

Dietary nutrient status modulates nutrient regeneration in the marine ciliate *Euplotes vannus*

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Abstract

Marine ciliates play important roles not only in linking the microbial loop to the classic pelagic and benthic food chains but also in regenerating nutrients, yet how dietary nutrient imbalance impacts their nutrient regeneration has not been thoroughly addressed. The growth and physiological responses of *Euplotes vannus* to low dietary nitrogen (LN) and low dietary phosphorus (LP) conditions were studied, with the bacterium *Pseudomonas putida* as prey. Feeding on LN prey reduced the growth rate of *E. vannus*. Dietary nutrient limitation changed the types and quantities of nutrient recycling. Feeding on LP prey enhanced dissolved organic carbon excretion but reduced orthophosphate excretion, whereas feeding on LN prey generally resulted in decreases in the excretion rate in all N forms (ammonium, urea, and nitrate). In addition, the proportion of ammonium in regenerated N increased significantly under the LN condition. These findings indicate that a nutrient-imbalanced diet triggers *E. vannus* to retain limited macronutrients and promotes the recycling of excessive macronutrients, which may potentially form positive and negative feedback to ambient N and P limitations, respectively.

Key words: protist, excretion, bacteria, microbial loop, nutrient regeneration

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1 Introduction

Protists play crucial intermediate roles in pelagic and benthic food chains in aquatic ecosystems and transfer energy and nutrients from bacteria, flagellates, algae, and other protozoa to organisms at higher trophic levels. These microzooplankton are important nutrient regenerators with regeneration rates that are 1–2 magnitudes faster than those of larger zooplankton. Protists regenerate nutrients at very high efficiencies, and the proportion of regeneration to their daily nutrient intake can reach as high as >100% and 91% for nitrogen (N) and phosphorus (P), respectively (Gast and Horstmann, 1983; Jürgens and Güde, 1990). Therefore, protists contribute quickly and efficiently to primary production in aquatic ecosystems (Gast and Horstmann, 1983; Jürgens and Güde, 1990).

N is potentially the most limiting macronutrient in brackish and marine ecosystems (Elser and Hassett, 1994; Sterner and Hessen, 1994; Hassett et al., 1997). On the other hand, in estuarine and coastal waters around the world, disproportionate loading of anthropogenic nutrients causes an imbalance between P and N (Bricker et al., 2008; Olsen et al., 2011; Glibert et al., 2014). Under these nutrient imbalanced circumstances, bacteria, the main decomposer in ecosystems, potentially suffer nutrient limitation (Elser et al., 1995; Danger et al., 2008). As they represent important prey for protists in the microbial loop (Lawrence and Snyder, 1998), nutrient imbalances may change the elemental composition of bacteria and degrade their quality as prey for protists. Several studies have been conducted on the effects of

elemental ratios of resources on bacterial stoichiometry, and controversial results have been obtained. Makino et al. (2003) found that *Escherichia coli* strictly maintained stoichiometric homeostasis; however, other studies demonstrated that the carbon (C) : N : P ratio of most aquatic bacteria changed when the bacteria were grown in media with varying nutrient compositions (Tezuka, 1990; Nakano, 1994; Chrzanowski and Kyle, 1996; Scott et al., 2012). Ciliates, which are important constituents of pelagic and benthic system protist communities in freshwater and marine systems (Pierce and Turner, 1992; Finlay and Esteban, 1998; Lei et al., 2014), nevertheless, usually act as strict stoichiometric regulators among a variety of protists and sustain their stoichiometric elemental ratio in a narrow range (Chrzanowski et al., 2010). Therefore, a nutrient mismatch possibly exists between the nutrient provision by bacteria and the nutrient demand of ciliates, which may constrain the energy transfer through the food chain and alter the types and quantities of nutrient recycling.

The effects of elemental limitations on physiology, metabolism and performance have been intensively studied in mesozooplankton, but much less is known about microzooplankton, especially ciliates. A few previous studies have revealed that a nutrient-imbalanced diet leads to retarded growth and an altered ingestion rate in ciliates with algal prey (Golz et al., 2015; Siuda and Dam, 2010; Ng et al., 2017). Although ciliate-bacterial interactions are important in aquatic systems, we are not aware of previous relevant studies using bacteria as prey.

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According to the ecological stoichiometry theory, nutrient regeneration depends on both the elemental ratio of intake and the strength of stoichiometric homeostasis in consumers (Sterner et al., 1992), which implies that the shortage of a certain element in prey provokes stoichiometric homeostatic regulation in consumers to retain the limited element and release excessive elements. As revealed in many previous studies, mesozooplankton and a few flagellate protists regulate their stoichiometry mainly through excretion, which generates positive or negative feedback to ambient nutrient limitations and eventually affects the bacterial or plankton communities and functions of the ecosystem (Elser et al., 1998; Rothhaupt, 1997; Grover and Chrzanowski, 2009; He and Wang, 2008). This subject has not been addressed in the literature on ciliate-bacteria interactions.

The protist-bacteria system was constructed in this study using the marine bacterium *Pseudomonas putida* and the ubiquitous marine ciliate *Euplotes vannus* to determine the growth and nutrient regeneration of ciliates under different N and P conditions with the objective to explore the influences of dietary nutrient imbalance on physiology of *E. vannus*. Two hypotheses were proposed: (1) low dietary N and P impact growth of *E. vannus* differently; (2) dietary nutrient imbalance alters nutrient regeneration of *E. vannus*. The ecological implications of altered nutrient regeneration under nutrient imbalanced conditions are discussed.

