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Phytodetrital quality (C:N ratio) and temperature changes affect C and N cycling of the intertidal mixotrophic foraminifer *Haynesina germanica*

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ABSTRACT: The combination of lower diet quality and increased metabolic rates is assumed to cause cascading effects on organismic C cycling. Future changes in CO_2 levels or terrestrial nutrient discharges in marine ecosystems can lead to increased phytoplankton C:N ratios relative to consumer C:N ratios, lowering the quality of the food source. In this study, we compared the single and interactive effects of diet quality and temperature on the feeding behavior and C and N intake and release of a common and abundant intertidal mixotrophic protist, the foraminifer *Haynesina germanica*. Two batches of artificially produced and dual isotope-labeled ($^{13}C/^{15}N$) chlorophyte detritus with different C:N ratios (5.6 and 7.1) were fed to the foraminifer at 3 different temperatures (15, 20, 25°C). We observed a strong interactive effect of temperature and diet. A very strong increase in feeding rates was observed at 20°C for the low-quality food source. Respiration rates of carbon derived from the low-quality diet (C:N ratio of 7.1) were lower than those of the high-quality diets and increased at 25°C. This indicates that a high C content of the diet might be of advantage in calcifying mixotrophs, since respired excess C could be advantageous for test calcification. Additionally, respired excess C could be a useful resource of CO_2 for kleptoplast photosynthesis and functionality in the mixotrophic lifestyle of *H. germanica*. Further, the observed effects of diet and temperature could impact nutrient fluxes in the habitat of *H. germanica*, possibly leading to food-web shifts in the future.

KEY WORDS: Benthic foraminifera · Food intake and turnover · Carbon and nitrogen stoichiometry · *Haynesina germanica*

1. INTRODUCTION

Foraminifera are key players in marine benthic food webs (e.g. Lipps & Valentine 1970, Moodley et al. 2000, Nomaki et al. 2008). These highly abundant protists are ubiquitous primary consumers and detritivores that play a critical role in the processing of marine particulate organic matter (POM). Foraminifera strongly contribute to benthic ecosystem metabolism and biogeochemical cycling, including ecosystem respiration and C and N cycling (e.g. Moodley et al. 2000, 2002, Witte et al. 2003, Geslin et al. 2011, Enge et al. 2014, 2016, Cesbron et al. 2016). *Haynesina germanica* is a benthic foraminifer with a calcified shell (test) that commonly inhabits coastal soft sediments in high abundances (e.g. Alve & Murray 1994, 2001, Mojtahid et al. 2016, Müller-Navarra et al. 2017, Wukovits et al. 2018).

Benthic foraminifera adapt a wide range of strategies for nutrient supply, including herbivory, detrivory,

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bacterivory, deposit feeding, carnivory, photosymbiosis and inorganic nutrient uptake (Murray 1991). The feeding strategy of *H. germanica* was investigated earlier in laboratory observations (Austin et al. 2005). *H. germanica* actively graze on the diatom *Pleurosigma angulatum*, by cracking its frustules outside of the foraminiferal test and absorbing the diatom content into the foraminiferal cytoplasm (Austin et al. 2005). Kleptoplasts, i.e. functional chloroplasts sequestered from algae, also play an important role in the life strategy of *H. germanica* (Jauffrais et al. 2017, 2018). A characteristic of the species is a mixotrophic lifestyle by using kleptoplast photosynthesis accompanied by O_2 production, driven by light supply, inorganic carbon and ammonium uptake from the surrounding seawater and subsequent storage of photosynthetically derived organic carbon in lipid droplets (Lopez 1979, Cesbron et al. 2016, LeKieffre et al. 2018). *H. germanica* sequesters chloroplasts exclusively from pennate diatoms, including chloroplasts derived from the diatom *Phaeodactylum tricornutum* (Pillet et al. 2011), which is also considered to be a suitable food source for *H. germanica* cultured under laboratory conditions (Ward et al. 2003).

D. tertiolecta and *P. tricornutum* detritus was used in feeding experiments with *H. germanica* (Wukovits et al. 2017, 2018). These experiments showed that the feeding activity of *H. germanica* is significantly affected by increasing temperature. Due to the high abundance of this species in temperate mudflats, it is of high interest to evaluate its feeding response to changes in phytodetrital C:N ratios, or diet quality, and to different temperatures. Heterotrophic consumers strongly depend on the quantity and quality of their diets, in all aspects of individual development, population growth or community composition (Sterner & Elser 2002 and references therein). Temperature also has a strong effect on herbivorous consumers, specifically on metabolic rates and feeding activities (Brown et al. 2004). To date, there has been a lack of research considering phytophagous or detrivorous mixotrophic protists with respect to their response to diet quality in combination with changing environmental factors such as temperature.

Diet quality can be expressed as the nutrient stoichiometry or the ratio of C to limiting nutrients (e.g. N or P, which occur at lower concentrations in the environment). A high C:N ratio indicates a lower nutritional value due to the relative lack of N and therefore indicates lower diet quality (Elser et al. 2000). Cellular C:N ratios of marine autotrophic organisms, like microalgae, are controlled by the nutrient concentrations in their environment, resulting in fluctuations between C:N ≤ 6 and C:N ≥ 10 (for examples for marine phytoplankton, see Levitan et al. 2007, Li et al. 2012, Clark et al. 2014). Future changes in CO_2 levels or terrestrial nutrient discharges in marine ecosystems can lead to increased imbalances in phytoplankton C:N ratios relative to consumer C:N ratios. For example, elevated CO_2 levels and decreased nutrient supply to surface waters can promote rising C:N ratios in phytoplankton (Urabe & Waki 2009, Steinacher et al. 2010, Tagliabue et al. 2011, Sardans et al. 2012, Diez et al. 2013, Clark et al. 2014, Eberlein et al. 2016). Further, an increase in global warming can lead to increased C:N ratios in phytoplankton (De Senerpont Domis et al. 2014). Moreover, future shifts in ocean currents and stratification caused by global warming can lead to nutrient imbalances, which may have a strong effect on microalgal nutrient stoichiometry (Makino et al. 2011, Sardans et al. 2012). In contrast, heterotrophic organisms show low variations over intra- and interspecific ranges in cellular or somatic C:N ratios (C:N ~ 3–6, Sterner & Elser 2002, Frost et al. 2005, 2006). Therefore, primary consumers (herbivores and detritivores) are often exposed to a stoichiometric mismatch between their own somatic (or cytoplasmic) C:N ratio and the nutrient ratio of their diet (stoichiometric imbalance).

