A critical assessment of the stoichiometric knife-edge: no evidence for artifacts caused by the experimental P-supplementation of algae

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Received: 23 October 2020 / Accepted: 27 April 2021 / Published online: 20 May 2021
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Abstract The stoichiometric knife-edge refers to the reduced performance of consumers encountering food with excess phosphorus (P) relative to carbon (C) or nitrogen (N). Studies that provide evidence for such knife-edge in aquatic systems often apply phosphate supplementation to create P-rich food treatments. However, this method may suffer from artifacts, because after uptake algae may store P in a form different from the P-rich biomolecules typically consumed by zooplankton. Our aim was to test if P supplementation results in potential biases. We experimentally exposed populations of the herbivore rotifer species, *Brachionus calyciflorus* (Pallas), to four different food quality treatments: algae grown under P-saturating (HP_{chem}, molar C:P ratio = 59.7 ± 2.7)

and P-sufficient (MP_{chem}, molar C:P = 116.3 ± 5.2) conditions in chemostats, and algae grown under P-limiting conditions, but with molar C:P ratios equal to HP_{chem} and MP_{chem} treatments, respectively (HP_{LP+P}, molar C:P = 59.8 ± 0.14 ; MP_{LP+P}, molar C:P = 121.0 ± 4.3). The latter two treatments were achieved through P-supplementation of P-limited algae. Results show that for rotifers fed algae with either excess or intermediate P content, population growth rates were consistently higher on algae grown in chemostats than algae treated with the P supplementation method. Importantly, growth rates were also consistently lower in HP than in MP treatments and the magnitude of this negative impact was independent on algal growth history. The latter result confirms the existence of a stoichiometric knife-edge and indicates that P supplementation is a reliable method to study the relative effect of excess P on zooplankton performance in a standardized way.

Handling Editor: Nandini Sarma.

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Keywords Ecological stoichiometry · Phosphorus · Zooplankton · Rotifera · *Brachionus calyciflorus*

Introduction

Recently, human activities have strongly altered the biogeochemical cycles of key elements, such as carbon (C), nitrogen (N), and phosphorus (P), either

via nutrient enrichment (Smith and Schindler 2009) or, conversely, oligotrophication (Stockner et al. 2000; Elser et al. 2009). Such anthropogenic changes have far-reaching consequences for aquatic biota. Organisms consist of various types of crucial biomolecules (e.g., proteins, lipids, nucleic acids...) with specific elemental ratios (Sternern and Elser 2002; Hessen et al. 2013). Generally, the elemental composition of autotrophs differs from that of consumers while it also exhibits a much wider range (e.g., van de Waal 2010). Mismatches of elemental ratios between producers and consumers have repeatedly been shown to cause negative impacts on the performance of consumers and to affect the relative ratios at which nutrients are recycled (Sternern 1990; Sternern et al. 1992). Ultimately, they have a great potential to impact food web structure and ecosystem functioning (Hall 2009; Hillebrand et al. 2009).

Ecological stoichiometry (ES) provides a powerful framework to study the consequences of stoichiometric imbalance in natural systems (Sternern and Elser 2002). It specifically links essential elements, such as C, N, P to biomolecules, and therefore the growth of organisms (Elser et al. 2003). Whereas most ES studies in terrestrial systems focus on N, studies regarding freshwater systems often emphasize the importance of P (Hessen et al. 2013). Currently, there is a long tradition of studies that address the impact of P-limitation in phytoplankton on the performance of zooplankton. For example, P-limited food has been shown to affect the life strategy (Jensen and Verschoor 2004; Zhou et al. 2018), feeding behaviour (Suzuki-Ohno et al. 2012; Urabe et al. 2018), somatic growth rate (Urabe et al. 1997; Sherman et al. 2017) and ultimately also the population growth rate of zooplankton consumers (Lemmen et al. 2020).