2 Materials and methods

2.1 Preparation of experimental organisms

An initial inoculum of *E. vannus* was obtained from the Laboratory of Trace Metal Ecotoxicology and Biogeochemistry in the Department of Life Sciences at the Hong Kong University of Science and Technology and then maintained for 4 years in our laboratory. Cells of *E. vannus* were grown in sterilized artificial seawater (Cavanaugh, 1956) as batch cultures under a 12 h : 12 h (L : D) cycle with a light intensity of 100 $\mu\text{mol}/(\text{m}^2\cdot\text{s}^{-1})$ at 20°C. *Euplotes* were supplied with sterilized rice grains, which were used for fermentation to produce bacterial prey.

The marine prey bacterium *P. putida* was isolated from surface water collected from the Daya Bay and kept in a VNSS solid medium (Mårdén et al., 1985). To prepare the prey for the experiments, bacterial cells were transferred to SMB medium (White and Hegeman, 1997) and grown under a 12 h : 12 h (L : D) cycle at 25°C. When the cells reached the stationary phase, they were harvested by centrifugation at 11 000 r/min for 20 min at 4°C, and the supernatants were resuspended in sterilized artificial seawater as stock for the experiments. One milliliter of stock was sampled, preserved in 5% formalin, stained with DAPI, and finally filtered on a 0.22 μm black polycarbonate membrane (Millipore, $\phi = 25$ mm) for bacterial enumeration. Under a fluorescence microscope, 20 fields were selected for counting. The cells were inoculated into a new SMB medium at an initial concentration of 10^6 cells/mL. These processes were repeated 2 or 3 times to activate the bacteria. Then, the activated bacteria were inoculated into

media modified to attain different nutritional conditions (Table 1), and the concentrations of vitamins and trace elements for the different culture conditions were the same as those of *f/2* medium (Guillard and Ryther, 1962). The differentiation cultivation provided nutrient-repleted (HNP), low N (LN) and low P (LP) prey (Table 1). After enrichment and resuspension, the bacterial stock was thermally inactivated in a water bath at 60°C for 2 h to obtain thermally inactivated bacterial prey.

2.2 Experimental design

In 12 sterilized 500 mL flasks containing 400 mL of sterile artificial seawater and HNP, LN, or LP bacteria at a C mass concentration of 4.17 $\mu\text{g}/\text{mL}$, 4 000 individuals of *E. vannus* at a cell density of 10 ind./mL were added. Three flasks containing only bacteria were used as controls for each treatment. Incubation proceeded at a constant temperature of 20°C in the dark for 7 days.

During the incubation, duplicate 1 mL aliquot samples were taken every day and transferred into two 1 mL centrifuge tubes; one was fixed with acid Lugol solution at a final concentration of 1% for determination of *E. vannus* density and the other was preserved with 5% formalin, followed by DAPI staining, for the determination of bacterial abundance. These samples were stored at 4°C, and the enumerations were completed within 24 h after sampling. Thirty individuals of *E. vannus* were randomly selected for each treatment and photographed under a fluorescence microscope (OLYMPUS BX61) every day to determine the length and width of the cells. The volume was calculated according to the formula: volume = $1/3 \times [\pi (\text{width}/2)^2] \times (\text{length})$ (Jeong et al., 2004). Three 100 mL aliquot samples were taken from each flask at 72 h (exponential growth phase), 120 h (stationary phase), and 168 h (recession phase) during the incubation. Samples were filtered through glass-fiber filters (Millipore, GF/F), and the filtrates were stored at -20°C for the determination of phosphate and N release, with the latter including ammonium, urea, and nitrate. All aliquot samples were placed in the dark for later measurements. A total of twenty-four 50 mL subsamples were taken both at the beginning and end of the incubation and filtered through a sieve with a mesh size of 10 μm , and *E. vannus* were collected to determine the elemental composition. After incubation, another 20 mL aliquot was sampled and filtered through a glass-fiber filter (Millipore, GF/F). Then, the filtrates were stored at -20°C for the subsequent determination of dissolved organic carbon (DOC). DOC samples were stored in precombusted (450°C, overnight) and acid-washed 20-mL vials.

2.3 Parameter calculations

The average excretion rates of DOC, N, and reactive P (E_C , E_N , and E_P ; nmol/(ind.·d)) of *E. vannus* were calculated with Eq. (1):

$$E = (N_t - N_0)/(t \times A_1), \quad (1)$$

where N_0 and N_t are the concentrations of excreted C, N or reactive P ($\text{mol}\cdot\text{mL}^{-1}$) at the end of incubation in the experimental and

Table 1. Macronutrient compositions of media to culture *Pseudomonas putida* under nutrient-repleted (HNP), low N (LN) and low P (LP) conditions and elemental molar ratios of bacterial prey

Medium	Macronutrient concentration /($\text{mmol}\cdot\text{L}^{-1}$)			Molar C : N : P ratio	Elemental molar ratio of prey		
	Glucose-C	NaNO_3	KH_2PO_4		C : N	C : P	N : P
HNP	7.50	1.80	0.075	600 : 24 : 1	4.00 ± 0.01	56.0 ± 1.0	14.0 ± 0.3
LN	7.50	0.09	0.075	600 : 1.2 : 1	5.5 ± 0.5	62 ± 4	11.0 ± 1.2
LP	7.50	1.80	0.003 75	120 000 : 480 : 1	4.8 ± 0.2	242 ± 27	51.0 ± 7.6

control groups, respectively, A_1 is the average cell density of *E. vannus* over the incubation period (ind./mL), and t is the incubation duration (h).

The instantaneous population growth rate (g) of *E. vannus* was calculated with Eq. (2):

$$g = \ln(C_{Et} - C_{E0})/t, \quad (2)$$

where C_{E0} and C_{Et} are the initial and final cell densities of *E. vannus*, respectively.