A common response to low diet quality is to increase feeding rates and compensate for the lack of the limiting nutrient, i.e. compensatory feeding (Cruz-Rivera & Hay 2000), whereas excess nutrients are released by excretion or mineralization (e.g. Anderson et al. 2005, Jensen et al. 2006, Jensen & Hessen 2007). Additionally, increased water temperatures raise the metabolic rate in heterotrophic ectotherms and protists (e.g. Bradshaw 1961, Brown et al. 2004). In this way, the nutrient turnover and demand is stimulated and results in higher feeding rates (Chen et al. 2012, Carr & Bruno 2013). A strong interdependence of nutrient stoichiometry (or ecological stoichiometry, Sterner & Elser 2002) and the temperature-driven metabolic rates (cf. metabolic theory of ecology in Brown et al. 2004) is therefore thought to amplify the respiratory CO_2 release and protist feeding rates in future ocean scenarios. Recent predictions state that this will lead to food web shifts and will affect ecosystem C and N fluxes (Anderson et al. 2005, Boersma et al. 2016).

Most studies on combined effects of diet stoichiometry and temperature fluctuations have focused on higher, multicellular organisms. However, fluctuations and interactions of these parameters are highly relevant across all scales of organismic organization and

evolution. Therefore, we tested the single and combined effects of diet stoichiometry and temperature on the foraminifer *H. germanica*. In this study, laboratory cultured *H. germanica* were used to investigate the interactive effect of the food C:N ratio and temperature on feeding rates in short-term (24 h) laboratory incubations. We aimed to identify the response of this mixotrophic organism, which acquires nutrients via both heterotrophic food ingestion and photosynthesis, to a combination of changes in diet quality and temperature in terms of diet-derived C and N intake and C and N loss. We carried out experiments with ^{13}C- and ^{15}N-double-labeled phytodetritus of different quality in the laboratory, to track the uptake of phytodetrital C and N in *H. germanica* at 3 different temperatures (15, 20, 25°C). Two different phytodetritus diets were offered for 24 h to the foraminiferal specimens: (1) *D. tertiolecta* (chlorophyte), C:N = 5.6, and (2) *D. tertiolecta*, C:N = 7.1. This approach allowed us to quantify the budgets of phytodetritus-derived C ($_{phyto}$C) and N ($_{phyto}$N) for *H. germanica*. We expected that the C:N ratio of the food source has a significant effect on the phytodetritus-derived C and N budget in *H. germanica*. We tested 2 main hypotheses: (1) We expected an increase in feeding rates with increasing temperature and decreasing diet quality (higher C:N ratio). We also expected a strong, interactive effect of temperature and diet, resulting in an amplification of the temperature-driven feeding compensation at higher temperatures. (2) We expected an elevated loss of $_{phyto}$C relative to $_{phyto}$N in the foraminifera when fed with the high-C diet (C:N = 7.1) and specifically an increased respiratory loss of $_{phyto}$C as dissolved inorganic C ($_{phyto}$DIC) at higher temperatures.

2. MATERIALS AND METHODS

2.1. Sampling site and material collection

Surface sediments were collected from 28 April to 1 May 2016 during low tide in the intertidal mudflat of the German Wadden Sea near Friedrichskoog (Germany). Water temperature and salinity at the sampling site were 19.9°C and 31 PSU. The average annual sea surface temperature for 2016 was 9–12°C during the night and 12–15°C during the day, the monthly sea surface temperature in 2016 was 12–15°C in April, 21– 24°C in May and 21–24°C (data from GIOVANNI Modis Aqua Satellite, https://giovanni.gsfc.nasa.gov/giovanni/). The projected temperature increase will be +3.7°C until the end of this century under the RCP8.5 scenario (van Vuuren et al. 2011, IPCC 2013, Hofstede & Stock 2018). Sediment was collected and pre-sieved at the sampling site to remove larger meiofauna (>500 µm) and organic particles (<125 µm). In the laboratory, samples were sieved again to concentrate adult foraminifera in the size fraction >250 µm, kept within glass aquariums (20 × 20 × 30 cm, filled with sieved sediment [<250 µm] and filtered seawater [43 µm] from the sampling site; ~7 cm height of the water column at 20°C) for up to 6 wk prior to the experimental incubation, and fed weekly with a mixture of live *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*. Individuals of living *Haynesina germanica* (Fig. 1) were removed and kept within glass crystallizing dishes (DURAN®, 9.5 cm diameter, 350 ml volume) containing a thin layer of sediment from the sampling site (<63 µm, dried at 50°C prior to use) to track traces of movement as proof of vital signs. Living individuals were further identified under the microscope based on intact protoplasm and particle accumulation around the aperture (Moodley et al. 1997, Nomaki et al. 2005, 2006).

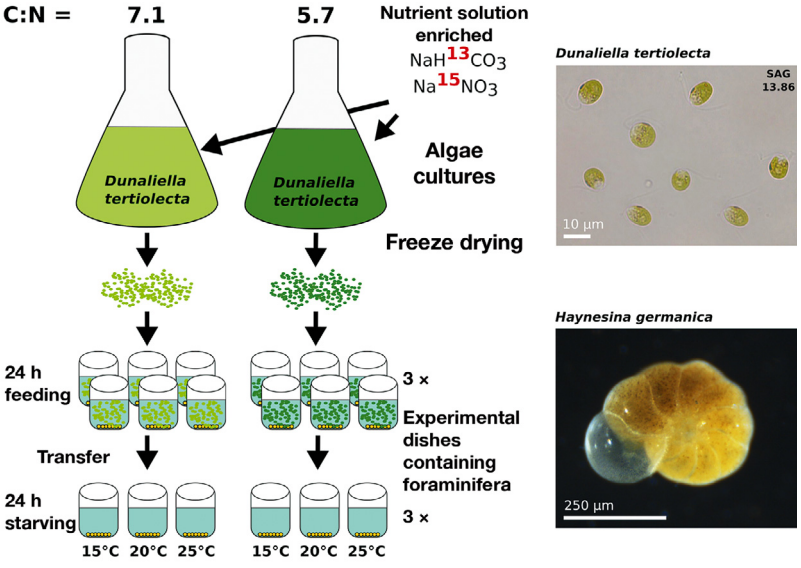

Fig. 1. Experimental setup of the feeding experiment. Two batches of dual-isotope-labeled *Dunaliella tertiolecta* phytodetritus were fed to 2 triplicate batches of the foraminifer *Haynesina germanica* (~50 specimens each) at 3 different temperatures. One triplicate of the 'feeding' series was terminated after 24 h for elemental and isotope analysis, the second triplicate was incubated for a further 24 h in fresh filtered seawater without food ('starving' series) and terminated after another 24 h

Table 1. Diets used in the present study. 7.1 D was a low-quality diet, whereas both 5.7 D and 5.5 P were considered high quality

