Response to the review comments on „Salinity-depending carbon and nitrogen uptake of two intertidal foraminifera (*Ammonia tepida* and *Haynesina germanica*)“ by Lintner et al.

ad Anonymous Referee #1

major points:

1: We agree with your suggestion– only values of the 1d samples are now compared.

2: There are several explanations for that listen here and we included them in the discussion.

- An important aspect to consider is the method used when processing the samples. Foraminiferal tests are dissolved with hydrochloric acid and due to that carbonate is lost, but also new mineral phases are formed which influence the total weight of the sample. This step is needed to remove the 13C, which may be bound in the test. Comparing with other studies like Wukovits et al. 2017, it can be seen, that the uptake values of our experiments lay in the same order of magitude.
- Another aspect is, that foraminifera are stressed during experimental conditions and therefore may have a lower turnover. It should be noted, that the food uptake is determined by the isotope content in the cytoplasm, which, as recommended in major point 1, can also vary over time.
- Since these experiment were all carried out under laboratory conditions, we would not consider pC and pN as absolute values, due to seasonal and environmental fluctuations. However, a difference in food uptake can be shown in our experiments with varying salinity.

Specific comments:

Title: Good point, title has been changed.

Lines 74-75: We added the tested temperature range (line 77).

Line 94: The meaning is the same; it only serves to avoid repetition. But we changed it now and wrote “salinity of 24” to avoid confusion.

Line 108: In the method used here, the algae were processed in the „most gentle“ way, which means that no further degradation of algae or gross damage of the cells occured. During the „normal“ freezing, the algae could be decomposed by further microbacterial activities or burst and therefore lose cytoplasm, which would lead to a change of the 13C and 15 N content.

Line 124: No, the algae powder was added directly into the crystallization dishes and mixed there. The algae settled down on the bottom of the dish and were available for foraminifera. We added this information now.

Line 135: Potential effect was added to the discussion. In general, all samples were always treated the same way, which means they were all washed with the same volume of distilled water. This way any impact that may have arisen from using the distilled water has the same effect on all samples.

Line 229: It has been changed and all graphs are plotting in Fig. 1 now.

Line 275: The content of at%^{13}C and at%^{15}N was added in line 109. This helps to calculate the atomic C:N ratio. We added this information.

Line 310: “Labeling” was added.

Line 317: We corrected the sentence.

Line 326: 33 is the salinity used in the experiments. 37 PSU are extreme values, that Maywald (1991) has found in the North Sea.
Line 338: They used d13C to estimate the origin of OM and as a consequence they could differentiate between a brackish and marine milieu. Below, an example is described in detail, that can be directly related to our experiments (Mackie et al. 2005).
Line 341 and 349: We improved the text here in accordance to the reviewer.
Line 367: We added this information.
Line 371: We would suggest to keep the structure here and add only interesting aspects, that have not been mentioned yet. In our opinion, a new chapter would disrupt the flow of reading.
Line 377: . was replaced with ,
Line 395: We improved the text here.

Response to the review comments on „Salinity-depending carbon and nitrogen uptake of two intertidal foraminifera (*Ammonia tepida* and *Haynesina germanica*)“ by Lintner et al.

