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Stoichiometric regulation in micro- and mesozooplankton

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Aquatic ecosystems experience large natural variation in elemental composition of carbon (C), nitrogen (N) and phosphorus (P), which is further enhanced by human activities. Primary producers typically reflect the nutrient ratios of their resource, whose stoichiometric composition can vary widely in conformity to environmental conditions. In contrast, C to nutrient ratios in consumers are largely constrained within a narrow range, termed homeostasis. In comparison to crustacean zooplankton, less is known about the ability of protozoan grazers and rotifer species to maintain stoichiometric balance. In this study, we used laboratory experiments with a primary producer (*Nannochloropsis* sp.), three different species of protozoan grazers and one mesozooplankton species: two heterotrophic dinoflagellates (*Gyrodinium dominans* and *Oxyrrhis marina*), a ciliate (*Euplotes* sp.) and a rotifer (*Brachionus plicatilis*) to test the stoichiometric response to five nutrient treatments. We showed that the dependency of zooplankton C:N:P ratios on C: nutrient ratios of their food source varies among species. Similar to the photoautotroph, the two heterotrophic dinoflagellates weakly regulated their internal stoichiometry. In contrast, the strength of stoichiometric regulation increased to strict homeostasis in both the ciliate and the rotifer, similar to crustacean zooplankton. Our study further shows that ciliate and rotifer growth can be constrained by imbalanced resource supply. It also indicates that these key primary consumers have the potential to trophically upgrade poor stoichiometric autotrophic food quality for higher trophic levels.

KEYWORDS: homeostasis; nutrient limitation; heterotrophic dinoflagellate; ciliate; rotifer

INTRODUCTION

Aquatic ecosystems experience large variability in the amount of available nutrients, such as carbon (C), nitrogen (N) and phosphorus (P) on account of natural environmental factors and human activities (Gruber and Galloway, 2008; Taylor and Townsend, 2010). Variation in C:N:P stoichiometry consequently affects the C-to-nutrient ratio available for primary producers. Autotrophic organisms, such as phytoplankton and vascular plants have relatively weak stoichiometric homeostasis regulation capacity and typically reflect the elemental

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ratios of their resource (Sterner *et al.*, 1992; Persson *et al.*, 2010). Thus, their elemental composition can vary considerably among ecosystems, depending on mineral nutrients availability as well as CO_2 and solar radiation (Sterner *et al.*, 1992; Meunier *et al.*, 2014). Additionally, the stoichiometric match of the nutrient supply ratio and primary producer depends on the state of the growth phase, with weak regulation capacity at low growth rate and less stoichiometric variation within a narrower range at high growth rate (Ågren, 2008; Hillebrand *et al.*, 2013; Meunier *et al.*, 2014). The physiological plasticity in elemental composition of primary producers affects their quality as a food resource for heterotrophic herbivores, which require uptake of macronutrients from their prey for metabolic growth. In contrast to primary producers, elemental ratios of heterotrophs are generally more constrained within a relatively narrow taxon- and stage-specific range (Sterner and Elser, 2002; Persson *et al.*, 2010). While this generalized framework holds across many autotrophic and heterotrophic organisms, the degree of homeostasis varies widely as a function of species composition and environmental conditions (Hessen *et al.*, 2013). Regulation of elemental composition and nutrient cycling has been well demonstrated for crustacean zooplankton, particularly for cladocerans and copepods (Kiørboe *et al.*, 1985; Sterner, 1993). However, comparatively little is known about whether microbe zooplankton (protozoa and rotifers), which play a key function in pelagic food web processes (Landry and Calbet, 2004), maintain stoichiometric balance similar to crustacean plankton.

The degree of maintaining constant elemental ratios has important consequences for consumer–resource interactions, the supply of elemental composition to upper trophic levels and for nutrient recycling. While C is an important element of organic macromolecules, N and P are important compounds for specific macromolecules. P is an essential building block for RNA, phospholipids and DNA, and N for proteins (amino acids) and nucleic acids (Sterner and Elser, 2002; Hessen and Anderson, 2008). Consumers have developed physiological mechanisms to maintain homeostasis (Frost *et al.*, 2005), such as adjusting net assimilation or excretion of elements to match metabolic and somatic requirements. Even though the benefits of homeostasis are not clearly understood, it is thought to be more common in heterotrophs due to their high energetic requirements for nutrient storage (Persson *et al.*, 2010). Heterotrophic organisms have to build N and P into complex macromolecules such as amino acids, proteins or polyphosphates, which require high amounts of C as a structural component (Persson *et al.*, 2010). Therefore, accumulation of N and P in heterotrophic organisms might be constrained when C is limited, which in turn would keep their body C:N and C:P ratios relatively constant (Persson *et al.*, 2010).

The degree of mismatch between the C:nutrient ratios of consumers and their prey will further determine internal nutrient recycling and the elemental nutrient quality for higher trophic levels, which is a key determinant for trophic efficiency. Deviations in the elemental food resource ratio from a consumer requirement may constrain growth fitness of herbivores, which may propagate up the food chain (Hessen *et al.*, 2013). However, homeostasis in food chains also means that food quality effects are buffered in primary consumers and not transferred to higher trophic levels (Saikia and Nandi, 2010), and thus dampen the effect of nutritional imbalances on primary producers. For example, protists have the potential to transfer bacterial C into a better quality food resource for zooplankton and may fuel higher trophic levels through trophic upgrading and repacking within the microbial loop (Faithfull *et al.*, 2011). Utilization of bacteria by protozoans can further substitute potentially limiting nutrients, particularly P, that are often several orders of magnitude more concentrated in bacteria than in the dissolved phase (Faithfull *et al.*, 2011). While the role of heterotrophic microbes for trophic upgrading biochemical compounds is highly species-specific, their elemental composition may be less variable as a consumer's demand for N relative to P is generally homeostatic (Sterner and Elser, 2002).

Microbial zooplankton such as heterotrophic nanoflagellates, ciliates and rotifers are a primary link of carbon and nutrient transfer from bacteria and small-sized primary producers to mesozooplankton (Stoecker and Capuzzo, 1990). Due to their high metabolic rates and short generation times, microbial zooplankton play a central role in the pelagic food web for grazing, secondary production and likely nutrient regeneration (Landry and Calbet, 2004). The goal of our study is to investigate the homeostatic ability in terms of C:N:P ratios of microbial zooplankton (protozoa and rotifers) and inter-taxonomic variability. We cultured two heterotrophic dinoflagellates, one ciliate and one rotifer species under varying nutrient ratios (i.e., different food quality regimes) and compared our results of homeostasis regulation with published literature on micro- and mesozooplankton species.