Diet	Species	Diet C:N	Growth medium		Amount fed to *Haynesina germanica*	
			C µmol l^{-1}	N µmol l^{-1}	mg C m^{-2}	mg N m^{-2}
7.1 D	*Dunaliella tertiolecta*	7.1	1500	800	600	85
5.7 D	*D. tertiolecta*	5.7	600	800	645	115
5.5 P[a]	*Phaeodactylum tricornutum*	5.5	600	800	545	100

[a]Data from Wukovits et al. (2018)

2.2. Production of ^{13}C- and ^{15}N-labeled phytodetritus

D. tertiolecta was grown in f/2 medium (Guillard & Ryther 1962, Guillard 1975), which was prepared with filtered North Sea water and enriched with 98 atomic % (at%) ^{13}C ($NaH^{13}CO_3$, Sigma-Aldrich) and 98 at% ^{15}N ($Na^{15}NO_3$, Sigma-Aldrich). *D. tertiolecta* cultures were grown at 2 different C:N nutrient conditions via the addition of distinct ratios of $NaHCO_3$ and $NaNO_3$ to the f/2 growth medium (for final content of DIC and dissolved inorganic nitrogen in the culture media, see Table 1) to obtain phytodetritus batches with distinct C:N ratios (Table 1). Cultures were incubated for 16–20 d at 20°C (light:dark = 16:8 h) with sterile filtered (22 µm) ambient air via an aquarium pump. Algae were harvested by centrifugation (800 × *g*), washed 3 times with filtered sea water with intermittent centrifugation and finally freeze dried at –55°C and 0.180 mbar to produce artificial phytodetritus. The final C:N ratios were 5.7 ('5.7 D' = high-quality diet) and 7.1 ('7.1 D' = low-quality diet). Final isotope enrichments were 5.7 D = 15.6 at% ^{15}N and 2.4 at% ^{13}C and 7.1 D = 55 at% ^{15}N and 11 at% ^{13}C.

2.3. Experimental setup

Cleaned, living individuals of *H. germanica* were placed in polystyrene 6-well plates containing filtered North Sea water in triplicate for each treatment per well (ca. 50 individuals in 12 ml of seawater). To each well, we added 1.4 mg (dry weight) of one sort of artificial phytodetritus. The amount of added C and N per well slightly differed between the 2 diets (cf. Table 1). The well plates were incubated at 15, 20, or 25°C with a 12:12 h light:dark cycle (25–50 µmol photons m^{-2} s^{-1} photosynthetically active radiation). For the 'feeding' series, 'fed' foraminifera were removed from the experimental dishes after 24 h and frozen at –20°C to halt metabolic activity. For the 'starving' series, specimens were transferred after the 24 h feeding period from the experimental dishes to glass dishes containing only filtered North Sea water (250 ml per replicate) and reintroduced into the incubation chambers for another 24 h at the same temperature as that they were kept at before. Then, 24 h later, the 'starving' series was also terminated by freezing the specimens (Fig. 1). The 'starving' series was implemented to calculate the release of C and N as the difference of the cytoplasmic content of phytodetritus-derived C and N over 24 h. The experimental setup is shown in Fig. 1. The experimental setup of Wukovits et al. (2018) was exactly the same as that in the present study, but with high-quality *P. tricornutum* detritus ('5.5 P'). The data of Wukovits et al. (2018) were visually compared with the results of the present study and, additionally, release rates for $_{phyto}$C and $_{phyto}$N and $_{phyto}$C:$_{phyto}$N ratios were calculated from Wukovits et al. (2018) to get an impression of the effect of diet quality and temperature when feeding on different food sources. Additionally, water samples were taken from the glass dishes of the 'starved' treatment and preserved for DIC analysis.

2.4. Sample preparation

Tests of 'fed' and 'starved' specimens were cleaned of adhering particles with a hair brush and carefully washed in artificial seawater, containing 4 different salts (Enge et al. 2011). For each replicate, all individuals of the same series which showed healthy intact cytoplasm were transferred to tin capsules and dried for several hours at 50°C. The survival rate was 88.20 ± 3.64 (SD)% and similar in all treatments, and all surviving specimens were analyzed; thus, ~50 individuals per replicate were analyzed. The tests were decalcified with 4% HCl in 2 steps of 5 µl HCl each and dried for 1 d at 50°C between the 2 steps. The remaining cytoplasm was dried in a final drying step for 3 d at 50°C. All glassware used for preparation was combusted at 500°C for 5 h, and tools and tin capsules were cleaned in a solution of CH_2Cl_2 and CH_4O (1:1, v:v) to be organic free.

Water samples were transferred to 50 ml headspace vials. A few drops of $HgCl_2$ (15–30 µl) were added to stop respiratory activity and biological production of CO_2 (Kroopnick et al. 1972). The vials

were sealed airtight and stored at 4°C. To determine $\delta^{13}C$ of DIC ($_{phyto}$DIC) in the samples, 12 ml Exetainer® vials were flushed with He, injected with 0.5 ml of 85% H_3PO_4 and then 2 ml water samples, and kept to equilibrate for 48 h at room temperature for conversion of DIC to CO_2 (Li et al. 2007, Taipale & Sonninen 2009). Note that the results of our $_{phyto}$DIC measurements do not represent the exact quantities of respired diet-derived C because the experimental dishes were not sealed airtight to ensure oxygen availability for the specimens. A considerable amount of respired CO_2 will have been lost to the atmosphere, specifically at higher temperatures, and atmospheric CO_2 will exchange with seawater DIC, lowering the ^{13}C enrichment of DIC. Therefore, we set up an additional incubation of artificial seawater containing the natural enrichment and 4 distinct elevated $^{13}C/^{12}C$ enrichment levels (1.101, 1.125, 1.133, 1.308, 4.314 at% ^{13}C). By applying regression models to these data, a correction factor for temperature-dependent $^{13}C/^{12}C$ fractionation was obtained (Fig. 2). The $\delta^{13}C$-DIC data obtained from the experimental incubation with the foraminifera were multiplied by the correction factor for the respective temperature. Subsequently, the fraction of algae-derived $\delta^{13}C$-DIC of the total DIC was used to calculate the individual respiration rate of diet-derived C from the labeled algae ($\delta^{13}C_{algae}$), from seawater sampled from the experimental incubations ($\delta^{13}C_{SWsample}$) and from the natural abundance $\delta^{13}C$ values from North Sea water ($\delta^{13}C_{SWBG}$) from the concentration of DIC and the number of individuals (n_{ind}) as follows:

$$_{phyto}DIC = \frac{\left(1-\left[\frac{\delta^{13}C_{algae}-\delta^{13}C_{SWsample}}{\delta^{13}C_{algae}-\delta^{13}C_{SWBG}}\right]\right)\times DIC}{n_{ind}} \quad (1)$$

The result was subsequently multiplied by 12 (atomic weight of carbon) to transform the result into g DIC ind.$^{-1}$.

