrede et al., 1999). However, thanks to the ability of P uptake from dissolved phase(Boersma, 2000; Urabe et al., 1997), *Daphnia* is not only reliant on algal P but also on the P compounds dissolved in the water. Phosphorus is known to have regulatory effects on energy transfer within food webs (Hessen, 2008; Welti et al., 2017), however in our study PO_4^{3-} concentration was significantly correlated only with $\delta^{13}C$ in *Daphnia*. PO_4^{3-} concentration was also retained in the final model for calanoid copepods, however it's effect was marginal. Copepods are recognized to store lower amounts of phosphorus in dry weight (P:DW) than *Daphnia* (Vrede et al., 1999) and nitrogen : phosphorus ratio (N:P) is higher in copepods than in *Daphnia* (Sommer and Sommer, 2006). Growth rates of copepods are slower than *Daphnia*'s therefore their P requirement is lower (Lorenz et al., 2019), which explains no effects of PO_4^{3-} concentration on $\delta^{13}C$ values of both copepod groups.

Beyond phytoplankton, which is substantial part of planktonic crustaceans' diet, also bacterial communities play an important role in cycle of carbon in shallow freshwater ecosystems. Bacterial communities are recognized to acquire majority of carbon from phytoplankton (Lammers et al., 2017), and can serve as a food source for crustacean zooplankton, directly (filter-feeders) or through trophic cascades (via protozoans; predatory animals) (Kalinowska et al., 2015; Vrede and Vrede, 2005; Work and Havens, 2003). In our studies, we found presence of several groups of bacteria: Proteobacteria, α-proteobacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria and "Other bacteria". The models established for calanoid copepods and *Daphnia* indicated positive effects of "Other bacteria" density on $\delta^{13}C$ values. This indicates that "Other bacteria" constitute a pathway (direct or indirect) of incorporation of photosynthetically fixed carbon to diet of these animals, since photosynthesis tends to drive $\delta^{13}C$ to less negative values in autotrophs and therefore in all consumers relying on them (Finlay and Kendall, 2007). The final model showed that $\delta^{13}C$ values in *Daphnia* underwent significant negative effect of β-proteobacteria density. β-proteobacteria are able to quickly utilize organic matter and thus significantly contribute element cycling in the freshwater ecosystems and are often related to nitrogen cycle (Bouvier and Del Giorgio, 2002; Hayden and Beman, 2014; Schulhof et al., 2020) which explains similar negative effects of NO_3^-, NH_4^+ and β-proteobacteria on crustaceans' $\delta^{13}C$. However, β-proteobacteria are also recognized as methanotrophs (Amin et al., 2017; van Grinsven et al., 2020; Kuloyo et al., 2020; Redmond et al., 2010; Somers et al., 2020) and multiple β-proteobacteria members have the genomic capacity to the biological cleavage of methylphosphonate, which simultaneously releases phosphate and methane (Peoples et al., 2023). Methane-oxidizing bacteria can be grazed by zooplankton and therefore constitute a conduit for methane incorporation into the food web (Agasild et al., 2014; Jones and Grey, 2011). Sanseverino et al. (2012) demonstrated that δ^{13}C of grazers foraging on methane oxidizing bacteria can be driven to low values (-42.0‰), which is comparable to the lowest δ^{13}C values found in *Daphnia* spp. (-42.5‰) in our study. Methane oxidation is also identified in α- and γ-proteobacteria (Borrel et al., 2011; Op den Camp et al., 2009), but our analyses showed no relationship between densities of these groups and δ^{13}C in planktonic crustaceans considered in present study.

Overall, our study highlights the complex interplay of environmental conditions, microbial processes and functional trait-driven response of δ^{13}C values in planktonic crustaceans. A clear limitation of the study is the lack of δ^{13}C measurements in crustacean dietary sources (e.g. algae, bacteria), which appear as a future research direction. Insight into the diets of planktonic crustaceans and linking δ^{13}C values found in our samples to specific food sources would require subsequent measurements of δ^{13}C in lower trophic levels and application stable isotope mixing models. This approach was not considered here and is a clear limitation of this study but will be examined in the future research. Nevertheless, this study involves integrative perspective improving the understanding of carbon flow in shallow freshwater ecosystems.

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Competing interests

The authors declare that they have no conflict of interest.