ad Anonymous Referee #2

Main point:
Line 93: For our experiments, we only used foraminifera with densely filled cytoplasm. In addition, we only used individuals with an intense yellowish color of the cytoplasm. The incubation time of foraminifera in the crystallization dishes before feeding was used for the „crawling test“. Foraminifera were placed in the center of the crystallization dish immediately after removal from the cultures. After 24 hours individuals could be identified that have moved away from the center. Accordingly, these individuals have active pseudopodia and are alive. After finishing the experiments, the individuals were also checked for the intense yellowish color, but no „crawling test“ has been carried out, since additional stress and time (with continuing breathing and excretion) could strongly affect the isotope content and therefore the results. In all our experiments is was very rare (below 4%), that single individuals did not show colored cytoplasm and they were therefore counted as „survive“. Completely empty tests, which would clearly stand for dead individuals, were not found. We added this information to the methods.
General Questions:
1, 2 & 4: In order not to disturb the experiments, no water change was carried out. O_2 and pH were also not measured, since we did not expect a significant change due to the small amount of added food.
3: The crystallization dishes were sealed tightly with parafilm. The water level and regular control of the salinity showed, that there was no evaporation. We added this information to the methods.
Specific comments:
Line 91: We used water from the location. We added this information to the methods.
Line 94 and 104: ad 94: The 21 °C correspond to the room temperature of the laboratory, where the foraminiferal cultures were placed. Ad 104: the 20°C refer to the temperature in the

incubator, where algae were cultivated. Experience shows, that algae grow very well at 20°C. The feeding experiments were also carried out at 20°C in the incubator, for optimal food uptake conditions. This experience is based on the experiments of Wukovits et al. (2017).

Line 121: 0.45 µm – We added this information to the manuscript.

Line 125: No, the food was directly put into the crystallization dishes and mixed there. See response to Referee #1.

Line 126: "Untreated" means not fed. Foraminifera were removed directly from the stock culture and processed. We improved this sentence to avoid confusion here.

Line 141: It is difficult to say what is inside of the foraminifera. But before foraminifera were further processed, they were cleaned with a brush to remove any organic or inorganic residues that were visible on their test.

Line 377: . was replaced with ,

Line 400: We agree with the reviewer. However, in this chapter we only refer to the availability of food.

Salinity-depending algae uptake and subsequent carbon and nitrogen metabolisms of two intertidal foraminifera (*Ammonia tepida* and *Haynesina germanica*)

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Abstract

Benthic foraminifera are abundant marine protists which play an important role in the transfer of energy in the form of organic matter and nutrients to higher trophic levels. Due to their aquatic lifestyle, factors such as water temperature, salinity and pH are key drivers controlling biomass turnover through foraminifera. In this study the influence of salinity on the feeding activity of foraminifera was tested. Two species, *Ammonia tepida* and *Haynesina germanica*, were collected from a mudflat in northern Germany (Friedrichskoog) and cultured in the laboratory at 20 °C and a light / dark cycle of 16: 8 h. A lyophilized algal powder from *Dunaliella tertiolecta*, which was isotopically enriched with ^{13}C and ^{15}N, was used as a food source. The feeding experiments were carried out at salinity levels of 11, 24 and 37 practical salinity units (PSU) and were terminated after 1, 5 and 14 days. The quantification of isotope incorporation was carried out by isotope ratio mass spectrometry. *Ammonia tepida* exhibited a 10-fold higher food uptake compared to *H. germanica*. Furthermore, in *A. tepida* the food uptake increased with increasing salinity but not in *H. germanica*. Over time (from 1-5 d to 14 d) food C retention increased relative to food N in *A. tepida* while the opposite was observed for *H. germanica*. This shows, that if the salinity in the German Wadden Sea increases, *A. tepida* is predicted to exhibit a higher C and N uptake and turnover than *H. germanica*, with accompanying changes in C and N cycling through the foraminiferal community. The results of this study show how complex and differently food C and N processing of foraminiferal species respond to time and to environmental conditions such as salinity.

keywords: benthic foraminifera, feeding experiments, salinity, isotope tracing

1. Introduction

The intertidal zone is one of the most extreme habitats on earth. This ecotone, also known as the foreshore or seashore, is determined by tidal activity. It is an important habitat for various living organisms like starfishes, sea urchins, corals and foraminifera (Allen 2000). Due to the alternating presence/absence of water, organisms living here must adapt to the specific environmental conditions. Important factors shaping the intertidal environment are the fluctuating water temperature and salinity, pH, available food sources, sediment organic matter

content and fresh water supply. These environmental factors significantly influence the activity of foraminifera (e.g. Schafer et al. 1996, Caldeira and Wickett 2005, Keul et al. 2013, Wukovits et al. 2017).