However, one potential obstacle that may bias our evaluation of elemental mismatch is that P-limitation does not only alter the elemental composition but also other non-stoichiometric traits of phytoplankton, such as biochemical composition (Spijkerman and Wacker 2011; Challagulla et al. 2015) and morphology (van Donk and Hessen 1995; van Donk et al. 1997). The importance of biochemical quality for the growth and reproduction of zooplankton is well-established (Wacker and von Elert 2001). Morphological changes due to nutrient stress in algae have also been found to affect consumer performance, e.g., via a change in palatability or digestibility (van Donk and Hessen

1993; van Donk et al. 1997). Indeed, the negative influence of elemental limitation of phytoplankton on the life history, growth and reproduction of zooplankton has to a large extent been found to be caused by non-stoichiometric aspects of nutrient limitation in addition to their direct effects (Rothhaupt 1995; Zhou et al. 2018).

One powerful experimental tool that allows to control for such indirect non-stoichiometric effects of P limitation is the P supplementation approach (Rothhaupt 1995; Plath and Boersma 2001; Zhou et al. 2018). This method relies on the fact that phytoplankton is able to quickly absorb inorganic dissolved P from their ambient environment (Lehman and Sandgren 1982), whereas responses in non-stoichiometric traits, such as biochemical composition or morphology are much slower (Plath and Boersma 2001; Elser et al. 2001). Therefore, with supplementation of P one can drastically modify the molar C:P ratio of P-limited algae on a very short time span while keeping any other P-dependent modifications in biochemical composition or morphology to a minimum.

P-supplementation to algae has been applied for two major aims. In one type of studies, the method has been used to compare the relative impact of direct with indirect, non-stoichiometric impacts of P-limitation (Rothhaupt 1995; Zhou et al. 2018; Zhou and Declerck 2020). Typically, two types of algal cultures are set up under P-sufficient (HP) and P-limited (LP food) conditions. Using the P supplementation method, a third food quality treatment is then created by spiking LP food with inorganic P, in such a way that algae are obtained with a low-P growth history but with molar C:P and N:P ratios equal to that of HP. The direct and indirect effects of P limitation on consumers can then be derived from a comparison of consumer performance in these three food quality treatments (Zhou et al. 2018). Another type of studies uses the method to evaluate the effects of various degrees of P limitation on consumer performance while controlling for indirect, non-stoichiometric effects. Whereas numerous studies in the framework of ES have addressed the effects of P-limitation, few have addressed the response of consumers along very broad ranges of stoichiometric food quality, including treatments with excess P (i.e., food with very low C:P ratios, Plath and Boersma 2001; Zhou and Declerck 2019). Intriguingly, several of these studies have reported unimodal

responses to broad food C:P gradients with reduced consumer performance not only at high but also at very low food C:P levels (coined as the ‘stoichiometric knife-edge’; Plath and Boersma 2001; Elser et al. 2006, 2016; Bullejos et al. 2014). Performance reductions associated with excess P have been attributed to increased costs associated with the excretion of or toxicity by P (Elser et al. 2016; Zhou and Declerck 2019). Although underexplored, such a knife-edge could have important implications for the way how phosphorus pollution might affect aquatic food webs dynamics and associated ecosystem functions (Elser et al. 2016).

Despite its frequent application, the reliability of the P supplementation method has never been critically evaluated. Artifacts resulting from its use could potentially mislead the interpretation of how food C:P ratios affect consumer performance. One potential problem with P supplementation may be that shortly after its addition to algae the inorganic P is temporarily stored instead of being metabolized. Consequently, the supplemented P may be taken up by zooplankton consumers under a different chemical form (e.g., as polyphosphates, Kenney et al. 2001; Eixler et al. 2006) than the typical naturally occurring P-rich biomolecules (e.g., nucleic acids, phospholipids or ATP, Elser et al. 1996). A straightforward interpretation of the stoichiometric knife-edge would be hampered if this chemical form is toxic or if it is assimilated with a different efficiency.