METHOD

Experimental design and nutrient manipulation

The marine Eustigmatophyte, *Nannochloropsis* sp., was cultured as food source under five different nutrient regimes from low N (4:1 N:P; N_{Low}) to medium N (10:1 N:P;

N_{Med}) and replete (20:1 N:P ratio; $NP_{Repl.}$) as well as low and medium P (P_{Low}: 205:1; P_{Med}: 102:1 N:P) levels, respectively (for concentrations see Table I). This primary producer was chosen as food source because of its relatively high food quality and small size to facilitate separation from the consumer. *Nannochloropsis* sp. was grown in artificial seawater (salinity of 20) enriched with f/2 medium following Guillard and Ryther (1962) and Guillard (1975), while the N and P concentrations were doubled following the original f medium (Guillard and Ryther, 1962). To ensure a stable nutrient regime, the algae were cultured in continuous flow through systems (chemostats). The dilution rate for the nutrient-limited treatments was set to 0.15 d^{-1} and to 0.36 d^{-1} in the replete (control) treatment to account for higher growth rates. The density and stability of the chemostat cultures was assessed by daily measurements of carbon concentrations based on photometric light extinctions (at 750, 664, 647, 630 nm) using carbon-extinction equations determined from dilution series. Four zooplankton species were chosen as grazers due to their common occurrence in natural systems and for use in aquaculture: two heterotrophic dinoflagellates *Gyrodinium dominans* and *Oxyrrhis marina* Dujardin 1841 (SCCAP K-175), the ciliate *Euplotes* sp., and the rotifer *Brachionus plicatilis* (L-strain). Stock cultures were reared under replete conditions at 18°C and a 12:12 light:dark cycle.

The experiment was carried out in glass culturing flasks filled with 500 mL of the nutritionally manipulated artificial seawater. Prior to transferring the heterotrophic organisms into the experimental containers, they were washed through plankton-nets with a mesh size of 10 μm for the heterotrophic dinoflagellates and 20 μm for the ciliate and the rotifer. The separation procedure worked well since the algae were 3 μm and the heterotrophic dinoflagellates 14–20 μm, the ciliate 98 μm, and the rotifer 260 μm in size. Zooplankton were fed with chemostat-grown food algae at a target value of 1 mg C L^{-1} and at the same temperature and light regimes as the stock cultures. The experiment was designed as a full-factorial design, where each treatment was set up in triplicate over 6 d. The food concentrations were kept constant by measuring the carbon content of aliquots every 24 h using the photometric light extinction described above, and by inoculating each replicate with fresh medium and food.

Table I: Nitrogen to phosphorus (N:P) molar supply ratios and N and P concentration in the culture medium used to grow Nannochloropsis *sp. for the different nutrient treatments*

Treatments	N:P supply ratio	N (μmol L^{-1})	P (μmol L^{-1})
N_{Low}	4	297	72
N_{Med}	10	742	72
$NP_{Repl.}$	20	1484	72
P_{Med}	102	1484	14
P_{Low}	205	1484	7

Low and Med refers to low and medium N and P limitation, respectively, Repl. refers to replete NP nutrient concentration.

At the end of the experiment, the zooplankton were washed with artificial seawater through plankton-nets and kept in food and media-free artificial seawater for at least 1 h to ensure food vacuole/gut clearance. While we assume that most of the food should have been digested during this time, we cannot exclude the possibility that this procedure has not fully cleared all food particles in the vacuoles or gut. Thus, our results resemble natural field conditions, as consumers typically do not differentiate between the organisms and particles left in the digestive track. Food algae and zooplankton of each treatment were filtered onto pre-combusted Whatman GF/F filters for elemental analysis. Particulate C and N were measured using a CHN Analyzer (LECO CHNS-932). Particulate P was analyzed by combusting the samples at 500°C in the presence of $MgSO_4$ and KNO_3 followed by a digestion in an acid persulfate solution ($K_2S_2O_8$). The amount of phosphate was determined by Segmented Flow Analysis (ALPKEM O. I. Analytical Flow Solution IV, modified method # 319528). All nutrient ratios are reported as molar elemental ratios.

Population growth rates were calculated for *Euplotes* sp. and *B. plicatilis* under replete and nutrient-limited conditions by taking subsamples at the first and last (sixth) day of each experiment. Population density of both species and lorica length and width of *B. plicatilis* were measured by microscopic counts, using an inverted microscope following Utermöhl (1958). Population growth rates were calculated as $\ln(T_6 - T_1)$/time, where T_1 and T_6 are the abundances at Days 1 and 6, respectively, and time is the experimental duration of 6 days.

Cell and vacuole biovolume of *Euplotes* sp. for nutrient-limited treatments were calculated from cell length and width as well as vacuole diameter measurements from photographs of preserved specimen using ImageJ (Rasband 1997–2014). The geometric form of a rational ellipsoid was used for cell and a sphere for vacuole biovolume following Olenina *et al.* (Olenina *et al.*, 2006). Biovolumes for *Euplotes* sp. grown under replete conditions were not measured because vacuoles were either absent or not prominent enough. Lorica biovolume (v) of *B. plicatilis* was calculated as $v = 0.52 \times a \times b \times c$; where 0.52 is a constant; a, b and c, are length, width and depth ($c = 0.4 \times a$), respectively (McCauley, 1984), which takes into account that cell dimensions change disproportionately during growth according to Kennari *et al.*

(Kennari *et al.*, 2008). The geometric shape of a sphere was used to calculate egg biovolume.

Data analysis

One-way analysis of variance (ANOVA) and Tukey's HSD post hoc tests were used to assess differences in stoichiometric, growth rate and biovolume responses among experimental treatments. Data were either log- or square root transformed to satisfy equal variance and normality assumptions.

To describe the relationship between the nutrient ratios of the resource and the consumers we applied a homeostatic regulation coefficient model using a linear relationship between the consumer and resource stoichiometry. A slope equal to or >1 indicates poor homeostasis, while a slope approaching 0 indicates homeostatic regulation. The homeostatic regulation coefficient H is calculated as 1/slope. The stoichiometric regulation is proportional to H, as an increasing value indicates stronger stoichiometric regulation of internal elemental composition (Sterner and Elser, 2002). The culture medium was used for *Nannochloropsis* sp.'s resource stoichiometry while *Nannochloropsis* sp. was the resource for the zooplankton species. Statistical analyses were performed using the R software environment 2.14.1 (R Core Team, 2012).

RESULTS

Elemental composition

N:P ratios of *Nannochloropsis* sp. mirrored N:P ratios of its resource, the nutrient manipulated media (Fig. 1, Table I).