Foraminifera are unicellular organisms, which live predominantly in marine environments. A recent field study showed that benthic foraminifera can account for up to 84% of total protozoan biomass in mudflats (Lei et al. 2014). Many foraminifera feed on phytoplankton (algae, diatoms) and thus play an important role in passing on energy in form of organic matter to higher trophic levels (Azam et al. 1983, Beringer et al. 1991). Due to the large quantity of foraminifera in the deep and shallow ocean waters and their large contribution to the uptake of primary produced organic material, foraminifera significantly contribute to the global marine carbon and nitrogen cycles (Altenbach 1992, Graf 1992, Gooday et al. 1992, Nomaki et al. 2008, Glock et al. 2013).

Foraminifera can even change between active feeding and passive ingestion diets depending on how much food is available (Sliter 1965). Some foraminifera can retain organelles (chloroplasts) from certain food sources and integrate them into their own metabolic cycle. This process is commonly referred to as kleptoplastidy. Currently nine benthic foraminiferal genera are known to follow this lifestyle: *Bulimina*, *Elphidium*, *Haynesina*, *Nonion*, *Nonionella*, *Nonionellina*, *Reophax*, *Stainforthia* und *Virgulinella* (Lopez 1979, Lee et al. 1988, Cedhagen 1991, Bernhard & Bowser 1999, Correia & Lee 2000, Grzymski et al. 2002, Goldstein et al 2004, Pillet et al. 2011, Lechliter 2014, Tsuchiya et al. 2015). In the temperate Wadden Sea, being a part of the North Sea, two foraminifera species occur most frequently, *Ammonia tepida* and *Haynesina germanica*, and have been relatively well studied in terms of trophic ecology. While *Ammonia* does not seem to be able for kleptoplastidy (Jauffrais et al. 2016), *H. germanica* possesses chloroplasts which are absorbed from food (microalgae) and are retained as organelles (Lopez 1979). Cesborn et al. (2017) demonstrated that the plastids in *H. germanica* are photosynthetically active, based on changes in O_2 consumption rates during dark-light transitions. *Haynesina germanica* therefore follows a mixotrophic lifestyle, with autotrophic and heterotrophic nutrition (Cesborn et al. 2017). While *Ammonia* can rapidly ingest organic carbon (Moodley et al. 2000) and *A. tepida* has a higher potential to convert algal organic matter into cellular biomass in a short time frame compared to *H. germanica* (Wukovits et al. 2018), the latter species (*H. germanica*) can eventually reduce its dependency on external food due to the presence of kleptoplasts.

The uptake of food by foraminifera depends on several factors such as food quality and quantity, temperature and salinity (Lee et al. 1966, Dissard et al. 2009, Wukovits et al. 2017). Past experiments with *A. tepida* and *H. germanica* showed that increasing temperature had a negative effect on food uptake of foraminifera (Wukovits et al. 2017). Highest food uptake rates were recorded at 20 °C in comparison the other tested temperatures 25 and 30 °C. As the temperature increased foraminifera of both species consumed less food (Wukovits et al. 2017). Today not only increasing temperature but also salinity changes play an important role in the oceans, mainly because of anthropogenic influence, however effects of salinity on food uptake and digestion by foraminifera have not yet been studied. Based on the strong variability and fluctuations in salinity levels in the intertidal systems we studied food uptake of *A. tepida* and

H. germanica at different salinity levels to provide a better understanding of the turnover of phytoplankton by foraminifera with changing physical conditions (salinity).