2.5. Sample analysis

The samples were analyzed at the Large-Instrument Facility for Advanced Isotope Research at the University of Vienna (SILVER). Cytoplasmic contents of organic C or N and ratios of ^{13}C:^{12}C and ^{14}N:^{15}N (and respective $\delta^{13}C$ and $\delta^{15}N$ values) were determined with an isotope ratio mass spectrometer (IRMS; DeltaPLUS, Thermo Finnigan) with interface (ConFlo III, Thermo Finnigan) coupled to an elemental analyzer (EA 1110, CE Instruments).

At% values of the samples were derived from isotope ratio data and were calculated using the Vienna Pee Dee Belemnite standard (R) for C (R_{VPDB} = 0.0112372) and atmospheric N_2 for N (R_{atmN} = 0.0036765), where X is ^{13}C or ^{15}N:

$$at\%X = \frac{100\times R_{standard}\times\left(\frac{\delta X_{sample}}{1000}+1\right)}{1+R_{standard}\times\left(\frac{\delta X_{sample}}{1000}+1\right)} \quad (2)$$

Uptake of phytodetrital C and N was calculated by determining the excess (E) isotope content within the samples against the natural abundance of the isotopes in the foraminiferal cytoplasm (Middelburg et al. 2000), where X is ^{13}C or ^{15}N:

$$E = \frac{(at\%X_{sample} - at\%X_{background})}{100} \quad (3)$$

Excess and total organic C (TOC) and total N (TN) were used to calculate the amount of incorporated isotope $I_{iso} = E \times$ TOC (TN) to obtain the amount of $_{phyto}$C and $_{phyto}$N within the foraminiferal cytoplasm defined as I_{phyto} (Hunter et al. 2012):

$$I_{phyto} = \frac{I_{iso}}{\left(\frac{at\%X_{phyto}}{100}\right)} \quad (4)$$

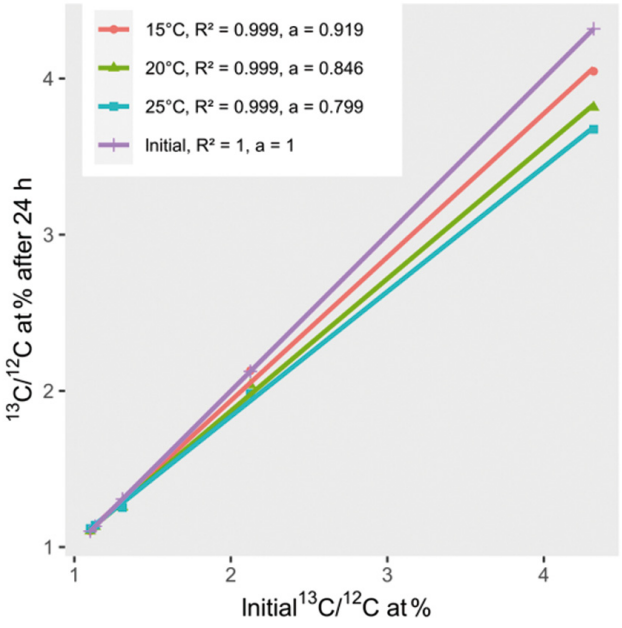

Fig. 2. Calibration for temperature-dependent $^{13}C/^{12}C$ fractionation of artificial seawater containing 5 distinct enrichment levels of ^{13}C, after a 24 h incubation period at 15, 20 and 25°C (regressions with mean values from experimental data, n = 3). Correction factors used to calculate the release of foraminiferal dissolved inorganic carbon from experimental incubations, as the ratio of the initial slope to the slope after 24 h of incubation, are: 15°C = 1.08804, 20°C = 1.181892, 25°C = 1.251926

The $\delta^{13}C$ values of the seawater DIC were measured after release by H_3PO_4 addition as CO_2 using a headspace gas analyzer (GasBench II, Thermo Fisher) coupled to an IRMS (Delta Advantage V, Thermo Fisher). The total amount of diet-derived C in seawater DIC (C respired by all incubated individuals, nmol l^{-1} d^{-1}) was calculated using E and I_{iso} values derived from seawater DIC and correcting for algal isotope enrichment (Sweetman & Witte 2008), using values of 2.264 mmol l^{-1} DIC for North Sea water (Stoll et al. 2001).

2.6. Data analysis

The experiment was set up in a 2 factorial design (factor 1: temperature, factor 2: diet). Due to the limited replication per treatment (n = 3) and the heteroscedasticity of the data, nonparametric statistics with randomization (resampling) were applied to examine the effect of temperature, diet and their interaction on the cytoplasmic content of $_{phyto}C$ and $_{phyto}N$ within the foraminifera. Nonparametric PERMANOVA was applied to compare the experimental output (number of computed permutations was 100). This approach provides a good alternative to parametric ANOVA (Feys 2016) for data sets of similar structure and replication to the one generated in our study (Manly 2007). Pairwise permutation tests were performed as post hoc tests for significant PERMANOVA results. Data analysis was done using R statistics (version 4.0.4. with packages 'ggplot2' for graphical output and 'plyr,' 'ez,' 'doBy,' 'rcompanion' and 'multcompView' for data manipulation and statistics; graphic user interface: R Studio). There were 5 missing values in the dataset. Missing values were replaced by mean imputation, which was carried out after verifying homogeneity of variances (Fligner-Killeen test) within the corresponding analogous sample series.

Weight-specific $_{phyto}C$ and $_{phyto}N$ and phytodetrital C:N ratios were used to estimate the individual uptake of total phytodetritus expressed in nanograms of phytodetrital dry weight per individual (ng DW ind.$^{-1}$). Additionally, the release of diet-derived total C ($_{phyto}C$) and total N ($_{phyto}N$) was calculated as the difference between the $_{phyto}C$ or $_{phyto}N$ content of the fed and starved individuals, to estimate the bulk release of the 2 elements into the surrounding water. Due to the different methods used for the analysis of C loss as $_{phyto}C$ (including $_{phyto}DIC$) and $_{phyto}DIC$, we expected an offset between $_{phyto}C$ and $_{phyto}DIC$ release values. Ultimately, C:N imbalances, or the stoichiometric mismatch, respectively, were calculated as ratios of diet C:N over foraminiferal cytoplasmic C:N ratios, and between $_{phyto}C$:$_{phyto}N$ and foraminiferal C:N ratios.