2.4 Analytical measurements

Nutrient concentrations were determined by spectrophotometric methods. Orthophosphate level was determined by applying a reaction that produced a blue complex of elemental P, and then the absorbance values of the complexes were measured at 885 nm (Strickland and Parsons, 1972).

Concentration of dissolved organic carbon was measured using a Shimadzu TOC VCPH analyzer. Ammonium salt level was determined by oxidizing ammonium salt to nitrite under alkaline conditions, and the absorbance value was determined at 543 nm (Matsunaga and Nishimura, 1974). Nitrate concentration was determined by reducing nitrate to nitrite in the presence of copper and cadmium, and its absorbance was measured at 543 nm (Grasshoff et al., 1983). Urea was measured according to Revilla et al. (2005).

Bacteria and predators were filtered through precombusted (450°C, 2 h) GF/C glass-fiber filters (1.2 µm) for measurement of C, N, and P contents. The C and N contents were analyzed with a CHN elemental analyzer (Perkin-Elmer Series 2200, America) after the filter samples had been dried in an oven at 60°C for 48 h, and the cellular P content was analyzed as orthophosphate after acidic oxidative hydrolysis with 1% HCl (Solórzano and Sharp, 1980). These elemental measurements were performed in triplicate.

2.5 Statistical analysis

One-way ANOVA was applied to compare the differences in growth rate, excretion rate, and volume of *E. vannus* among treatments, followed by Tukey's post hoc test. Two-way ANOVA was used to analyze the interaction between the effects of prey nutrient condition and time on *E. vannus* volume and nutrient excretion rates, followed by Tukey's post hoc test. The percentage data were square-root inverse sine transformed before statistical analysis. The correlation between nutrient excretion of *E. vannus* and prey stoichiometry was analyzed with Pearson's correlation. R 3.6.2 software was used for all analysis, and the significance level was set to <0.05.

3 Results

3.1 Elemental composition of *E. vannus*

The P content of *E. vannus* varied with the elemental compos-

ition of the bacterial prey (Table 2). *Euplotes vannus* fed LP prey had the lowest P content ((0.17 ± 0.09) ng/cell, $p < 0.001$) and, correspondingly, the highest molar C : P ratio ($p = 0.031$ 6; one-way ANOVA and Tukey's post hoc test). In contrast, the C and N contents remained generally consistent in *E. vannus*, and therefore, the molar C : N ratio varied slightly and insignificantly in the narrow range of (4.1 ± 1.8)–(5.5 ± 2.4) (Table 2).

3.2 Growth rate and cell volume

The bacterial biomass in the treatments gradually decreased, and after 120 h, the bacteria were depleted in the HNP and LP treatment groups but not in the LN treatment group (Fig. 1).

Euplotes vannus in the HNP and LP treatment groups reached the stationary phase after 72 h of incubation, whereas those in the LN treatment group did so after 120 h of incubation, but with comparable maximum cell densities of (215 ± 8) ind./mL to (240 ± 20) ind./mL among all treatments ($p < 0.05$; one-way ANOVA). The instantaneous population growth rate was lower in the LN treatment group than in the other two treatment groups ((0.77 ± 0.08) d⁻¹ vs. (1.08 ± 0.07) d⁻¹ and (1.10 ± 0.09) d⁻¹, Fig. 2A). The average volume of *E. vannus*, ranging from (3.48 ± 0.39) × 10⁴ µm³ to (4.51 ± 1.08) × 10⁴ µm³, was independent of the nutrient status of the prey. Nevertheless, it varied slightly at different growth phases under HNP and LN conditions but not under the LP condition (Fig. 2B). The volume of *E. vannus* was higher during the first 72 h than during the last 48 h in the HNP treatment group. For those in the LN treatment group, the cell volume during the final 48 h was larger than that during the middle 48 h ($p < 0.001$; two-way ANOVA and Tukey's post hoc test; Table 3).

3.3 Nutrient excretion

During the 168 h of incubation, *E. vannus* in the LP treatment group exhibited a significantly higher DOC excretion rate than those in the HNP treatment group ((0.184 ± 0.105) nmol/(ind.·d) vs. (0.088 ± 0.029) nmol/(ind.·d)); $p = 0.013$, one-way ANOVA and Tukey's post hoc test; Fig. 3A). No difference was detected between the LN treatment group and the HNP treatment group or LP treatment group ($p > 0.05$; one-way ANOVA and Tukey's post hoc test; Fig. 3A).

The phosphate excretion rate of *E. vannus* varied within the range of 1.58–10.5 pmol/(ind.·d) during incubation and decreased with incubation time. *Euplotes vannus* in the HNP treatment group generally exhibited the highest excretion rate compared with those in the LN and LP treatment groups ($p < 0.001$), and with incubation time, the P excretion rate slowed down ($p < 0.001$; two-way ANOVA and Tukey's post hoc tests; Table 3, Fig. 3B). The interaction of growth phase and food quality showed that the difference between different treatments narrowed with incubation time (Fig. 3B). Overall, the highest rate was present in the HNP treatment group during the initial 72 h ((10.5 ± 1.47) pmol/(ind.·d)), whereas the lowest rate was present in the LP treatment group during the final 48 h ((2.63 ± 0.28) pmol/(ind.·d), Fig. 3B). The average P excretion rate followed a similar trend in

Table 2. Carbon (C), nitrogen (N) and phosphorus (P) contents and molar elemental ratio of *Euplotes vannus* fed with nutrient-repleted (LNP), low N (LN), and low P (LP) preys. Means ± SD ($n = 6$); superscript letters indicate significant differences between nutrient treatments ($p < 0.05$; one-way ANOVA)