With this study, we aimed to test the working hypothesis that observations of a stoichiometric knife-edge in zooplankton studies have resulted from artifacts associated to the P-supplementation method rather than from true negative effects of excess P in naturally occurring food. More specifically, Zhou and Declerck (2019) applied P-supplementation to create a broad gradient of food C:P-ratios and studied the population growth response of experimental populations of the planktonic rotifer *Brachionus calyciflorus* (Pallas) to this gradient. They demonstrated a knife edge response: population growth rates showed a unimodal response to the food quality gradient reaching a maximum at a food C:P ratio of approximately 170 and declining as food C:P deviated from this optimum. With the present study, we aimed at evaluating to what extent artifacts associated with P-supplementation may have caused this response pattern, more specifically the performance reduction

observed with decreasing food C:P. For this, we compared the growth performance of populations in response to food with excess P versus food with sufficient P (food C:P ratios of 60 and 115, respectively). Both food quality levels were created by two different methods, i.e., through P supplementation of P-limited algae and by manipulating P-availability to algae in continuous cultures. Absence of differences in population growth responses to excess P between these two types of food would indicate that responses are not confounded by artifacts of the P supplementation method. Conversely, differences in the responses of consumers between the different algal pre-treatments would indicate problems with the interpretation of results obtained by P supplementation.

Materials and methods

Algae and rotifer cultures

For this study, we used a *B. calyciflorus* clone obtained from the dormant egg bank of a Dutch lake (52° 50'26.50" N; 4° 20'018.40" E). The rotifer species *B. calyciflorus* is known to consist of a species complex, and has been recently described as four species based on a combination of phylogenetic and morphological analyses (Papakostas et al. 2016; Michaloudi et al. 2018). Based on ITS1-sequence information, the clone used in the present study was identified as *B. calyciflorus* s.s. (Michaloudi et al. 2018). Stock cultures were maintained at room temperature under continuous light conditions and fed with nutrient replete green algae *Chlamydomonas reinhardtii* (1000 $\mu\text{mol C L}^{-1}$). Animals were transferred to new containers with fresh medium every 3 days.

We cultured *C. reinhardtii* in nine continuous 2L-chemostats at 23 ± 1 °C using modified WC (Woods Hole Chu-10) medium (Guillard and Lorenzen 1972) at a dilution rate of 0.33 day^{-1} . More specifically, we created algae with contrasting C:P ratios by culturing them under different combinations of nutrient and light conditions: (1) high P supply (120 $\mu\text{mol P L}^{-1}$, using K_2HPO_4) at 40 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ continuous light, resulting in phytoplankton cultures with a molar C:P ratio of 60, further referred to as ‘HP_{chem}’; (2) intermediate P supply (65 $\mu\text{mol P L}^{-1}$) at 40 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ of continuous light, yielding algae

with a molar C:P of 115 ('MP_{chem}'); (3) low P supply ($15 \mu\text{mol L}^{-1}$) at $120 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ continuous light, resulting in algae with a C:P of 600 ('LP_{chem}'). Zhou and Declerck (2019) studied the growth response of one clone of the same rotifer species along a broad food C:P gradient of the same food alga and observed a unimodal response with optimal growth at a food C:P ratio of 170 and substantially reduced growth reductions at food C:P levels below approximately 100 and above 300. The MP_{chem} algae in this study can therefore be considered as good quality food, whereas the LP_{chem} and HP_{chem} algae contain limiting and excess amounts of P, respectively. Each type of algal cultures was replicated three times. All chemostats were at steady state for at least one month prior to the experiment.

Preparation of the food quality treatments

During the experiment, we created two food quality treatments through P-supplementation. One treatment was created by enriching the LP_{chem} algae with an amount of inorganic phosphate so that it achieved a molar C:P ratio similar to that of the MP_{chem} algae (further referred to as the 'MP_{LP+P}' treatment). The other treatment was created by enriching the LP_{chem} algae so that they achieved a molar C:P ratio similar to that of the HP_{chem} algae ('HP_{LP+P}'). Together with the MP_{chem} and HP_{chem} algae, the MP_{LP+P} and HP_{LP+P} algae provided four different food quality treatments for the rotifer growth rate experiment with the MP_{LP+P} and HP_{LP+P} treatments combining a history of growth under P-limited conditions with an artificially elevated cell P content through P supplementation. The algae for HP_{chem} and MP_{chem} treatments were harvested daily from the chemostats and diluted with nutrient free WC medium. To create the MP_{LP+P} and HP_{LP+P} treatments, we first harvested algae from LP chemostats and then added inorganic phosphate (K_2HPO_4 , 0.05 mol L^{-1}) to LP algae 90 min before feeding them to experimental rotifer cultures. Estimates of the amount of P needed for the algae to obtain the target molar ratios were based on the algal C content estimated from cell counts (Multisizer 3TM Coulter Counter, Beckman Coulter) and a C—biovolume regression relationship. For all the four food quality treatments, algae were kept in the dark for 90 min before being fed to the rotifers.