3. RESULTS

There was a significant interactive effect of diet and temperature on the intake and the release of phytodetritus-derived carbon ($_{phyto}C$ and $_{phyto}DIC$) and nitrogen ($_{phyto}N$) and on the ratios of $_{phyto}C$:$_{phyto}N$ release in *Haynesina germanica* (Table 2). The results are presented together with the results from Wukovits et al. (2018) in Fig. 3.

Phytodetritus-derived nitrogen intake ($_{phyto}N$) was between 0.05 and 0.09 ng ind.$^{-1}$, and pairwise permutation tests did not show significant differences across the treatments between diets (Fig. 3G). There was no effect of the temperature treatments for the low-quality food source (7.1 D, Table 2), but the $_{phyto}N$ intake was significantly elevated at 15°C compared to 20 and 25°C for the high-quality food source (5.7 D, Table 2). The intake of $_{phyto}C$ ranged between 5 and 12 ng ind.$^{-1}$ and was significantly affected by the 2 different diets at 15 and 20°C, with higher intake of the high-quality food source (5.7 D) at 15°C and lower intake of 5.7 D compared with the low-quality food source (7.1 D) at 20°C (Fig. 3F). There was no effect of the diets on $_{phyto}C$ intake at 25°C (Fig. 3F). There was no temperature effect on the $_{phyto}C$ intake in the low-quality diet (7.1 D), but the $_{phyto}C$ intake was significantly higher at 15°C compared with the intake at 20 and 25°C (Fig. 3F, Table 3). Roughly half of the $_{phyto}N$ was metabolized after the 24 h starvation period, and there was no significant effect of the different diet treatments on the $_{phyto}N$ release (Fig. 3E). Phytodetritus-derived N release was not affected by the temperature treatments for the high-quality diet (5.7 D), but for the

Table 2. Results of factorial PERMANOVAs for both diets showing p-values for the experimental factors temperature (T) and diet (D), and their interactive effects (T:D), of treatments on phytodetritus-derived C and N ($_{phyto}C$, $_{phyto}N$), and foraminiferal release of phytodetritus-derived dissolved inorganic C ($_{phyto}DIC$), $_{phyto}C$, $_{phyto}N$ and $_{phyto}C$:$_{phyto}N$ ratios. Significant p-values ($p < 0.05$) are in **bold**

	Feeding		Release			
	$_{phyto}C$	$_{phyto}N$	$_{phyto}DIC$	$_{phyto}C$	$_{phyto}N$	$_{phyto}C$:$_{phyto}N$
T	**0.020**	0.130	0.670	**<0.001**	**<0.001**	**0.010**
D	**<0.001**	**0.010**	**<0.001**	**0.020**	**<0.001**	**<0.001**
T:D	**<0.001**	**0.010**	0.370	**<0.001**	**0.020**	**0.030**

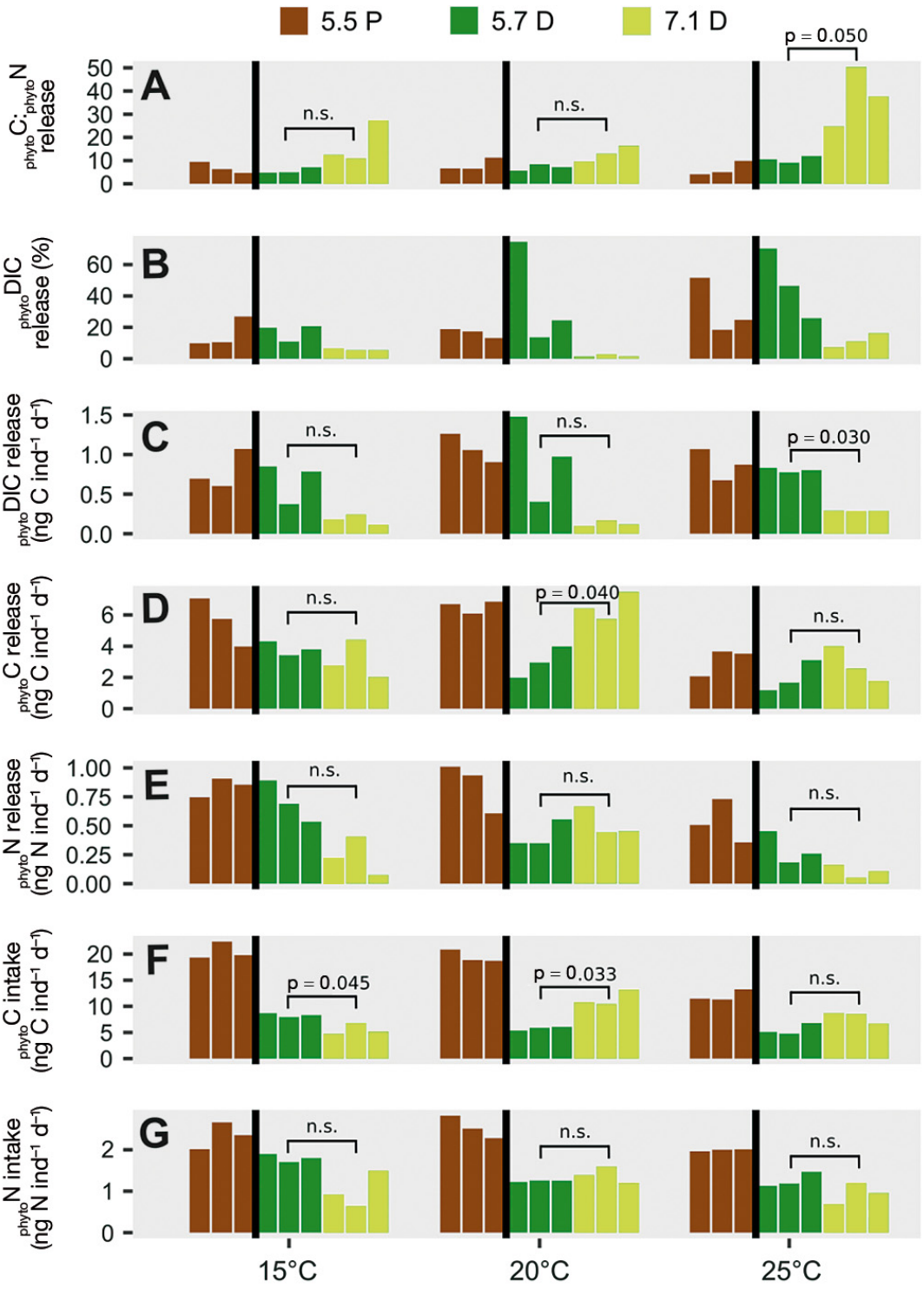

Fig. 3. (A) Ratios of phytodetritus-derived C and N ($_{phyto}$C, $_{phyto}$N) release. (B) Release of phytodetritus-derived dissolved inorganic carbon ($_{phyto}$DIC) relative to bulk $_{phyto}$C. (C) Release of $_{phyto}$DIC (ng ind.$^{-1}$ d^{-1}). (D) Release of bulk $_{phyto}$C (ng ind.$^{-1}$ d^{-1}). (D) Release of bulk $_{phyto}$N (ng ind.$^{-1}$ d^{-1}). (F) Intake of $_{phyto}$C (ng ind.$^{-1}$ d^{-1}). (G) Intake of $_{phyto}$N (ng ind.$^{-1}$ d^{-1}). Results of pairwise permutation tests for the distinct food sources are shown above the bars; temperature treatments did not show significant results. Brown bars (5.5 P) to the left of the black bars are data from Wukovits et al. (2018). n.s.: not significant