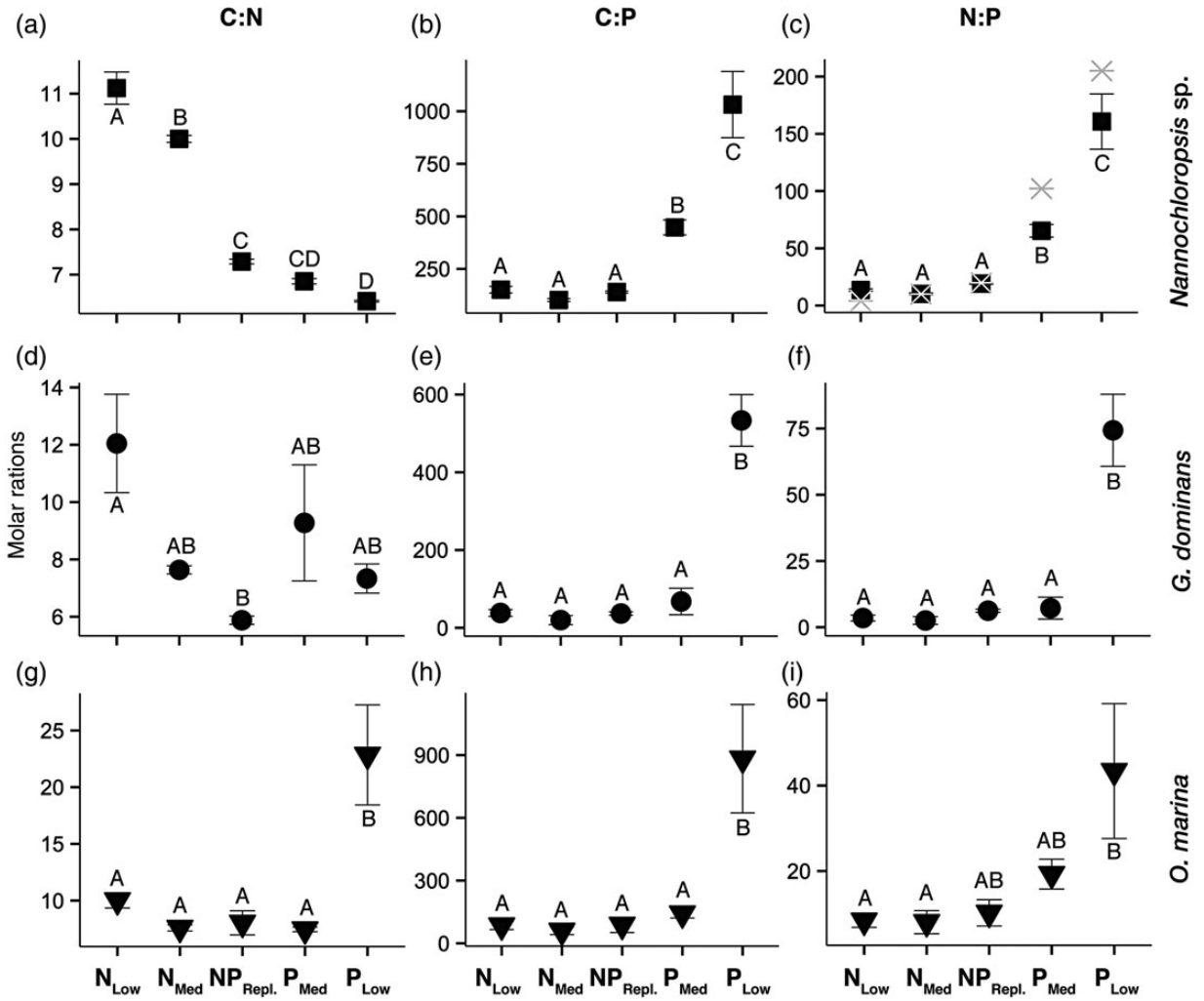

Fig. 1. Mean molar (μmol) C:N, C:P and N:P ratios (±SE) of the algae *Nannochloropsis* sp. (**a**–**c**), and the dinoflagellates *Gyrodinium dominans* (**d**–**f**) and *Oxyrrhis marina* (**g**–**i**) across the five nutrient treatments (Low and Med refers to low and medium N and P limitation, respectively, Repl. refers to replete NP nutrient concentration). Letters from A–D indicate significant differences among treatments and the grey crosses in c indicate the medium supply ratios.

The replete C:N molar ratio of *Nannochloropsis* sp. was 7.3 and increased significantly to 10 and 11 at N_{Med} and N_{Low}, respectively, and decreased slightly at P_{Med} and P_{Low} (Fig. 1). The replete C:P ratio was 140 and was not affected by N limitation, and increased up to 3.3 times at P_{med} and 7.4 times at P_{Low}. *Nannochloropsis* sp. reached a N:P ratio of 19 under replete nutrient levels, which was slightly lower in the N-limited treatments and increased to 65 and 161 at P_{Med} and P_{Low}, respectively. Similar trends between nutrient treatments were observed in the absolute elemental composition, with lowest average nutrient concentrations (N and P) in the corresponding nutrient-limited treatments (Table II).

C:N ratios of the heterotrophic dinoflagellate *G. dominans* reared on N_{Low} algae increased from the $NP_{Repl.}$ treatment of 5.9−12 and were not affected by P_{Med} and N limitation (Fig. 1d). Similar to the food resource, C:P ratios of *G. dominans* were not affected by N limitation with an average C:P value of 35, but C:P increased from 37 in the $NP_{Repl.}$ to 68 and 533 at P_{Med} and P_{Low}, respectively. The N:P ratio of *G. dominans* reflected their higher sensitivity to P limitation, which increased from 6 to 7 and 74 at $NP_{Repl.}$, P_{Med} and P_{Low}, respectively (Fig. 1f). Similar to their food resource, average absolute elemental concentrations of N and P were lowest when *G. dominans* was reared on N and P limited algae, respectively (Table II). Similar to *G. dominans*, nutrient ratios of the heterotrophic dinoflagellate *O. marina* were most strongly affected by P limitation (Fig. 1g−i) and their N:P ratio increased 2- and 4-fold at P_{Med} and P_{Low} from 10.2 at $NP_{Repl.}$. Surprisingly, their C:N ratio quadrupled at P_{Low} compared with the other nutrient treatments. A comparable pattern was observed in the absolute nutrient concentrations, and the P_{Low} treatment had the lowest average N concentration (Table II).

Elemental ratios of the ciliate *Euplotes* sp. were not affected by the stoichiometric ratios of their food (Fig. 2a−c). Both C:N and C:P ratios tended to be higher in the nutrient-limited treatments compared with the control, although differences were not significant. Similarly, the rotifer *B. plicatilis* was marginally affected by the stoichiometric ratios of their food (Fig. 2d−f). C:N ratios increased when reared on N_{low} and P_{low} algae within a narrow range (from 5 to 5.5 and 5.4, respectively), and both C:P and N:P ratios of *B. plicatilis* were higher at N_{Med} compared with all other treatments, which was however not significantly different (Table III).

Homeostatic regulation

The relationship between the nutrient ratios of the resource (nutrient manipulated media and food algae) and the corresponding consumers (algae and zooplankton) was satisfactorily explained by the homeostatic regulation coefficient model. *Nannochloropsis* sp. weakly regulated its internal N:P ratio over varying resource ratios, indicated by the low $H_{N:P}$ coefficient ($H = 1.5$, Table IV, Fig. 3). Similarly, H indicated weak internal stoichiometric regulation for the heterotrophic dinoflagellates, *G. dominans* and *O. marina*, with coefficient values <1.8 (Table IV). In contrast, both the ciliate, *Euplotes* sp. and the rotifer, *B. plicatilis* strongly regulated their internal stoichiometry as indicated by their higher $H_{N:P}$ values of 4.8 and 69, respectively (Fig. 3, Table IV). H coefficients showed that both *Euplotes* sp. and *B. plicatilis* regulated N more strongly compared with P ($H_{C:N} = 53$ and 23.5; $H_{C:P} = 3.4$ and

Table II: Algae and zooplankton stoichiometric composition in the replete (Repl.), low (Low) and medium (Med) nitrogen (N) and phosphorus (P) treatments