Prey	C/(ng.ind. ⁻¹)	N/(ng.ind. ⁻¹)	P/(ng.ind. ⁻¹)	Elemental molar ratio		
				C : N	C : P	N : P
HNP	6.77 ± 2.55 ^a	2.28 ± 0.50 ^a	0.66 ± 0.16 ^a	4.1 ± 1.8 ^a	26 ± 12 ^a	7.6 ± 2.5 ^a
LN	7.96 ± 1.50 ^a	1.96 ± 0.78 ^a	0.65 ± 0.04 ^a	5.5 ± 2.4 ^a	31.0 ± 6.4 ^a	6.6 ± 2.7 ^a
LP	4.74 ± 0.97 ^a	1.32 ± 0.59 ^a	0.17 ± 0.09 ^b	4.9 ± 2.4 ^a	71 ± 15 ^b	17 ± 8 ^a

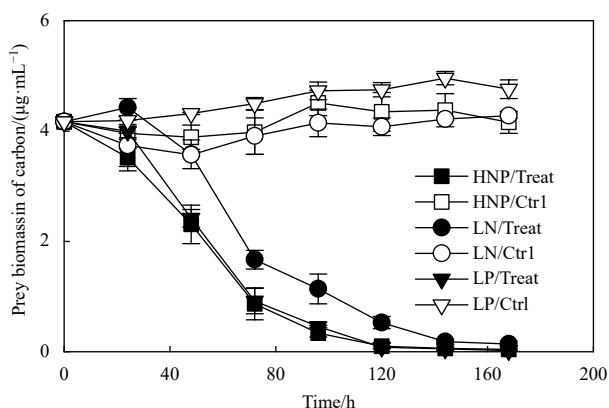


Fig. 1. Changes in bacterial biomass during the 168 h of incubation of *Euplotes vannus* and nutrient-repleted (HNP), low N (LN), and low P (LP) prey, *Pseudomonas putida* (Treat: the treatment with both bacteria and *E. vannus*; Ctrl: the control with only bacteria). Error bar indicates means \pm SD, $n = 6$.

that the animals in the LP treatment group had a significantly lower excretion rate than those in the LN and HNP treatment groups ($p = 0.013$), between which no difference in excretion rate was found ($p = 0.077$; one-way ANOVA and Tukey's post hoc tests; Fig. 3C).

N excretion was measured in the forms of ammonium, urea, and nitrate during the 168 h of incubation. Ammonium, urea, and nitrate excretion rates varied within the ranges of (8.60 ± 1.57) – (120.0 ± 6.07) pmol/(ind. \cdot d), (0.00 ± 0.00) – (5.63 ± 1.05) pmol/(ind. \cdot d), and (0.00 ± 0.00) – (1.38 ± 0.37) pmol/(ind. \cdot d), respectively, and decreased with incubation time in most cases, paralleling the trend of the ingestion rates (Figs. 4A–C). Ammonium and urea excretion rates were consistently lower in the LN treatment group than in the other two treatment groups during incubation, except that no difference was detected between the three groups during the last 48 h (Fig. 4A; $p < 0.05$, two-way ANOVA and Tukey's post hoc tests; Table 3). Animals in the HNP treatment group had higher or equal ammonium and urea excretion rates than those in the LP treatment group (all $p > 0.05$ except for $p = 0.034$ for urea excretion rate during 0–72 h) (two-way ANOVA and Tukey's post hoc tests; Fig. 4A, Table 3). The nitrate excretion rate was highest in the HNP treatment group, followed by the LN treatment group and then the LP treatment group, which had the lowest nitrate excretion rate ($p < 0.001$, two-way

ANOVA and Tukey's post hoc tests; Fig. 4C, Table 3). Ammonium and urea excretion rates generally decreased with incubation time except in the LN treatment group ($p < 0.001$; two-way ANOVA, Table 3). Additionally, there were interactions between the effects of time and prey nutrition state on the excretion rates of all N forms ($p < 0.001$; two-way ANOVA, Table 4). The strength of the effect of time on the excretion rate was different for different N forms, and furthermore, in the LN treatment group, ammonium and urea excretion showed an increasing trend with time (Figs. 4A–C); two-way ANOVA and Tukey's post hoc tests, Table 4).

The average N excretion rate was calculated by summing the rates of all N forms. The average excretion rate was comparable in the HNP and LP treatment groups (35.0 nmol/(ind. \cdot d) vs. 38.3 nmol/(ind. \cdot d), $p = 0.245$) and was significantly lower in the LN treatment group (18.9 nmol/(ind. \cdot d); $p = 0.003$, one-way ANOVA and Tukey's post hoc tests; Fig. 4D).

Ammonium was the most important route for N excretion, with high contributions of up to $95.4 \pm 1.26\%$, followed by urea, with contributions of $(2.31 \pm 0.53\%)$ – $(18.1 \pm 4.54\%)$, and nitrate, with a negligible contribution of $(2.08 \pm 0.46\%)$ – $(2.33 \pm 0.18\%)$ (Fig. 4E). Food quality influenced the proportions of ammonium and urea in the total N excretion. The animals in the LN treatment group had the highest proportion of ammonium but the lowest proportion of urea, whereas those in the HNP treatment group showed the opposite trend (all $p < 0.01$, one-way ANOVA and Tukey's post hoc tests; Fig. 4E). *Euplotes vannus* in the LP treatment group had moderate proportions of both ammonium and urea compared with the other two treatment groups ($p > 0.1$ for ammonium and $p < 0.05$ for urea; one-way ANOVA, Tukey's post hoc test; Fig. 4E).

The total N excretion rate of *E. vannus* was significantly positively correlated with the N : P ratio of prey ($p = 0.002$) but negatively correlated with the C : N ratio of prey ($p = 0.04$). Meanwhile, a significantly negative relationship existed between the P excretion rate and the C : P ratio of prey ($p = 0.02$), whereas the P excretion rate was correlated with the N : P ratio of prey, but not significantly ($p = 0.058$; Pearson's correlation; Table 4).