Author contributions

WK and EWW conceived the ideas and designed the study; WK and EW collected the data; WK and IG processed biological samples; WK analyzed the data; WK and EWW led the writing of the manuscript; EW and IG reviewed and edited the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability

The dataset used within this study will be made available on a reasonable request

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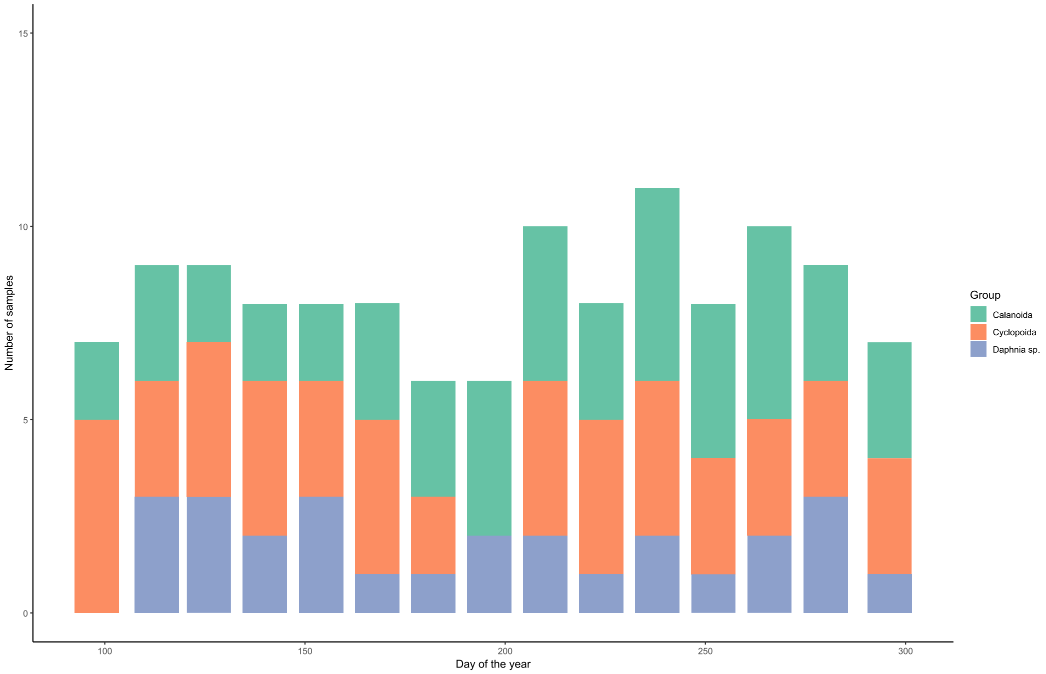

Fig. 1. Distribution of studied planktonic crustacean groups throughout the sampling period

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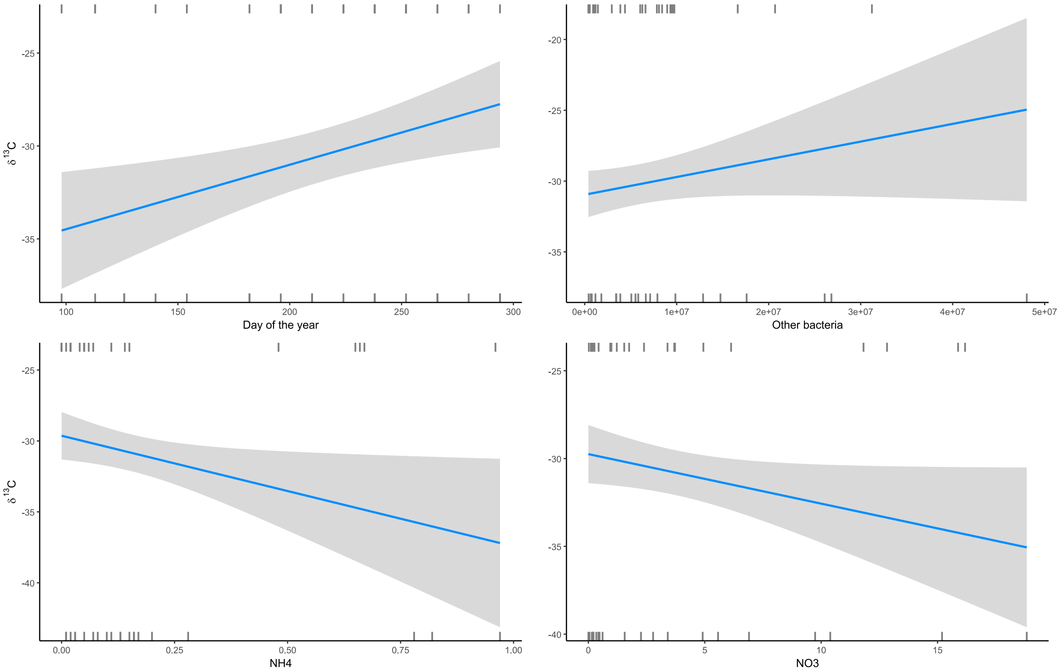

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565 Fig. 2. Visualization of stepwise-selected significant predictors of Calanoid copepods' δ^{13}C