Population growth rate experiment

We conducted an experiment to investigate the population growth response of rotifers to the four food quality treatments. This experiment consisted of 12 experimental units, with four food quality treatments being replicated by food from three independent chemostat replicates. The population growth rate experiment was initiated by seeding 4000 rotifer individuals to flasks filled with 200 ml culture medium containing ad libitum concentrations of the respective food treatment ($1000 \mu\text{mol C L}^{-1}$). The experimental units were incubated in a shaking incubator under continuous darkness at a temperature of $23 \pm 1^\circ\text{C}$. After 24 h, we estimated the density of each population by counting the number of rotifers in two separate 5 ml subsamples. Based on these counts we filtered a culture volume estimated to contain about 4000 rotifers over a $60 \mu\text{m}$ mesh. Rotifers that remained on the mesh were then collected and used to restart the population in a fresh food suspension. We repeated this culturing process for a period of 23 days.

Elemental composition of food quality treatments

For each food quality treatment, we measured the molar C:P ratio at three occasions during the population growth rate experiment. Algae were collected on GF/F filters and dried in an oven at 60°C . Algal C and N contents were determined using a FLASH 2000 organic element analyzer (Interscience B.V., Breda, The Netherlands), while P content was determined by a QuAAtro segmented flow autoanalyzer (Beun de Ronde, Abcoude, The Netherlands).

Data analysis

The exponential population growth rate of rotifer populations was repeatedly calculated for each experimental unit as $(\ln N_t - \ln N_0)/t$, where N_0 and N_t represent the population density at the start and end of each time interval and t refers to the length of the time intervals (i.e., one day). Growth rate for each unit was calculated as the mean growth rate for the last 8 days of the experiment, i.e., the moment at which population growth in all treatments had stabilized.

One-way ANOVA combined with post hoc Tukey'HSD comparisons was used to test for differences in molar C:P ratios between the different food

quality treatments. To investigate how the population growth rate of rotifers is affected by food stoichiometry and P-supplementation, we applied two-way ANOVA, with food C:P ratio and algal growth history as factors. Differences in responses between individual treatment levels were further studied in detail with a Tukey post hoc comparison. All analyses were performed in R (R Core Team 2016).

Results

The mean molar C:P ratios measured for the algal cultures in the intermediate (MP_{chem}) and high phosphorus (HP_{chem}) chemostats equaled 116.3 ± 5.2 (mean $\pm$ sd) and 59.7 ± 2.7 , respectively. Supplementation of algae from the low phosphorus chemostats (LP_{chem}) with inorganic P allowed us to create food quality treatments according to our targets: 1.5 h after P-addition, the molar C:P ratios of the MP_{LP+P} and HP_{LP+P} algae equaled on average 121.0 ± 4.3 and 59.8 ± 0.14 , being very close to (and not

statistically different from) the C:P ratios of the MP_{chem} and HP_{chem} algae, respectively (Fig. 1).

Both food C:P and algal growth history had strong effects on rotifer population growth rates (Tables 1 and 2, Fig. 2). Growth rates of populations fed with HP algae (i.e., HP_{chem} and HP_{LP+P}) were approximately 16.7% lower than those of populations fed with the respective MP algal treatments (MP_{chem} and MP_{LP+P}). Populations fed algae with an LP growth history (MP_{LP+P} and HP_{LP+P}) were characterized by population growth rates 36% lower than those fed with algae with the respective MP and HP growth histories (i.e., MP_{chem} and HP_{chem}). Importantly, we observed no significant interaction between food C:P and algal growth history, indicating that the magnitude of the negative effect of high food P-levels on rotifer population growth was unaffected by P supplementation.