The average molar C : N and C : P ratios of nutrients excreted by *E. vannus* did not vary with prey nutritional status except that the individuals feeding on LP prey released N and P at a N : P ratio of 28.7 ± 0.47 , which was 2.5–3.1-fold higher than that of those feeding on HNP and LN prey (8.2 ± 3.2 and 7.08 ± 3.21 , Fig. 5).

4 Discussion

Growth and nutrient regeneration were investigated in a cili-

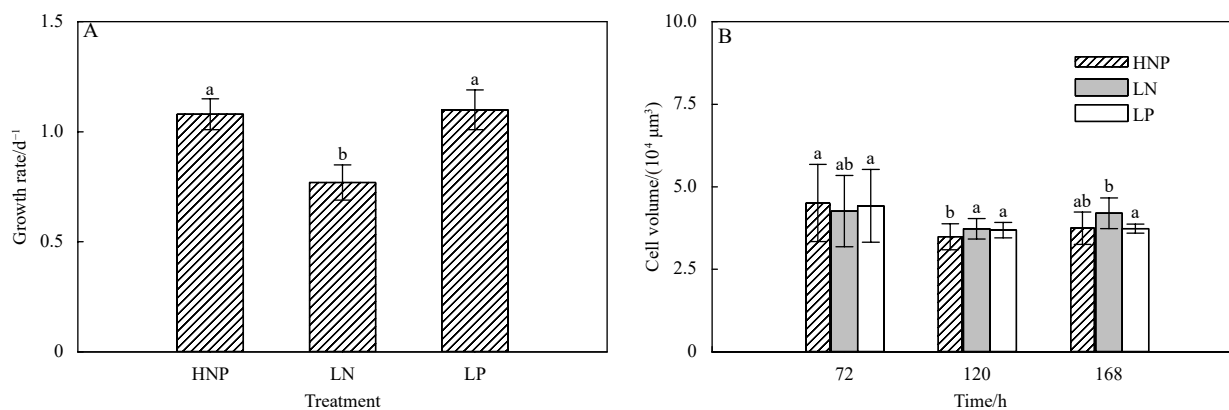


Fig. 2. Instantaneous growth rate (A) and volume (B) of *Euplotes vannus* during the 168 h of experimental feeding with nutrient-repleted (HNP), low N (LN), and low P (LP) prey, *Pseudomonas putida*. Error bars indicate means \pm SD, $n = 6$ and 30, respectively; different lowercase letters indicate significant differences among the treatments.

Table 3. Two-way ANOVA testing for the effects of incubation time and the prey nutritional status (NS) on prey concentration, *Euplotes vannus* volume, and nutrient excretion rates by *Euplotes vannus* (SS: sum of squares; df: degrees of freedom; MS: mean sum of squares; *F*: test statistic; *P*: probability).

Parameter	Source	SS	df	MS	<i>F</i>	<i>P</i>
<i>E. vannus</i> volume	Time	4 600 936 256.044	6	766 822 709.341	79.979	<0.001
	NS	53 266 685.012	3	17 755 561.671	1.852	0.148
	Time × NS	474 317 325.207	18	26 350 962.512	2.748	0.002
	Error	4 600 936 256.044	6	766 822 709.341	79.979	<0.001
	Total	5 991 830 739.700	85	-	-	-
Ammonia excretion	Time	18 680.693	2	6 226.898	46.325	<0.001
	NS	6 263.514	2	3 131.757	23.299	<0.001
	Time × NS	10 839.230	4	1 806.538	13.440	<0.001
	Error	2 957.212	9	134.419	-	-
	Total	48 480.030	33	-	-	-
Urea excretion	Time	7.518	2	3.759	12.951	<0.001
	NS	96.607	2	32.202	110.939	<0.001
	Time × NS	16.355	4	2.726	9.391	<0.001
	Error	7.257	25	0.290	-	-
	Total	133.248	36	-	-	-
Nitrate excretion	Time	0.049	2	0.024	0.562	0.579
	NS	1.492	2	0.497	11.489	<0.001
	Time × NS	2.857	4	0.476	11.001	<0.001
	Error	0.866	20	0.043	-	-
	Total	5.313	31	-	-	-
Phosphate excretion	Time	56.387	2	28.194	33.105	<0.001
	NS	106.103	2	35.368	41.528	<0.001
	Time × NS	44.771	4	7.462	8.762	<0.001
	Error	20.440	24	0.852	-	-
	Total	254.714	35	-	-	-

Note: - represents no data.