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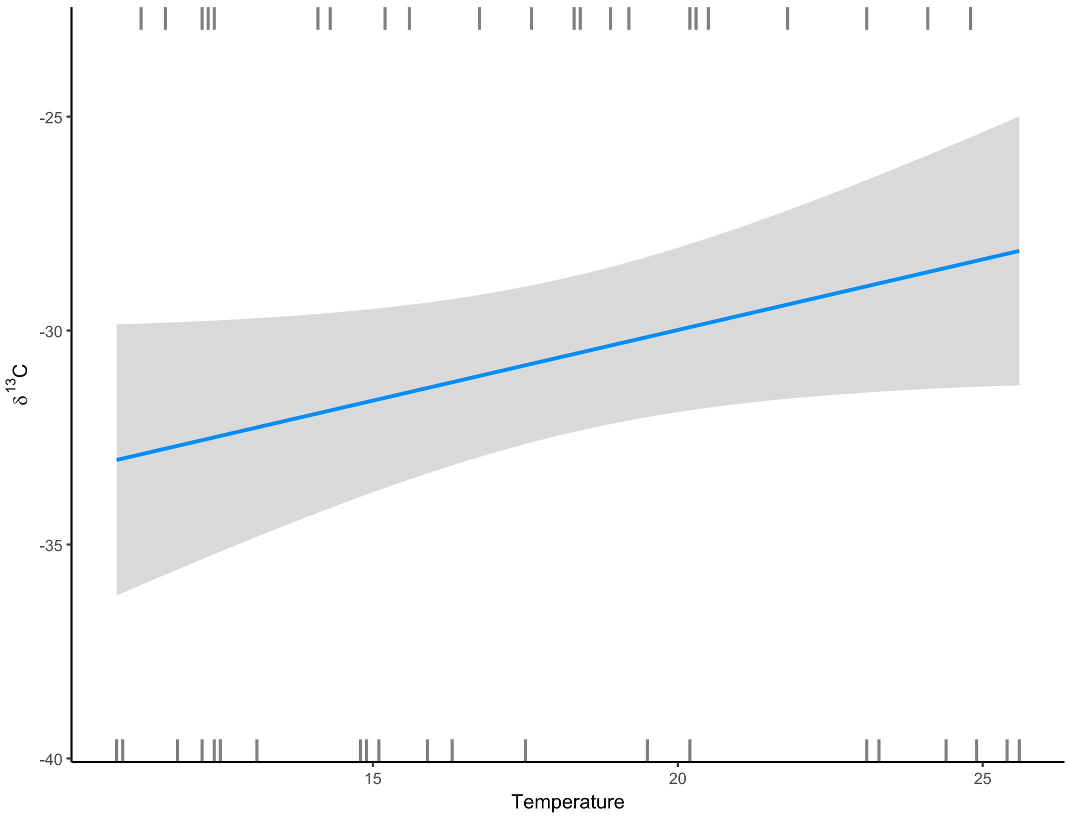

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569 Fig. 3. Visualization of stepwise-selected significant predictors of Cyclopoid copepods' $\delta^{13}C$

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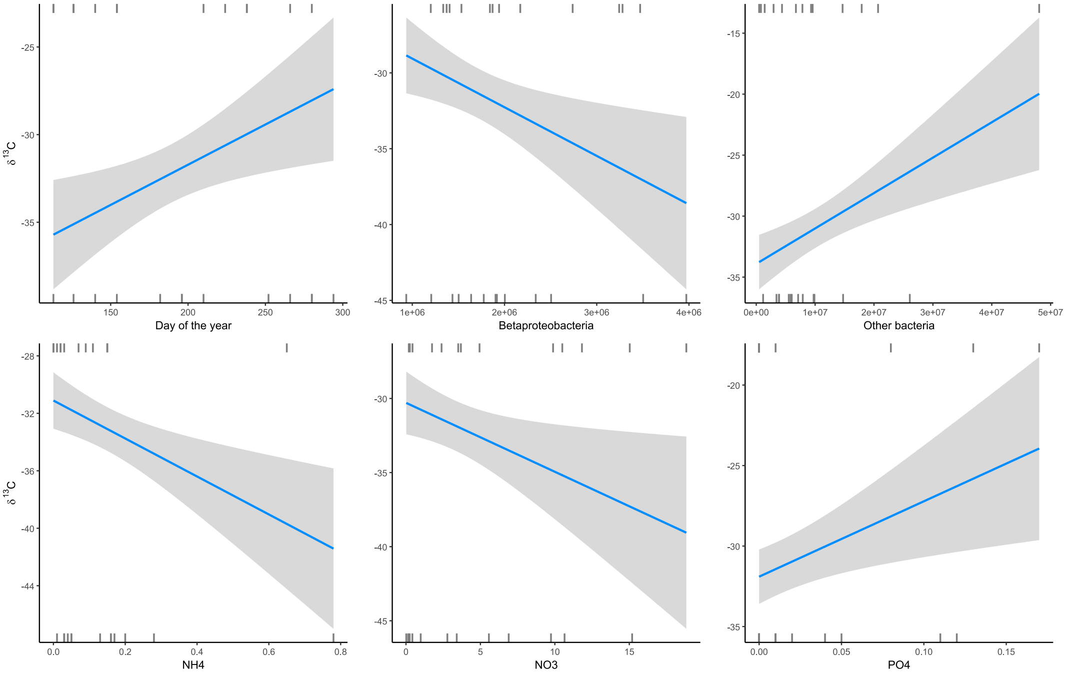

Fig. 4. Visualization of stepwise-selected significant predictors of *Daphnia* δ^{13}C