low-quality diet (7.1 D), the phytoN release was significantly elevated at 20°C (Fig. 3E, Table 3). The release of $_{phyto}$C was significantly affected by the distinct food C:N ratios at 20°C, with higher release from the low-quality food; the rates of $_{phyto}$C release ranged between 1 and 7.5 ng ind.$^{-1}$ (Fig. 3 D). There was no effect of temperature on the $_{phyto}$C release in the specimens incubated with the high-quality diet, but there was a significantly increased $_{phyto}$C release at 20°C compared with 25°C in the specimens fed with the low-quality diet (Fig. 3D, Table 3).

The release of $_{phyto}$DIC was significantly increased for the specimens previously fed with the high-quality diet (5.7 D) at 25°C (Fig. 3C). A significant effect of temperature on the $_{phyto}$DIC release was only observed for the low-quality diet (7.1 D) between 20 and 25°C, with higher release rates at 25°C (Fig. 3C, Table 3). The relative amount of $_{phyto}$DIC release compared with the total $_{phyto}$C release was generally higher for the specimens from the high-quality treatment where the data show a high spread at 20 and 25°C (Fig. 3B). The release ratios of $_{phyto}$C:$_{phyto}$N were in general slightly higher in specimens exposed to the low-quality food, with significantly higher ratios at 25°C, where the mean ratio was 38, whereas the lowest ratio was observed at 15°C for the high-quality food incubations (mean $_{phyto}$C:$_{phyto}$N release ratio = 5.6, Fig. 3A). There was a significantly higher $_{phyto}$C:$_{phyto}$N release ratio at 25°C compared with 15°C for the high-quality diet, but no significant temperature effect for the low-quality diet (Fig. 3A, Table 3). There was no effect of the treatments on the cytoplasmic C:N ratios in *H. germanica*.

Table 3. Results for pairwise permutation tests between temperature treatments (15, 20 and 25°C) for the high-quality (5.7 D) and low-quality (7.1 D) diets (see Table 1 for details); phyto: phytodetritus-derived; DIC: dissolved inorganic carbon. Significant p-values ($p < 0.05$) are in **bold**

	Temperatures (°C)	5.7 D	7.1 D
$_{phyto}$N intake	15–20	**0.029**	0.206
	20–25	0.867	0.085
	15–25	**0.041**	0.785
$_{phyto}$C intake	15–20	**0.030**	0.091
	20–25	0.710	0.269
	15–25	**0.044**	0.060
$_{phyto}$N release	15–20	0.091	0.086
	20–25	0.269	**0.040**
	15–25	0.060	0.235
$_{phyto}$C release	15–20	0.205	**0.045**
	20–25	0.248	**0.041**
	15–25	0.064	0.743
$_{phyto}$DIC release	15–20	0.397	0.261
	20–25	0.603	**0.031**
	15–25	0.365	0.065
$_{phyto}$C:$_{phyto}$N release	15–20	0.214	0.461
	20–25	0.064	0.058
	15–25	**0.042**	0.093

4. DISCUSSION

Changes in ecosystem stoichiometry, or fluctuations of environmental nutrient ratios (e.g. C:N:P) disrupt the dynamics in trophic interactions, specifically in primary producer and consumer relationships (Glibert et al. 2013). In a future warming world, the C:N ratios of phytoplankton will rise due to temperature increase and temperature increase combined with eutrophication in specific marine environments, which is expected to lead to shifts in food web structures (De Senerpont Domis et al. 2014). In general, primary consumers feeding on autotrophs with a high C content relative to e.g. N typically increase their feeding rates to cover their demand for limiting nutrients like N or P (Cruz-Rivera & Hay 2000, Hillebrand et al. 2009, Jochum et al. 2017). Therefore, we expected *Haynesina germanica* to increase its feeding rates when exposed to the low-quality diet (7.1 D). Elevated temperatures can cause increased feeding rates in herbivores due to higher metabolic activity (Carr & Bruno 2013), which can result in increased grazing pressure (Chen et al. 2012). Our laboratory study on the mixotrophic protist *H. germanica* showed a significant, interactive effect of temperature, diet type and diet stoichiometry (here diet C:N ratio) on the feeding behavior of our specimens. Although we used a relatively small range in diet C:N ratios (5.7 and 7.1), our specimens were not heterotrophic (*H. germanica* uses kleptoplasts to sequester C from the environment [LeKieffre et al. 2018]; and we provided live *P. tricornutum* as a food source, which is also a source for kleptoplasts of *H. germanica*, and the feeding rates were relatively low (cf. Wukovits et al. 2017, Goldstein & Richardson 2018), we nevertheless clearly observed a significant effect of the food source C:N ratio and temperature (Fig. 3, Table 2).

Further, this study also supports earlier findings that *H. germanica* prefers a diatom-based diet over a chlorophyte-based diet (Hohenegger et al. 1989, Wukovits et al. 2018; higher $_{phyto}$C and $_{phyto}$N intake when fed with high-quality diatoms, 5.5 P; cf. Fig. 3). The chlorophyte *Dunaliella tertiolecta* is only taken up in low amounts, but the presented results are still clear (Fig. 3, Table 2).

The results from our study only partly follow conventional models and observations from previous literature (compensatory feeding, release of excess C, see above in Section 4). Compensatory feeding, for example, only occurred at 20°C, and the release of $_{phyto}$DIC was higher after feeding on the high-quality diet (5.7 D) than on the low-quality diet (7.1 D). The mixotrophic and calcifying lifestyle of our specimens can be considered as a cause for these deviations, probably using respired excess carbon from a low-quality diet as a source for kleptoplast activity.