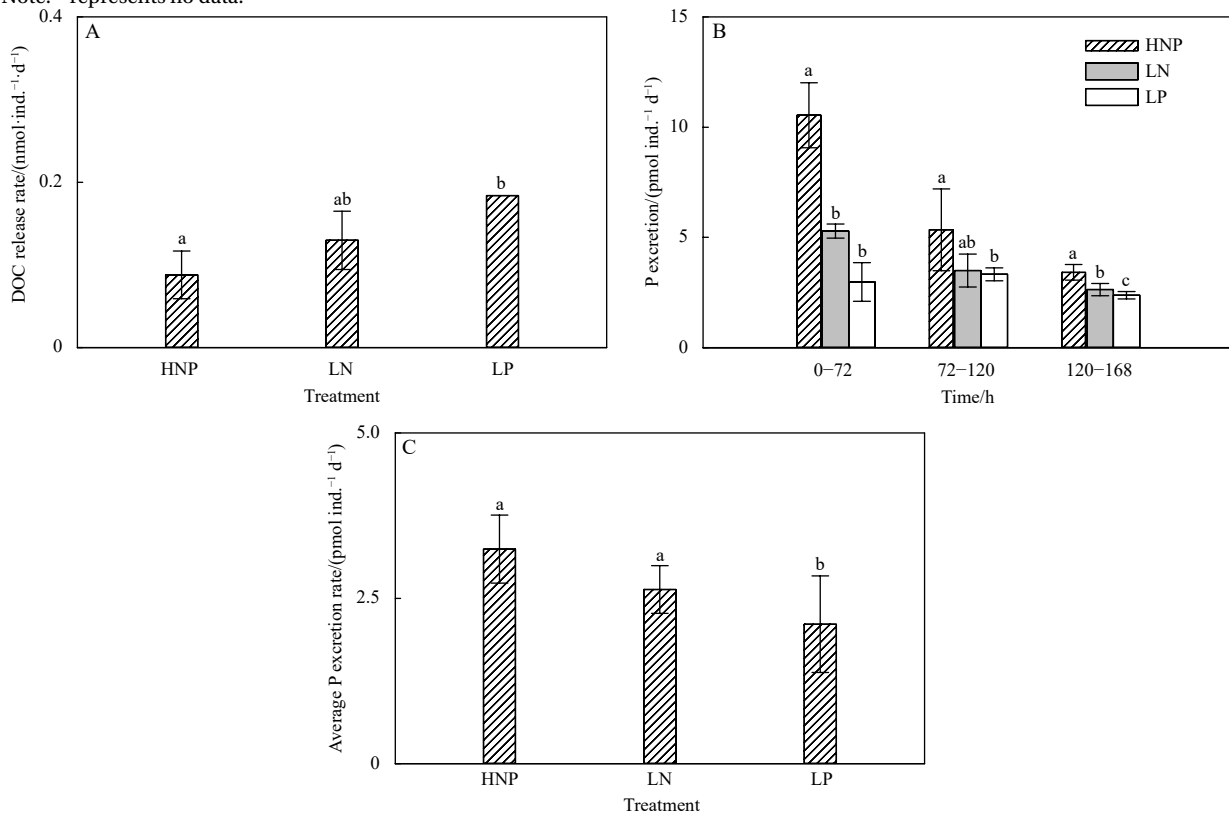


Fig. 3. Dissolved organic carbon (DOC, A), phosphate (P) excretion rates at different phases (B), and average P excretion rates (C) of *Euplotes vannus* fed nutrient-repleted (HNP), low N (LN), and low P (LP) prey, *Pseudomonas putida*, during the 7-day experiment. Error bar indicates means ± SD, *n* = 6; different lowercase letters indicate significant differences among the treatments.