Treatment	Nutrient	*Nannochloropsis* sp. (ng μg DW^{-1})	*G. dominans* (ng μg DW^{-1})	*O. marina* (ng μg DW^{-1})	*Euplotes* sp. (ng μg DW^{-1})	*B. plicatilis* (ng μg DW^{-1})
N_{Low}	C	76.62 ± 9.34	41.42 ± 2.56	86.07 ± 4.89	136.55 ± 43.37	239.07 ± 40.08
	N	8.00 ± 0.79	4.15 ± 0.54	10.04 ± 0.33	28.27 ± 8.69	50.30 ± 8.24
	P	1.30 ± 0.03	3.22 ± 0.92	2.85 ± 0.52	6.52 ± 0.73	8.97 ± 0.63
N_{Med}	C	123.34 ± 5.55	35.85 ± 6.72	35.14 ± 4.07	258.10 ± 37.40	395.49 ± 9.06
	N	14.39 ± 0.74	5.51 ± 1.08	5.46 ± 0.83	52.20 ± 6.83	91.24 ± 2.98
	P	3.14 ± 0.08	9.95 ± 4.71	1.68 ± 0.28	10.06 ± 2.05	8.83 ± 0.30
$NP_{Repl.}$	C	168.66 ± 6.88	37.96 ± 14.80	55.71 ± 5.25	207.72 ± 43.41	276.58 ± 18.19
	N	26.97 ± 0.94	7.69 ± 3.17	8.29 ± 1.09	44.83 ± 8.28	64.32 ± 4.59
	P	3.11 ± 0.04	2.91 ± 1.29	2.28 ± 0.79	23.84 ± 9.44	16.34 ± 6.77
P_{Med}	C	339.88 ± 21.91	16.98 ± 3.48	18.12 ± 7.81	262.51 ± 11.14	346.43 ± 85.13
	N	57.83 ± 3.74	2.20 ± 0.42	2.80 ± 1.16	54.03 ± 2.97	77.65 ± 18.72
	P	1.97 ± 0.10	1.63 ± 1.06	0.35 ± 0.16	7.06 ± 1.61	10.01 ± 2.67
P_{Low}	C	161.52 ± 12.42	54.06 ± 2.46	87.95 ± 14.22	131.15 ± 34.85	204.91 ± 62.05
	N	29.37 ± 2.23	8.66 ± 0.62	4.64 ± 0.53	26.83 ± 7.24	44.60 ± 13.78
	P	0.42 ± 0.06	0.27 ± 0.03	0.32 ± 0.12	5.87 ± 3.28	6.19 ± 1.45

Mean C, N and P concentrations per dry weight were calculated at the end of the experiment ($n = 3$, SE < 0.01).

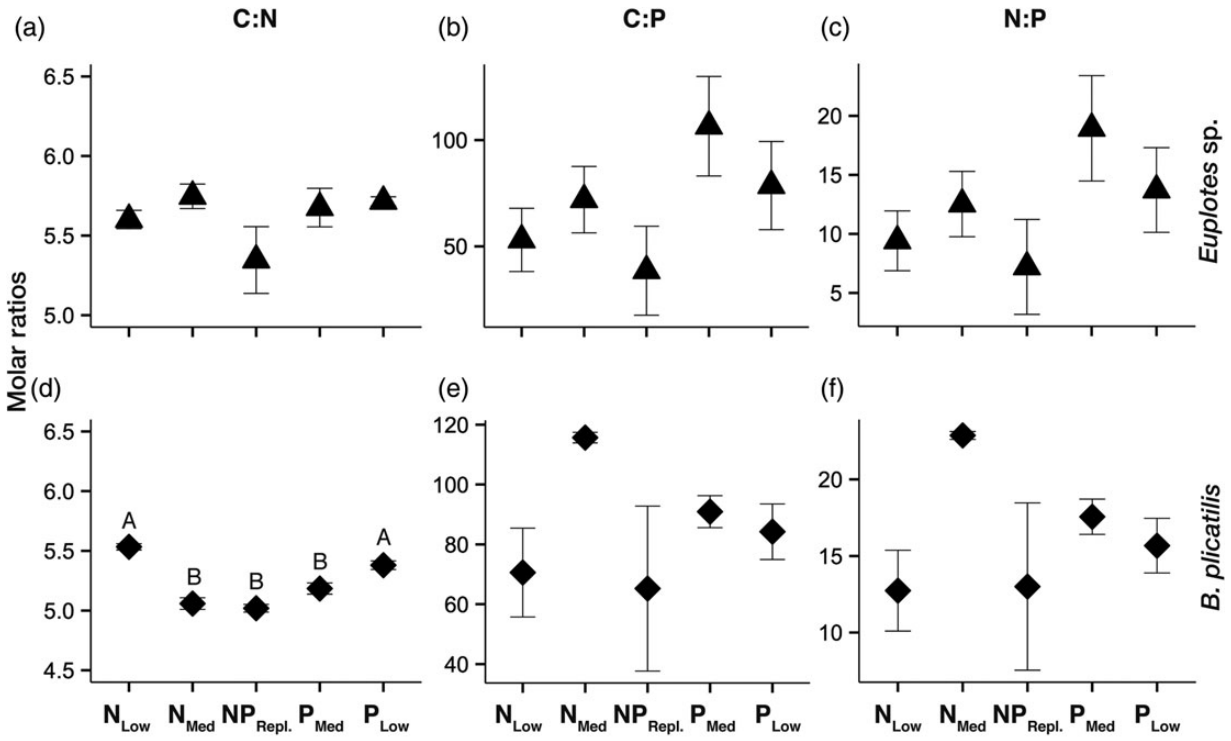

Fig. 2. Mean molar (μmol) C:N, C:P and N:P ratios (±SE) of the ciliate *Euplotes* sp. (**a**–**c**) and the rotifer *Brachionus plicatilis* (**d**–**f**) across the five nutrient treatments. For abbreviations see legend Fig. 1. Letters from A–B indicate significant differences among treatments.

Table III: Overview of published zooplankton and prey phytoplankton C:N, C:P and N:P molar ratios under varying experimental nutrient conditions

	Microzooplankton				Food				
Treatment	Species	C:N	C:P	N:P	Food type	C:N	C:P	N:P	Reference
N_{Low}	*O. marina*	8.5	217	25	*Rhodomonas salina*	13.4	278.5	18.8	Malzahn *et al.* (2010)
P_{Low}	*O. marina*	7.3	385	52	*R. salina*	8.7	847.9	98.9	Malzahn *et al.* (2010)
$NP_{Repl.}$	*O. marina*	7	180	25	*R. salina*	8.8	368.0	38.5	Malzahn *et al.* (2010)
N_{Low}	*B. calyciflorus*	5.6	121	21.7	*Selenastrum capricornutum*	16.3	76	4.7	Jensen *et al.* (2006)
P_{Low}	*B. calyciflorus*	7	105	15.1	*S. capricornutum*	12.9	484	38.1	Jensen *et al.* (2006)
$NP_{Repl.}$	*B. calyciflorus*	5.6	70	12.6	*S. capricornutum*	7.6	66	8.8	Jensen *et al.* (2006)
N_{Low}	*Acartia tonsa*	5.7	177.6	32.5	*R. salina*	13.4	278.5	18.8	Malzahn *et al.* (2010)
P_{Low}	*A. tonsa*	5.8	186.5	33.0	*R. salina*	8.7	847.9	98.9	Malzahn *et al.* (2010)
$NP_{Repl.}$	*A. tonsa*	5.1	245.3	46.9	*R. salina*	8.8	368.0	38.5	Malzahn *et al.* (2010)

For treatment abbreviations see legend of Table I.