Against our expectations, increased feeding rates, or compensatory feeding, while feeding on the low-quality chlorophyte diet (7.1 D) was only observed at 20°C and not across all tested temperatures (Fig. 3F). At 20°C, the interaction between the elevated temperature and the higher food C:N ratio caused an up-regulation of feeding rates on the high-C, low-quality diet (7.1 D) to maintain the cellular supply of the limiting nutrient N. This increased $_{phyto}$C intake could be explained with compensatory feeding to ensure the supply with limiting nutrients, as described by Cruz-Rivera & Hay (2000) and observed in macrofaunal detritivores (Ott et al. 2012) and herbivorous freshwater zooplankton (Hillebrand et al. 2009). As a consequence, an increased nutritional demand when feeding on a low-quality diet could cause increased grazing pressure on foraminiferal diet organisms with high cellular C:N, e.g. microphytobenthos at elevated temperatures. The N demand seems to be covered when feeding on high-quality chlorophytes at 15°C and on diatoms, since $_{phyto}$N intake and turnover were comparable for 5.7 D and 5.5 P (Fig. 3). The rather low intake of the low-quality food source (7.1 D) at 15°C could be caused by an avoidance of this low-energy food source, followed by an increased energy demand at 20°C, due to metabolic stress with increasing temperature. The decrease in the feeding activity and total C and N turnover with all diets at 25°C can be explained by generally high temperature stress (Fig. 3). Temperatures of 25°C and higher might therefore lead to increased competitive pressure and reduced survival rates of *H. germanica*, as previously stated by Wukovits et al. (2017). *H. germanica* is generally found in mudflats of the temperate zone, where tidal pools rarely reach temperatures that high. However, no studies have investigated the topic of temperature-related competition in foraminifera.

Besides compensatory feeding, a high-C diet typically causes a release of excess C relative to the limiting nutrient N (or P) in phytophagous organisms (e.g. Jensen et al. 2006, Jensen & Hessen 2007). Possible ways to dispose of C from a low-quality diet in aquatic organisms include respiration (DIC release) or other metabolic pathways resulting in the disposal of particulate organic carbon (POC) or dissolved organic carbon (DOC). In *H. germanica*, the release of C relative to N was significantly increased by feeding on the high-C low-quality diet (7.1 D). The major part of C appears to be released as POC or DOC and not as DIC after feeding on the low-quality chlorophyte diet (Fig. 3). This is in contrast to the high-quality diet (5.7 D) and the high-quality diatom diet (5.5 P), where a major part of phytoC is released as DIC (Fig. 3A), particularly so for 5.7 D. There are apparent species-specific and intra-specific size-related variations of favored excess C-release pathways in aquatic herbivores. For example, the polyphagous freshwater rotifer *Brachionus plicatilis* contributes to high concentrations of DOC when exposed to a microcosm with limiting P conditions and bacteria and algae as food sources (Olsen et al. 2002, Vadstein et al. 2003), and respiration is not employed for excess C removal by the herbivorous *B. calyciflorus* (Jensen et al. 2006). In herbivorous freshwater cladocerans (*Daphnia*), both DIC and POC/DOC release are important (Jensen & Hessen 2007). In marine copepods, the ratio of DOC:DIC release depends on the growth stage. Juveniles show a tendency to release more DIC than DOC, whereas adults release higher amounts of DOC relative to DIC (Schoo et al. 2013). Our specimens were advanced and homogeneous in their ontogenetic development (the size fraction >250 µm typically does not include juvenile individuals of *H. germanica*) and showed varying ratios of $_{phyto}$C:$_{phyto}$DIC release depending on food quality and temperature (cf. Fig. 3A, Table 3). Hence, the results of our study indicate a strong dependence of metabolic C release on diet source C:N ratio and temperature.

According to ecological stoichiometry theory, a positive relationship between diet quality and the ratio of the release of the abundant:limiting nutrient results in consumer-driven nutrient recycling (Elser & Urabe 1999, Sterner & Elser 2002). This implies that the ratio or the availability of dissolved nutrients like DIC, DOC or dissolved N for e.g. microalgae or prokaryotes in the foraminiferal microhabitat strongly depends on the rate and the efficiency of nutrient assimilation and accordingly on the ratio of released (excreted or remineralized) nutrients. A major reason for this positive relationship is the homeostasis of the consumer (stable ratios of the body or cellular C:N). Homeostasis with respect to cellular C:N ratios is maintained in *H. germanica*.

Therefore, it can be assumed that nutrient fluxes in the microhabitats surrounding *H. germanica* populations follow 'consumer driven nutrient cycling' (Elser & Urabe 1999, Sterner & Elser 2002). Hence, the availability of DIC and dissolved C and N for benthic microalgae and prokaryotes that share the same microhabitat as *H. germanica* might be strongly controlled by foraminiferal diet intake, when exposed to temperatures up to 20°C. In general, release ratios of $_{phyto}C_{:phyto}N$ were highest for the low-quality (7.1 D) diet (Fig. 3 A).

Depending on the predominant release pathway of diet-derived C, the growth of different microorganism groups sharing their habitat with *H. germanica* can be promoted. A higher DIC availability (e.g. after feeding on high-quality diatoms or chlorophytes) due to increased foraminiferal respiration could stimulate the growth of associated microalgae or diatoms in the micro-environment surrounding the foraminifera. Increased DOC, on the other hand, can serve as a nutrient source for heterotrophic prokaryotes and other microbes. Recycling of phytodetrital C, and subsequent POC release, could further cause an increase of sedimentary C flux or burial. The significant interactive effect of diet stoichiometry and temperature on C and N fluxes in *H. germanica* (Tables 2 & 3, Fig. 3) and specifically the high ratio of $_{phyto}C:_{phyto}N$ release when exposed to a high-C diet and a temperature of 25°C (see Fig. 3A) is therefore highly likely to affect closely associated microorganisms. This could possibly alter trophic connections in benthic communities in the course of environmental changes such as in nutrient concentrations or rising temperatures — in particular with regards to the high abundances of *H. germanica* in temperate tidal flats.