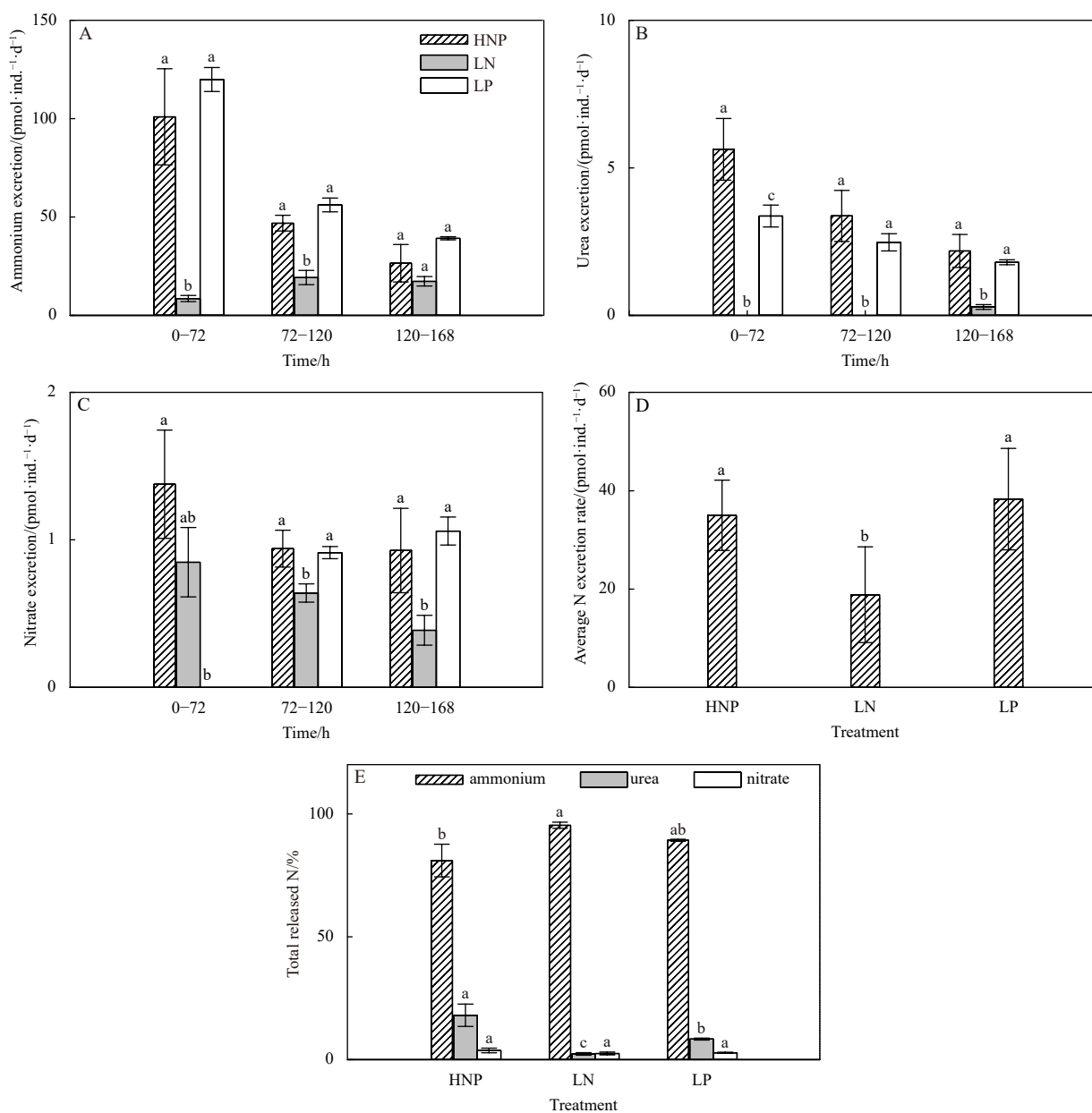


Fig. 4. Nitrogen excretion of *Euplotes vannus* fed nutrient-repleted (HNP), low N (LN), and low P (LP) prey, *Pseudomonas putida*, during 168 h of incubation (A: ammonium; B: urea; C: nitrate; D: average nitrogen excretion rate; E: contributions of different nitrogen forms to total nitrogen release). Error bar indicates means \pm SD, $n = 6$; different lowercase letters indicate significant differences among the treatments.

Table 4. Pearson correlation coefficients between carbon (C), nitrogen (N), and phosphorus (P) excretion rates of *Euplotes vannus* and prey stoichiometry. (C : N_{Prey}: C : N molar ratio of prey, C : P_{Prey}: C : P molar ratio of prey, N : P_{Prey}: N : P molar ratio of prey).

	C : N _{Prey}	C : P _{Prey}	N : P _{Prey}
C excr. rate	0.51	0.56	–
N excr. rate	–0.63 ^b	–	0.82 ^a
P excr. rate	–	–0.64 ^b	–0.56

Note: Superscript a and b represent statistical significance with $p < 0.01$ and $p < 0.05$, respectively.

ate-bacteria system constructed with *E. vannus* and *P. putida* in this study. We found that dietary nutrient imbalance enhanced the recycling of excessive nutrients and reduced the recycling of

limited nutrients in *E. vannus*, which facilitated consumers' adaptation to nutrient imbalanced conditions and created feedbacks to the ambient nutrient imbalance.

Feeding bacteria cultured in the low N medium led to a 28.7% decrease in the intrinsic growth rate of *E. vannus*, indicating that LN impaired the performance of *E. vannus*. Considering the extent of N- and P-limitation used in this experiment, it seems that *E. vannus* were more vulnerable to LN relative to LP. Golz et al. (2015) using a series of diets with broader elemental ratios also found that the population growth rate and stoichiometric elemental composition of *E. vannus* were more affected by LN than LP. In their study, cell size decreased significantly under the LP condition, which, was thought to indicate earlier cell division of *E. vannus*. Differently, this phenomenon didn't occur in our study possibly because their study used algae as prey and dietary

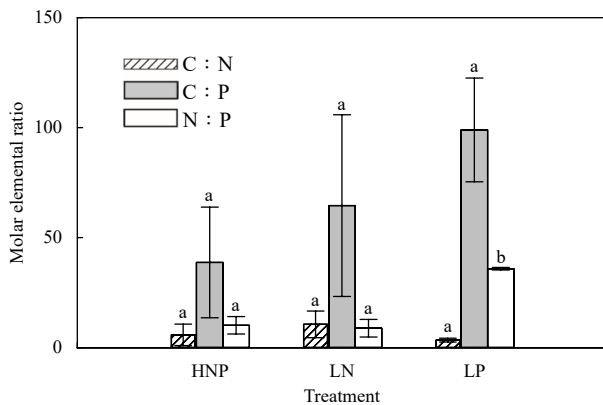


Fig. 5. Stoichiometry of nutrient release in *Euplotes vannus* fed nutrient-repleted (HNP), low N (LN), and low P (LP) prey, *Pseudomonas putida*, during the time period. Error bars indicate means \pm SD, $n = 6$; different lowercase letters indicate significant differences among the treatments.

C : P ratios varied in a wider range compared with our study. *Euplotes vannus* had lower P content under the LP condition compared with those under HNP or LN condition, but their growth rate remained unchanged. This appears inconsistent to the growth rate hypothesis (GRH), which relates high P requirement to fast growth in organisms (Sterner and Elser, 2002). We speculated that *E. vannus* probably overcame the LP condition and maintain the growth rate by increasing utilization efficiency of P, which possibly involves decreasing P allocation to P-rich RNA and increasing biomass production per unit P.

Orthophosphate was released by *E. vannus* at an average rate of 3.25 pmol/(cell·d) during incubation, which was in the range of P excretion rates for different ciliates (0 to 10.1 pmol/(cell·d)) reported in the literature (reviewed by Dolan, 1997). Although *E. vannus* can release P in the form of organic phosphorous (Johannes, 1965), orthophosphate is still the main form for P release in this species. Considering that *E. vannus* containing 0.02 nmol P per cell under nutrient-replete conditions, the P release rate accounted for 16% of the body P content per day, which suggests that *E. vannus* could be a noteworthy P regenerator in aquatic ecosystems. Johannes (1965) estimated that three species of *E. vannus* regenerate P extremely fast with P body-equivalent excretion times ranging 20 min to 160 min, which equals to release rates of 37.5%–300% body P per hour by each *E. vannus* individual. In this early study, the excretion experiment lasted 3–5 min and conducted in a small volume (5 mL), which may lead to overestimation of P excretion rate in *E. vannus*. P excretion was constrained by the dietary P condition and feeding on LP bacteria led to a 35% decrease in P excretion rate in our study. Jürgens and Güde (1990) also revealed that miscellaneous phagotrophic flagellates exhibited lower P regeneration efficiency when fed P-limited bacteria than C-limited bacteria (18%–90% vs. 50%–91%, respectively).