20, respectively), which was more pronounced in *Euplotes* sp. Our results are consistent with published data for *O. marina* with low *H* coefficient values (Table IV). Similar to *B. plicatilis*, the conspecies *Brachionus calyciflorus* showed strong internal stoichiometric regulation, however P regulation ($H_{C:P} = 11.9$) was greater than N regulation ($H_{C:N} = 1.2$) in *B. calyciflorus* (Table IV). *H* literature values for copepods also indicate strong internal regulation and are in the same range as the ciliate and rotifer species (Table IV).

Population growth rates and cell biovolumes

Euplotes sp. population growth rates were significantly higher in the replete and P_{Low} treatment compared with N_{Low} ($N_{Low} = 2.16$; $NP_{Repl.} = 2.27$; $P_{Low} = 2.19$) (Fig. 4a). For *B. plicatilis* population growth rates did not differ significantly ($F_{2,6} = 5.03$, $P = 0.06$) between nutrient treatments, but showed tendencies toward higher growth rates at P-limited conditions ($N_{Low} = 1.62$; $NP_{Repl.} = 1.59$; $P_{Low} = 1.65$) (Fig. 4b).

Cell biovolume of *Euplotes* sp. was significantly smaller at P_{Low} with a mean biovolume of 136×10^3 μm^3, compared with $>161 \times 10^3$ μm^3 at N_{Low}, N_{Med} and P_{Med} (Fig. 5a). Contractile vacuoles were largest in the nutrient-limited treatments (~500 μm^3) and decreased to half the volume at P_{Med} (Fig. 6). This resulted in a significantly higher vacuole-to-cell ratio at P_{Low}, with the vacuole contributing to 0.34% of the cell biovolume (Fig. 5c and e). Mean *B. plicatilis* biovolume was lowest in both N and P limited treatments ($<4709 \times 10^3$ μm^3), and almost a third larger at P_{Med} (Fig. 5b). Egg biovolume was not significantly different between treatments and showed a tendency of bigger eggs at N_{Low}. The egg-to-biovolume ratio was highest at N_{Low} and P_{Low} and lowest at P_{Med}, although not significantly different from the replete treatment (Fig. 5d and f).

Table IV: Regression equations of the resource and consumer nutrient ratios and the homeostatic regulation coefficient H *for zooplankton species from our study and the literature*

Species	Ratios	Regression equation	*H* (1/slope)
Nannochloropsis sp.	NP	$y = 0.67x + 0.51$	1.49
G. dominans	CN	$y = 0.62x + 0.35$	1.62
G. dominans	CP	$y = 1.28x - 1.35$	0.78
G. dominans	NP	$y = 1.09x - 0.81$	0.92
O. marina	CN	$y = -0.71x + 1.64$	1.41
O. marina	CP	$y = 1.04x - 0.39$	0.96
O. marina	NP	$y = 0.58x + 0.25$	1.72
O. marina[a]	CN	$y = 0.36x + 0.53$	2.78
O. marina[a]	CP	$y = 0.58x + 0.86$	1.72
O. marina[a]	NP	$y = 0.46x + 0.77$	1.72
Euplotes sp.	CN	$y = 0.02x + 0.73$	4.80
Euplotes sp.	CP	$y = 0.29x + 1.06$	3.42
Euplotes sp.	NP	$y = 0.21x + 0.70$	52.94
B. plicatilis	CN	$y = 0.04x + 0.68$	23.50
B. plicatilis	CP	$y = 0.05x + 1.78$	20.47
B. plicatilis	NP	$y = 0.02x + 1.16$	68.47
B. calyciflorus[b]	CN	$y = 0.81x + 0.25$	1.23
B. calyciflorus[b]	CP	$y = 0.08x + 1.80$	11.92
B. calyciflorus[b]	NP	$y = 0.12x + 1.33$	8.29
A. tonsa[a]	CN	$y = 0.11x + 0.64$	9.39
A. tonsa[a]	CP	$y = \lvert -0.04x \rvert + 2.41$	23.97
A. tonsa[a]	NP	$y = \lvert -0.01x \rvert + 1.59$	88.30

H is calculated as 1/slope.
[a]Malzahn *et al.* (2010).
[b]Jensen *et al.* (2006).

DISCUSSION

The regulation of elemental composition and nutrient cycling has been well demonstrated for heterotrophic organisms, and especially for crustacean mesozooplankton (Sterner *et al.*, 1992; Sterner and Elser, 2002). In comparison, little is known about whether protozoans and rotifers maintain stoichiometric balance. Our study indicates that the regulatory strength of internal stoichiometry in zooplankton species increases from unicellular osmotrophs to mesozooplankton. The two heterotrophic dinoflagellates, *G. dominans* and *O. marina* displayed weak homeostasis regulation, whereas the ciliate *Euplotes* sp. and rotifer *Brachionus* spp. regulated their internal stoichiometry over a large stoichiometric range in their resource.

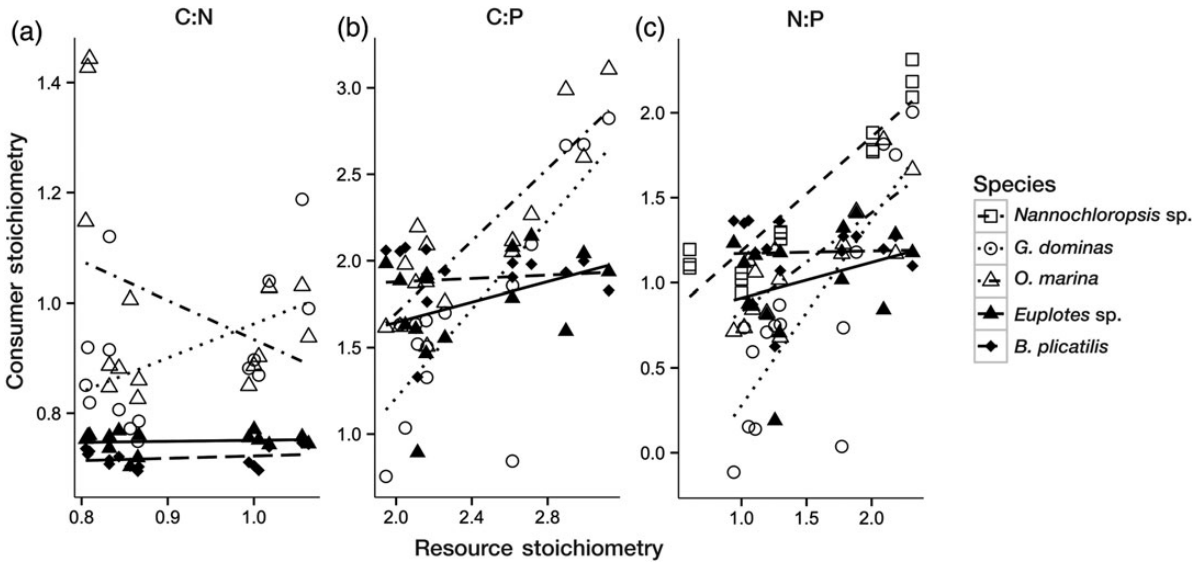

Fig. 3. Primary producer (N:P) and consumer stoichiometry (C:N:P) as a linear function of resource stoichiometry for mean values of log-transformed (**a**) C:N (**b**) C:P and (**c**) N:P ratios (in μmol) used to calculate the strength of homeostasis regulation. Regression coefficients are shown in Table IV. The resource stoichiometry of *Nannochloropsis* sp. was the culture medium and *Nannochloropsis* sp. was the food source for zooplankton species.