Discussion

We observed substantial effects of both food P-content and algal growth history on rotifer population growth. Irrespective of the molar C:P ratio of the food, rotifer populations fed P-supplemented algae with a growth history under P-limiting conditions (i.e., MP_{LP+P} and HP_{LP+P}) systematically underperformed compared to rotifers that had been fed algae grown under intermediate or high P levels (MP_{chem} and HP_{chem}). This observation is consistent with other studies (Rothhaupt 1995; Zhou et al. 2018; Zhou and Declerck 2020) that observed and explained such growth rate reductions by indirect non-stoichiometric changes in algal traits associated with P-limited growth, e.g., biochemical composition (Spijkerman and Wacker 2011; Challagulla et al. 2015) or morphology (van Donk and Hessen 1995; van Donk et al. 1997). Alternatively, such effect of growth history could be interpreted as the result of adverse effects of the P-supplementation treatment, but then one would expect that the magnitude of such effect should increase with increasing amounts of supplemented P. However, we found no empirical support for this expectation given the absence of a statistical interaction between food P content and growth history.

Irrespective of the effects of algal growth history, our results also confirmed the validity of previous experimental evidence supporting the existence of a

Fig. 1 Algal molar C:P ratios of the different food quality treatments in the experiment. Circles represent treatments with chemostat grown algae. Triangles represent treatments in which algal food from P-limited chemostats was supplemented with P before it was fed to rotifers. HP: algae with excess P content; MP: algae with intermediate P content. Treatments with different letters differ significantly according to Tukey post hoc tests. Symbols and error bars represent the mean $\pm$ 2 standard error, respectively. Means were calculated as the average of three independent chemostat replicates. Values for each of these replicates were obtained as averages of three consecutive measurements in time (once at the start, once in the middle and once at the end of the experiment)

Table 1 Summary of two-way ANOVA results testing for the effects of stoichiometric food quality and algal growth history on rotifer population growth rate. *P*-values in bold indicate significant effects

	df	SS	MS	<i>F</i> -value	<i>P</i> value
Stoichiometric food quality (SFQ)	1	0.02858	0.02858	48.804	< 0.001
Algal growth history (AGH)	1	0.17221	0.17221	294.089	< 0.001
SFQ*AGH	1	0.000006	0.000006	0.099	0.761
Residuals	8	0.00468	0.00059		

df degrees of freedom, *SS* sum of squares, *MS* mean square

Table 2 Summary of post hoc pairwise comparisons of rotifer population growth rates under the different food quality treatments. *P* adj-values in bold indicate significant effects

	diff	lwr	upr	<i>P</i> adj
MP _{chem} -HP _{Chem}	0.09320982	0.02993852	0.15648112	0.007
HP _{LP+P} -HP _{Chem}	- 0.24397688	- 0.30724817	- 0.18070558	< 0.001
MP _{LP+P} -HP _{Chem}	- 0.14198631	- 0.20525761	- 0.07871502	0.0004
HP _{LP+P} -MP _{Chem}	- 0.33718669	- 0.40045799	- 0.27391540	< 0.001
MP _{LP+P} -MP _{Chem}	- 0.23519613	- 0.29846743	- 0.17192483	< 0.001
MP _{LP+P} -HP _{LP+P}	0.10199056	0.03871926	0.16526186	0.0038

Fig. 2 Response of rotifer population growth rate to the four food quality treatments. Circles represent treatments with chemostat grown algae. Triangles represent treatments in which algal food from P-limited chemostats was supplemented with P before it was fed to rotifers. HP: treatments with excess P content; MP: treatments with intermediate P content. Treatments with different letters differ significantly according to Tukey post hoc tests. Each value represents the average of three independent replicates across the last 8 days of the experiment. Symbols and error bars represent the mean $\pm$ 2 standard error, respectively

stoichiometric knife-edge, given that rotifer population growth was consistently slower in algae with excess compared to intermediate P-content both in the treatments with chemostat grown algae and in the