Interestingly, our results show a very low release of $_{phyto}DIC$, or respiratory CO_2 for the high-C diet (7.1 D, Fig. 3B,C). Any DIC that was built into the foraminiferal test via calcification would have been lost during sample processing (decalcification), since $CaCO_3$-derived CO_2 from the foraminiferal test is lost to the atmosphere due to the acidification with HCl. Typically, foraminiferal growth, or the formation of new test compartments (chambers), respectively, takes a few hours and happens in intervals of several days or weeks in adult benthic foraminifera (Schnitker 1974, Stouff et al. 1999, De Nooijer et al. 2009). Therefore, it is highly unlikely that the majority of the specimens in the experiment started chamber formation simultaneously within the 24 h starvation period, using excess C from the high-C diet for chamber formation. On the other hand, one could suggest that excess C within a foraminiferal diet could stimulate chamber formation. This could be an important consideration, since foraminiferal calcification plays a strong role in the global marine carbonate production (Langer 2008, Titelboim et al. 2017, 2019). Future long-term feeding experiments should be designed to identify the connection between diet stoichiometry, foraminiferal DIC flux and chamber formation. For foraminifera, it could be beneficial to use excess diet-derived C as a resource for the construction of their calcified test. However, foraminiferal calcification is suggested to mainly use seawater DIC to build up internal DIC pools for calcification (Bentov et al. 2009, De Nooijer et al. 2009, 2014, Toyofuku et al. 2017). One of their proposed calcification mechanisms includes high-pH vesicles (via a proton pump, releasing H^+ and accumulating HCO_3^-), which concentrate intracellular DIC. Analogously, within the cytoplasm, respired CO_2 from diet-derived C sources (specifically with high-C content relative to limiting nutrients) could diffuse into these vesicles. Clearly, an increased feeding on a high-C diet in *H. germanica* is temperature driven but significant only at 20°C (Fig. 3, Table 3). The consideration of excess metabolic CO_2 built into the test of *H. germanica* would result in far lower ratios of $_{phyto}C:_{phyto}N$ release. Foraminifera could then benefit from a high C:N diet and would act as an important sink for excess C from high C:N primary productivity.

H. germanica is known for a mixotrophic lifestyle due to chloroplast husbandry, retaining kleptoplasts derived from specific diatoms (Lopez 1979, Pillet et al. 2011). The kleptoplast activity causes lower oxygen consumption rates during the day than at night (Cesbron et al. 2017). However, the quantity of respiratory CO_2 used by kleptoplast photosynthesis in *H. germanica* is unknown, whereas there is proof of the involvement of kleptoplasts in environmental C acquisition in *H. germanica* (LeKieffre et al. 2018). Our results for the $_{phyto}DIC$ within the seawater therefore do not represent the total respiratory release, but rather an average for day and night metabolic activity of the specimens. Respired excess C in *H. germanica* could be an excellent CO_2 source for kleptoplast photosynthesis and downstream biosynthesis of C compounds such as metabolic storage products or sugars for further ATP synthesis, but this could not explain the high rates of C release at 20°C and high-C diet (7.1 D). It could, however, explain the comparably very low ratio of $_{phyto}DIC:_{phyto}C$ release at 15°C while feeding on 7.1 D (Fig. 3), if diet-derived C was used for photosynthetic sugar production and recycled for renewed respiration. Mixotrophy might be an effective adaptation to

nutrient limitation or nutrient fluctuations. This can be an advantage for this species in coastal environments that are affected by present and future changes and will be increasingly exposed to high storm frequencies and resulting fluctuations in nutrient loads (Brierley & Kingsford 2009).

Anderson et al. (2017) hypothesized that increased feeding rates at high temperatures compensate for higher C respiration due to metabolic temperature stress in invertebrates. In our study, there was no simultaneous increase in feeding rates or $_{phyto}$C intake, when feeding on the high-quality diet (5.7 D), while $_{phyto}$DIC tended to increase with temperature (Fig. 3A). Results of the modeling exercise of Anderson et al. (2017) suggest that an increased diet C:N ratio is not beneficial, even when there is a higher demand for C at higher temperatures due to increased respiration. In case of the calcifying, kleptoplast-bearing foraminifer *H. germanica*, a high-C diet could be beneficial if excess C could be used for test construction and kleptoplast photosynthesis.

In general, low diet availability results in a low efficiency of the synthesis of cellular C compounds, since most C is used for energy metabolism (ATP synthesis) via respiration (Urabe & Watanabe 1991). We assume that a similar effect (diet limitation) might have been present for *H. germanica* when feeding on chlorophytes. A lack of affinity for chlorophytes as a food source could explain the decrease of feeding rates and the lower ratios of $_{phyto}$DIC:$_{phyto}$C release with a high-quality chlorophyte diet (5.7 D) at 20°C (Fig. 3). Additionally, the longer laboratory incubation period of the specimens before the start of the chlorophyte experiment (6 wk) in contrast to the shorter incubation period prior to the diatom experiment (3 wk) could have caused lowered metabolic activity in the chlorophyte setup. However, another feeding experiment with *H. germanica* carried out 4 wk after sampling (see Wukovits et al. 2017) showed values of 1.6–4.0 ng $_{phyto}$C ind.$^{-1}$ 48 h after incubation with *Dunaliella tertiolecta* detritus at 20 and 25°C, which is lower than the values observed herein (4.8–13.2 ng phytoC ind.$^{-1}$) after 24 h. The preferential feeding on diatoms as a nutrient resource for *H. germanica* might help to cover greater energy demands at increased temperatures to maintain feeding activity (Fig. 3). However, we speculate that low-quality diatoms, like low-quality chlorophytes, might not be sufficient to cover the energy demand at increased temperatures as well. Therefore, global warming combined with low diet quality of diatoms might be problematic for protists like *H. germanica*.

5. CONCLUSIONS

In summary, combined effects of diet C:N ratio (diet quality) and temperature impacted the feeding behavior of *Haynesina germanica*. Such effects on organismic C balances can have strong effects on C pathways and energy fluxes in the ecosystem, as pointed out by Hessen & Anderson (2008). For example, high $_{phyto}$C:$_{phyto}$N release ratios under availability of predominantly low-quality diet (Fig. 3, Table 3, $_{phyto}$C:$_{phyto}$N release at 25°C) might have a strong effect on the biogeochemistry of benthic microenvironments surrounding foraminifera and on microorganisms, specifically at strongly elevated temperature. Increased excretion of organic C (POC or DOC) due to low diet quality, for example, can cause an increased vertical flux of POC, or an increased recycling of DOC and an associated increase in bacterial production (cf. Olsen et al. 2002, Vadstein et al. 2003).

Observed changes in the feeding behavior of *H. germanica* include significantly increased feeding rates at 20°C, which could reinforce the grazing pressure on foraminiferal prey (e.g. microphytobenthos). Temperatures above 20°C caused lowered feeding rates on all diets. This could challenge the survival of *H. germanica* at prolonged exposure to elevated environmental temperatures. Further, a high C:N diet appears to have a distinct effect on the inorganic C metabolism in *H. germanica*. This is most likely related to the calcifying and mixotrophic lifestyle of this species and might in turn affect these mechanisms. Therefore, there is a strong need to increase experimental research on the combined effects of warming, feeding and nutrient fluxes in marine calcifiers and mixotrophs. A low diet quality also resulted in higher ratios of diet-derived C:N release. These factors could strongly impact nutrient fluxes and could play a role in future food web shifts, with respect to global warming and ecosystem nutrient shifts.

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