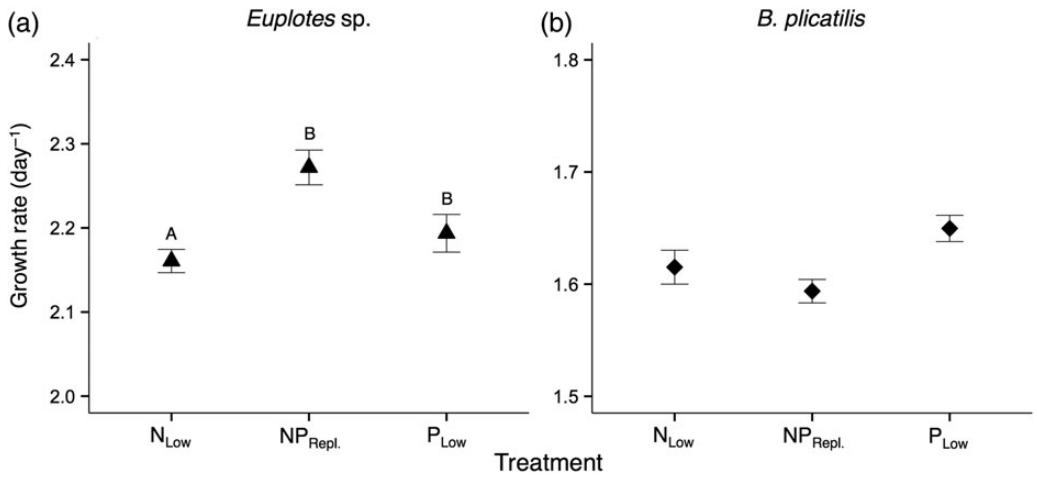

Fig. 4. Growth rates of *Euplotes* sp. (**a**) and *B. plicatilis* (**b**) calculated as mean $\ln(T_6 - T_1)$/time per treatment ($\pm$SE). For treatment abbreviations see legend Fig. 1. Letters from A–B indicate significant ($P < 0.05$) differences among treatments.

These metabolic mechanisms are supported by both the nutrient ratios as well as the homeostasis regulation coefficients.

The primary producer, *Nannochloropsis* sp., showed significant differences in its elemental composition depending on the nutrient supply of the culture media. The N:P ratio ranged from 10 to 161, the C:P from 100 to 1032 and C:N from 11 to 6 under nutrient limitation, and approached Redfield ratios of C_{106}:N_{16}:P_1 under nutrient-replete conditions. The weak internal stoichiometric regulation in *Nannochloropsis* sp. and high elemental cell plasticity over a wide elemental range is consistent with other studies (Geider and La Roche, 2002; Malzahn *et al.*, 2010) and the general assumption of weak homeostatic regulatory capacity in autotrophs (Sterner and Elser, 2002; Hillebrand *et al.*, 2013). It has been shown, however, that the degree of homeostasis in autotrophs can vary depending on species composition, growth phase and environmental conditions (Ågren, 2008; Hessen *et al.*, 2013). Further, the range of C:N and N:P ratios of *Nannochloropsis* sp. observed in our experiment is comparable with observed ratios of marine particulate matter, which in their extremes span can range from 5 to 316 for N:P and from 27 to 1702 for C:P ratios at the global scale (Martiny *et al.*, 2013), with the majority of pelagic systems having C:P ratios from 100 to 500 (Martiny *et al.*, 2013). Thus, our experiments were within realistic resource stoichiometric ratios experienced by herbivores.

The two heterotrophic dinoflagellates *O. marina* and *G. dominans* used in our experiments were not able to completely reduce the stoichiometric imbalance of their food source and the elemental nutrient patterns of their prey were traced in these consumers. This is further supported by the lower homeostasis coefficient, similar to the phytoplankton species. Our findings are comparable to other studies indicating that heterotrophic dinoflagellates are rather poor stoichiometric regulators for N and P (Goldman *et al.*, 1987; Malzahn *et al.*, 2010). A flexible cell stoichiometry may be of advantage for heterotrophic dinoflagellates, as they are often exposed to variation in food quality and quantity in their natural habitat (Meunier *et al.*, 2012). Both species can accumulate elements through luxury consumption when they are available in excess (Hantzsche and Boersma, 2010; Meunier *et al.*, 2012) and use these stores later to supplement growth, instead of using energy to dispose of excess material (Persson *et al.*, 2010). It may therefore be more energetically efficient to maintain a rather flexible cell stoichiometry when exposed to varying elemental food quality. Both dinoflagellates seemed to be better adapted to internal N regulation compared with P regulation, as suggested by the 8–10 times higher N:P and C:P ratios at P-limited compared with N-limited conditions. A more variable P content than N is consistent with other autotroph and consumer studies, which is in part linked to growth metabolism and the high amount of P in RNA (Sterner *et al.*, 1992).

Both heterotrophic flagellates had to cope with the excess C they ingested under nutrient limitation. Previous studies have shown that *O. marina* displayed higher respiration rates when reared on both N- and P-limited algae compared with replete food (Hantzsche and Boersma, 2010; Meunier *et al.*, 2012), suggesting that *O. marina* regulates its excess C via higher respiration and thus release of CO_2. However, respiration rates of *O. marina* are higher when fed with N-limited algae compared with P-limited algae (Meunier *et al.*, 2012), which is