treatments with P supplemented algae. Also here, it is important to note the absence of a statistical interaction between food stoichiometry and algal growth history. The magnitude of the growth reductions caused by excess P was independent of whether food quality treatments originated from chemostats or whether they had been created by P-supplementation of P-limited algae. This outcome leads to two main conclusions. First, our results reject our initial working hypothesis that evidence for the stoichiometric knife-edge is based on artifacts associated with the method of P supplementation as results with P-supplemented food show the same magnitude of rotifer growth reduction upon exposure to excess P as when algae are used that are grown under constant chemostat conditions for multiple generations. Second, our results show no evidence for the possibility that rotifer performance may be affected by non-stoichiometric alterations of algae continuously grown under very P-rich conditions. High levels of ambient nutrients have been reported to alter the biochemical composition (Gladyshev et al. 2007). Although we cannot exclude such alterations, they seem to not have affected the performance of our study organism, neither in a positive or negative way. To summarize, the outcome of our experiment shows that P-supplementation is a valid method that can be used to experimentally manipulate the P-content of food in

studies that aim at investigating the performance response of consumers to excess P while keeping other food quality aspects constant. Although P-supplementation of P-limited algae results in a consistent underestimation of zooplankton population growth rates due to indirect negative effects of P-limitation that act through changes in non-stoichiometric algal traits, population growth rates of zooplankton populations will respond to excess P in the same way as with non-treated algae.

The existence of a stoichiometric knife-edge implies an important impact of phosphorus pollution on producer-herbivore interactions (Elser et al. 2006; Zhou and Declerck 2019) and possibly also interactions between higher trophic levels (Laspoumaderes et al. 2015; Benstead et al. 2014). Its consideration may therefore contribute to a better understanding of the consequences of eutrophication. Pollution of freshwater bodies with phosphorus and associated changes in light climate result in increased phytoplankton P content worldwide (Sternner et al. 2008). Most of the work addressing the effects of eutrophication on zooplankton focus on indirect effects via toxic cyanobacterial bloom formation (Amorim and Moura 2021) or increased levels of fish predation pressure (He et al. 2020). Although the potential importance of the stoichiometric knife-edge is increasingly recognized among researchers in the field of ecological stoichiometry (Yang et al. 2016; Currier and Elser 2017), its potential consequences on aquatic food webs remains largely underappreciated among aquatic ecologists in general. Admittedly, more work needs to be done in order to establish its generality for a wider range of study organisms and environmental conditions. Several studies do provide convincing evidence (Elser et al. 2005, 2006, 2016; Boersma and Elser 2006; Laspoumaderes et al. 2015; Zhou and Declerck 2019) but exceptions exist. For example, Currier and Elser (2017) exposed three *Daphnia* species to lake seston enriched with inorganic P, and found that only one species (*D. magna*) showed strong growth reductions. Khattak et al. (2018) exposed *D. magna* to a range of food C:P ratios, but found no evidence for growth reductions at food C:P levels of 80. To upscale knife-edge effects to the food web level, it is important to realize that natural consumer communities consist of multiple species that differ in their responses to elemental mismatch (Currier and Elser 2017; Teurlinx et al. 2017). Furthermore,

consumers are exposed to a mixture of food source types that may vary in elemental composition (Schulhof et al. 2019) and on which they may feed selectively. For this reason, there is a need for additional empirical support from experiments performed in a multispecies community context under field conditions (Currier and Elser 2017). Our study suggests that the P supplementation approach provides a reliable tool for studies aiming at addressing the generality and implications of the stoichiometric knife-edge, although a confirmation of the generality of our current findings would also be beneficial.

Acknowledgements We would like to thank Laura Martin Cabrera for her laboratory assistance and Nico Helmsing for performing the chemical analyses and the China Scholarship Council for providing a PhD-Grant to Libin Zhou.

Author contributions LZ and SAJD developed the idea and designed the experiments. LZ carried out the experiments and performed the data analysis. LZ and SAJD wrote the manuscript.

Funding The China Scholarship Council provided a PhD Grant to Libin Zhou.

Availability of data and material Data have been made publicly available through the Dryad repository (<https://doi.org/10.5061/dryad.000000038>).

Code availability Not applicable.

Declarations

Conflict of interest Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Ethics approval Not applicable.

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