Ammonium, urea, and nitrate were considered in our study when measuring the N excretion rate of *E. vannus*. As a whole, the average N excretion rate varied within the range of 35.1–38.3 pmol/(cell·d) under nutrient-balanced and P-deficient conditions, which was at the lower end of the wide range of 0.0–141 pmol/(cell·d) reported for ciliates in the literature (Dolan, 1997). The LN condition reduced the N excretion rate by 46%, and a strong positive correlation existed between the N excretion rate and the N : P ratio of prey. Ammonium and urea, accounting for

81.0%–95.4% and 4.6%–19.0% of the total N excretion, respectively, were two important constituents of the N release of *E. vannus*. Ammonium is well accepted to be the most important form of N excretion in microflagellates and macrozooplankton (Steinberg and Saba, 2008). Urea is another important byproduct of N metabolism. Previous studies demonstrate that organic N forms, including urea and dissolved primary amines, collectively constituted 7%–89% of the N released by zooplankton (Steinberg and Saba, 2008; Goldman et al., 1985; Valdés et al., 2017), but less information is available on the excretion of urea by microzooplankton. Urea release accounted for 40% of the total N (ammonia + urea) release in tintinnid ciliates (Lee Johansen, 1976). L'Helguen et al. (2005) asserted that ciliates were the main urea regenerators in the well-mixed waters of the western English Channel, where corresponding urea regeneration made up 33% of the total regenerated N annually. Nitrate represents a minor form of N excretion with marginal proportions to the total N excretion, which are close to the lower end of the range reported for planktonic crustaceans, benthic organisms, and fish (~2% vs. 3%–20%; Atayde and Hansson, 1999; Nędzarek and Stepanowska, 2022). So far, potential metabolic pathways related to production of nitrate within organisms remain unsolved. The LN and LP conditions led to significantly decreased N excretion rates in all three N forms and altered the proportion of ammonium and urea in the present study. To our knowledge, this is the first report that prey nutritional status alters the partitioning of N excretion in ciliates. Miller and Roman (2008) reported a decrease in both ammonium and urea excretion in copepod fed a LN diet, but partitioning of N excretion was independent of dietary N : C ratio. A nutrient-imbalanced diet will reduce the availability of regenerated N by ciliates to ambient bacteria and phytoplankton and change the uptake rate by altering the relative proportion of different N forms (Antia et al., 1991). It is speculated that the LN cultivation condition reduced the synthesis of N-rich amino acids (e.g., arginine) by bacteria and consequently lowered the urea excretion and the proportion of urea in the total N excretion of *E. vannus*.

The DOC excretion rate was 0.09–0.18 nmol/(ind·d), and the LP condition led to a 1.1-fold increase in the DOC excretion rate, whereas moderate increases in the DOC excretion rate (48% and 21%) were achieved under the LN condition. Considering the decrease in bacteria biomass through *E. vannus* consumption at the end of the incubation, the proportion of bacterial prey C, which became DOC, was roughly calculated as 24.0%, 24.0%, and 42.6% with HNP, LN, and LP prey, but the differences between these three values were not significant. These regeneration efficiencies fell into the range of 3%–37% reported for protists in the literature (Caron et al., 1985; Taylor et al., 1985; Strom et al., 1997). Zhang et al. (2017) reported a dramatic increase in C excretion (3.40 folds) in the protist *Noctiluca scintillans* under P limitation. These results suggest that consumers tend to reduce utilization efficiencies of C by releasing more C for stoichiometric homeostasis under nutrient-imbalanced circumstances. The excretion route for disposal of excessive C in *E. vannus* was highlighted in our study, but respiration may be another important route for disposal of excessive C in zooplankton (He and Wang, 2008).

Comparing the C : N ratio of bacterial prey and excretion in the system indicated that the C : N ratio of excretion was significantly higher than that of bacterial prey under LN conditions (10.6 vs. 5.5; $p < 0.01$), whereas the N : P ratio showed the opposite trend (8.2 vs. 11.0; $p < 0.01$). Under the LP condition, *E. vannus* excreted nutrients at lower C : P and N : P ratios than the bacterial prey (99.0 and 28.7 vs. 242 and 51.0; $p < 0.01$). The relative and absolute nutrient excretion rates both showed that *E. vannus*

modulate nutrient release to retain limited nutrients but dispose excessive nutrients, which, eventually, establishes different feedbacks to the ambient nutrient state. As N was released by *E. vannus* at a lower proportion to C compared with the bacterial N : C ratio under the LN condition, it seemed that the presence of *E. vannus* intensified the existing N deficiency; however, the increase in the contribution of ammonium to the total N excretion may partially offset the negative effect (Andersson et al., 1985). In contrast, P-limitation may be alleviated to some extent by the presence of *E. vannus*. However, additional studies are needed to ascertain whether this result can be applied to other protist-bacteria systems.

5 Conclusions

Growth rates were reduced when *E. vannus* were fed with bacterial prey cultured under the LN condition. More generally, *E. vannus* reduced their loss of the limiting nutrient but increased their recycling of excessive nutrients through excretion under nutrient-limited conditions, which conforms to the ecological stoichiometry theory. Furthermore, the partitioning of N excretion was skewed to ammonium rather than urea under the LN condition. Interactions between *E. vannus* and bacteria under nutrient-imbalanced conditions may lead to different feedbacks to environmental N and P limitations in that the nutrient recycling by these organisms may enforce the ambient N-deficiency but alleviate the ambient P-deficiency.

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