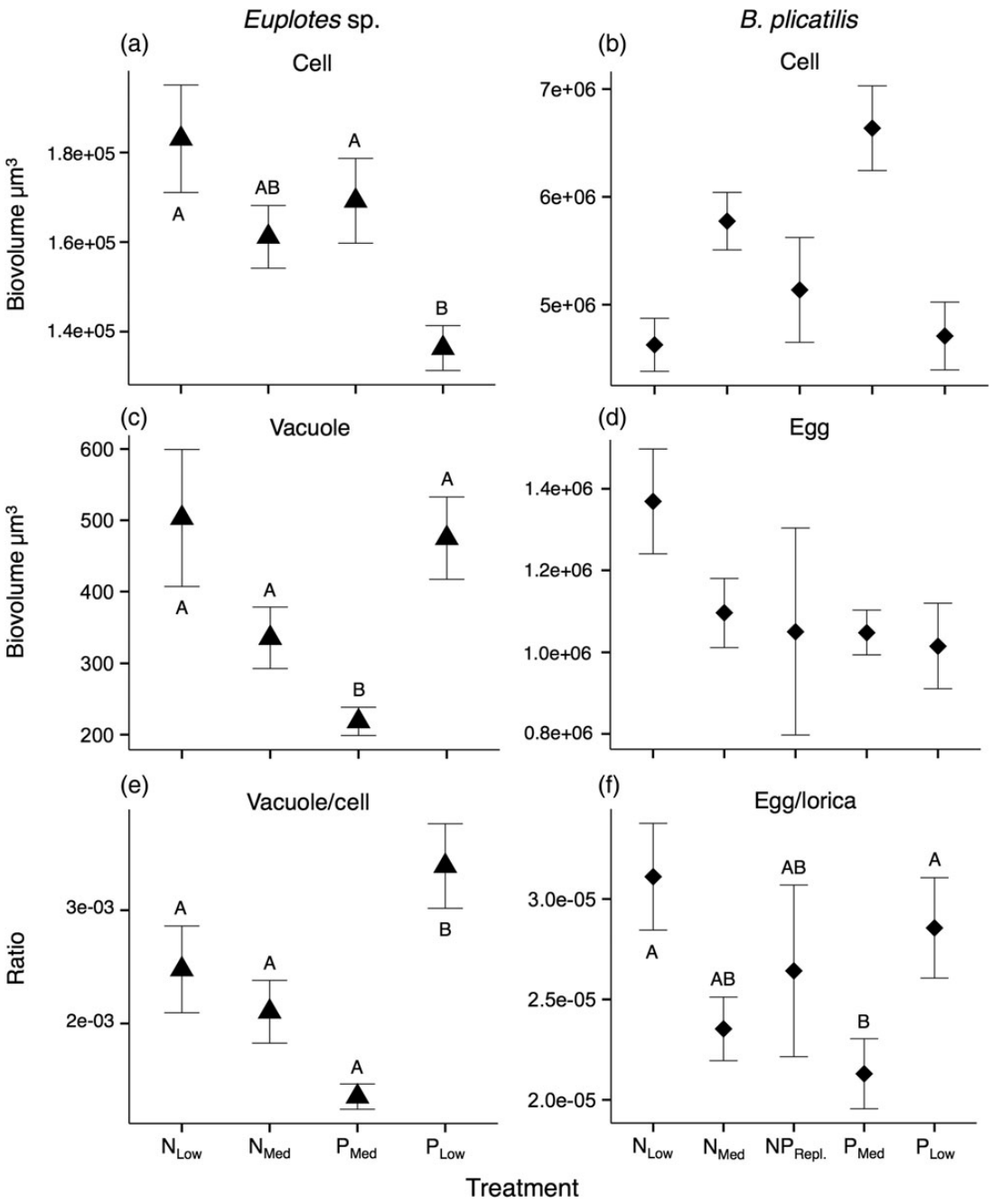

Fig. 5. Physiological response of *Euplotes* sp. and *B. plicatilis* to nutrient-limited treatments. Cell and vacuole biovolume and cell-to-vacuole biovolume ratio (**a**–**c**, respectively) for *Euplotes* sp. at low and med N and P treatments, respectively. Lorica biovolume, egg biovolume and egg-to-lorica biovolume ratio for *B. plicatilis* (**d**–**f**). Letters from A–B indicate significant differences ($P < 0.05$) among treatments.

likely due to the metabolizing of different macromolecules such as N-rich proteins. Proteins have a higher respiration quotient than lipids, as the amount of CO_2 eliminated per oxygen consumed is higher for lipids (Sakami and Harrington, 1963). Therefore, organisms that are metabolizing proteins have lower oxygen consumption, and thus lower respiration rates than those metabolizing P-rich lipids (Lusk, 1924; Weir, 1949). If nutrient limitation results in different substrates being metabolized during respiration, it will affect nutrient remineralization differently and therefore impact phytoplankton communities and nutrient cycling.

In comparison to dinoflagellates, the ciliate *Euplotes* sp. and rotifer *Brachionus* spp. showed higher regulation of

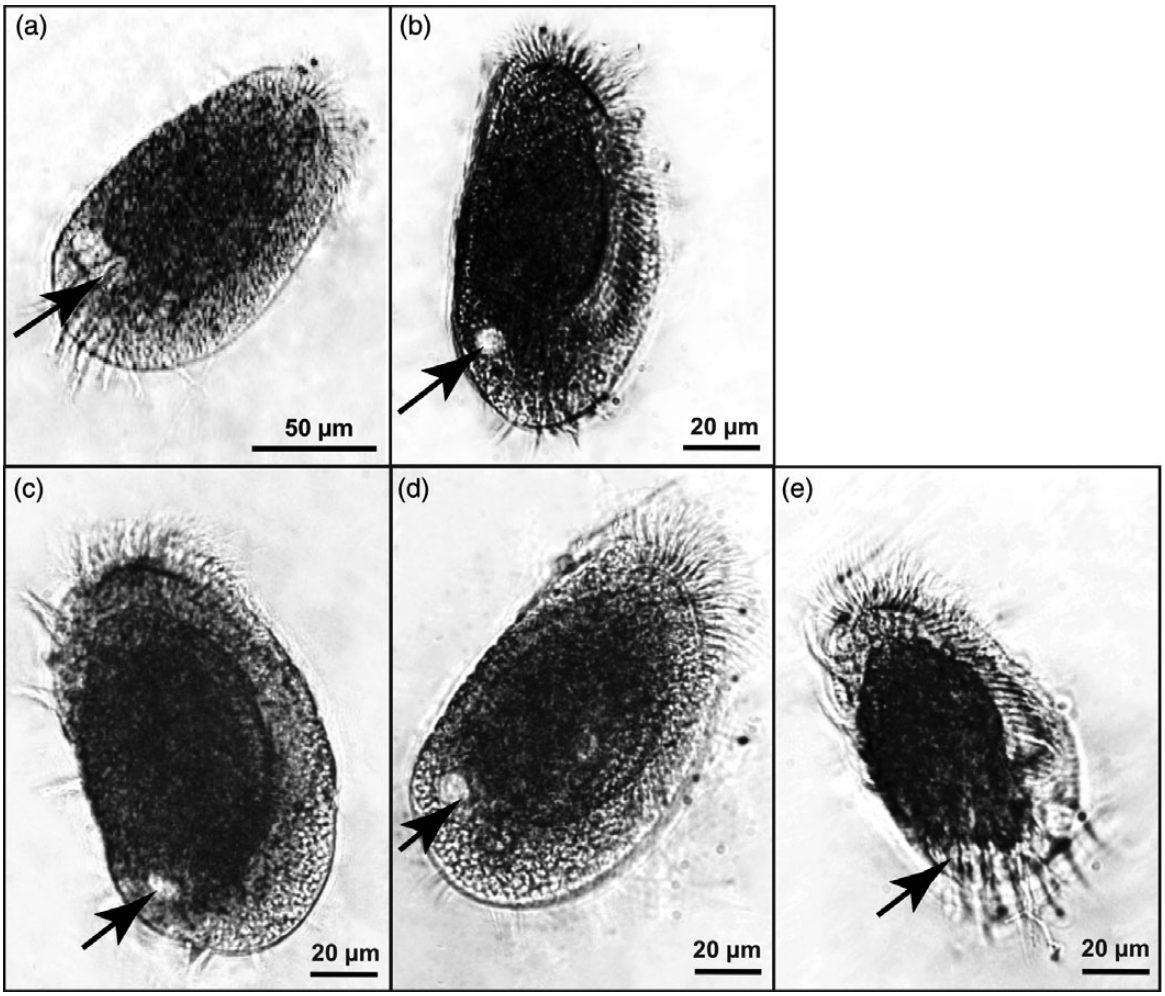

Fig. 6. Contractile vacuoles in the ciliate *Euplotes* sp. at different nutrient treatments: (**a**) N_{Low}, (**b**) P_{Low}, (**c**) N_{Med}, (**d**) P_{Med} and (**e**) NP_{Repl}. For treatment abbreviations see legend Fig. 1. Arrows point to the vacuoles.

their internal element concentration, as indicated by relatively higher homeostasis coefficients. A comparison of two rotifer species indicated differences in stoichiometric regulation strength between species, where *B. plicatilis* appeared to regulate its internal stoichiometry more than *B. calyciflorus*. Intra-specific variation in the ability to cope with elemental imbalance has been shown for a variety of consumer organisms (DeMott and Pape, 2005), suggesting that environmental stoichiometric variation can be an important factor favoring certain species traits in natural populations. Our results further indicate that *Euplotes* sp. regulated their internal N more strongly than their P content, similar to dinoflagellates. These findings are consistent with other studies showing that ciliates engage in compensatory feeding when faced with N limited food resources in order to derive enough N to keep their internal C:N ratio constant, which implies strong homeostatic regulation of internal elemental ratios (Siuda and Dam, 2010). For *B. plicatilis*, regulatory strength of internal N and P content were similar. Both the regulatory pattern and strength of the ciliate and rotifer are comparable with the regulatory strength of copepods found in literature studies. In copepods, C:N ratios are generally more constrained than C:P ratio, which is also consistent with other mesozooplankton (Hessen *et al.*, 2013).

Ecological stoichiometry theory predicts that a mismatch between resource and consumer stoichiometry results in reduced growth and/or fitness of the consumer (Sterner and Elser, 2002; Hessen and Anderson, 2008). Indeed, the ciliate *Euplotes* sp. in our study followed the expected growth trend under nutrient limitation. The population growth rate was highest under replete conditions and decreased under nutrient-limited conditions. Additionally, the organisms were smaller under nutrient limitation. While the growth rate was significantly lower

at N_{Low}, the cell biovolume was smallest at P_{Low}, which indicates that *Euplotes* sp. were more affected by N limitation, which was also evident from the stoichiometric response. One adaptation to P limitation might be a reduction in individual size and hence earlier cell division, while the population growth rate stays relatively high, which has also been observed in other zooplankton (Hessen and Anderson, 2008). A similar trend can be observed in *B. plicatilis* where the population growth rate is not affected by nutrient limitation, but the biovolume was the lowest and hence individuals were the smallest under nutrient limitation. While there was no significant difference in egg diameter, the egg-to-biovolume ratio indicated that the eggs produced under severe nutrient limitation were proportionally larger than under moderate nutrient limitation and replete conditions. This may indicate that the relative investment per offspring may be greater under unfavorable nutrient regimes.

Our results further indicate that ciliates have the capacity to buffer stoichiometric imbalances of primary producers for higher trophic levels. Additionally, these protozoan grazers can also feed on bacteria, which in general are more homeostatic than phytoplankton (Makino *et al.*, 2003). Therefore, if they utilize both bacteria and phytoplankton as a food source, the mismatch between the resource and the consumer can be reduced. Moreover, from a trophic transfer perspective this implies that protozoan grazers feeding both on phytoplankton and bacteria recover carbon fixed in the microbial loop and repackage macromolecules for higher trophic levels in the food web (Sherr and Sherr, 2002). This is consistent with observations showing that heterotrophic dinoflagellates and ciliates comprise a significant part of mesozooplankton diets (Calbet, 2008).

Our results of stoichiometric regulation are relevant in an ecological context, such as trophic transfer and nutrient recycling, and less for physiology given that we cannot exclude the possibility that undigested food left in the consumers is part of the measured values. However, the treatment signal within the consumers should be stronger than any food remains since the grazers were feeding on the manipulated food for over a week. Digestion rates are highly species specific and can vary considerably within taxa. For example, for heterotrophic flagellates digestion rates range between 19 and 28% h^{-1} (Bockstahler and Coats, 1993), and for *O. marina* highly digested material remained up to 24 h (Öpic and Flynn, 1989). For ciliates, digestion rates vary from 1 to 8% h^{-1} (Dolan and Coats, 1991), which suggests for our study that *Euplotes* sp. gut content ranged from completely to 60% emptied after one hour. Nevertheless, our results are applicable to natural conditions, as consumers do not discriminate between the organism consumed and the remains of food in the digestive track. Additionally, our results are comparable with stoichiometric studies of similar species, which show similar trends for heterotrophic dinoflagellates (Malzahn *et al.*, 2010) and it is evident from our study that the ciliate and rotifer have very different body stoichiometry compared with the algal food source. Thus we argue that the remaining food is only of minor importance.

The mechanisms involved in regulating stoichiometric homeostasis vary broadly among organisms, including food selectivity and post-ingestive processes by differential regulation of assimilation and excretion (Meunier *et al.*, 2011; Isari *et al.*, 2013). Post-ingestive differential acquisition of elements could be achieved by adjusting the assimilation efficiency of each element (Frost *et al.*, 2005). It has been suggested that crustacean zooplankton can differentially assimilate C relative to other elements (Anderson *et al.*, 2005; Frost *et al.*, 2005). This infers that herbivores can decrease the efficiency with which they assimilate C-rich compounds during digestion by altering production of digestive enzymes and excrete the excess C, store excess C as lipids, or increase metabolic rates and thus respire excess carbon in the form of CO_2 (Cease and Elser, 2013).

Post-ingestive regulation is also likely in the ciliate *Euplotes* sp. The biovolume of contractile vacuoles as well as the proportional size of the vacuoles compared with the cell size of *Euplotes* sp. increased under severe N and P limitation (for illustration see Fig. 6), which may indicate that ciliates use these vacuoles to excrete excess C, probably in the form of carbohydrates. Another possible explanation for the observed larger vacuoles under nutrient limitation can be related to increased feeding rates, as the contractile vacuoles function as a mechanism to excrete water that was taken up within the food vacuoles during feeding. Therefore, an increased feeding rate would result in an increased amount of water intake that has to be excreted, and hence increases the contractual vacuole size (Kitching, 1934; Patterson, 1980).

Weak homeostasis is likely better under variable environments and for organisms that can store elements when they are available in excess. Plastic organisms are also able to increase their nutrient use efficiency under nutrient imbalance conditions by adding a lower concentration of nutrients to new tissue (Elser *et al.*, 2003). Stoichiometric homeostasis, on the other side, allows organisms to function effectively over a broad range of environmental conditions, with the trade-off of increased costs associated with these physiological mechanisms. Stoichiometric imbalances between primary producer and consumer result in decreased growth, reproduction and survival of the consumer (Cease and Elser, 2013). It is therefore likely that plasticity in terms of stoichiometric

regulation is more advantageous under high environmental fluctuations, while homeostatic regulation is an advantage when environmental conditions are more stable.

In conclusion, this study shows that the strength of stoichiometric regulation in zooplankton increases with increasing from unicellular osmotrophs to mesozooplankton. While heterotrophic protists displayed weak homeostasis, ciliate and rotifer species showed strong homeostasis regulation in terms of their internal stoichiometry. Although homeostasis constrains consumer growth under imbalanced resource supply, our study indicates that these zooplankton species have the potential to trophically upgrade poor autotrophic quality for higher trophic levels. Climate change and eutrophication affect the C: nutrient ratios in aquatic ecosystems in various ways, and consequently the elemental ratio of autotrophs, which may reduce their food quality. Zooplankton may be able to buffer these effects for higher trophic levels to some extent.

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