2. Materials and Methods

2.1. Sampling

The sample material was collected in May 2018 during low tide at Friedrichskoog Spitze (German Wadden Sea, at 54° 02' N, 8° 50' E). At that time the seawater had a salinity of 24.2 PSU and a temperature of 13 °C, and the air temperature was 11 °C. The collected sediment was directly wet-sieved with seawater from the location at the site through a 125 and a 63 µm sieve to remove larger meiofauna and smaller organic particles. In the laboratory, the sediments (size class 63-125 µm) containing living benthic foraminifera were fed regularly with *Dunaliella tertiolecta* (green algae) until the start of the experiment and were kept at a temperature of 21 °C and a salinity of 24 PSU. 1 PSU (1 practical salinity unit) corresponds approximately to 1 g salt per kg seawater.

2.2. Preparation of $^{13}C^{15}N$-labeled phytodetritus

A f/2 medium (Guillard & Ryther 1962, Guillard 1975), enriched with ^{13}C (1.5 mmol $NaH^{13}CO_3$/L) and ^{15}N (0.44 mmol $Na^{15}NO_3$/L) was used as a nutrient solution for the cultivation and production of isotopically labeled *D. tertiolecta*, a common food source in laboratory experiments with benthic foraminifera (e.g. Heinz et al. 2002, Wukovits et al. 2017). It should be noted that *H. germanica* prefers to eat diatoms (Austin et al. 2005), however significant uptake of *D. tertiolecta* was also previously reported (Wukovits et al. 2017). The algal culture was kept in an incubator at 20 °C with a light/dark cycle of 16:8 h. Once the algae had grown to high density in the medium, they were collected by centrifugation at 800 x*g* for 10 minutes. The algal pellet was washed three times with artificial seawater (Enge et al. 2011). After each washing step the culture was centrifuged and the supernatant decanted. For the storage of the labelled algae, the pellet was shock frozen in liquid nitrogen and then lyophilized for 4 days at 0.180 mbar. The labeled algal powder was isotopically enriched at about 3.3 at%^{13}C and 32.3 at%^{15}N (C:N ratio is about 5,58).

3. Sample preparation and analysis

3.1. Sample preparation

The experiment was run in triplicates. For each salinity level (11, 24 and 37 PSU) and each time point of harvest (1, 5 and 14 days) three glass crystallization dishes were setup for *A. tepida* and for *H. germanica*. The selected salinities correspond to a brackish milieu (11 PSU), to the natural conditions in the North Sea (24 PSU) and to a highly saline basin (37 PSU). For *A. tepida* 55 individuals and for *H. germanica* 60 individuals were prepared per replicate to obtain a dry mass of cytoplasm between 1 and 2 mg. The crystallization dishes were filled with 280 ml of filtered (pore size: 0.45 µm) natural seawater from the sampling site. The salinity was previously adjusted to the desired PSU value by adding NaCl or distilled water. The

foraminifera were then placed in the dishes (without sediment) and acclimated at 20 °C and a light/dark cycle of 16:8 h for three days in an incubator. The crystallization dishes were sealed tightly with parafilm.

For our experiments, we only used foraminifera with densely filled cytoplasm. In addition, we only used individuals with an intense yellowish color of the cytoplasm. The incubation time of foraminifera in the crystallization dishes before feeding was used for the „crawling test". Foraminifera were placed in the center of the crystallization dish immediately after removal from the cultures. After 24 hours individuals could be identified that have moved away from the center. Accordingly, these individuals have active pseudopodia and are alive. In all experiments it was very rare (below 4%), that single individuals did not show colored cytoplasm and they were therefore counted as "survive". Completely empty tests, which would clearly stand for dead individuals, were not found.

In order not to disturb the experiments, no water change was carried out. O_2 and pH were also not measured, since we did not expect a significant change due to the small amount of added food.

Following the acclimation period, 5 mg lyophilized labelled algal powder was added as the only food source to each replicate and left in the incubator for the desired incubation time. The algae powder settled down on the bottom of the dish and was available for foraminifera. In addition, untreated (not fed) foraminifera were taken to obtain the natural abundance of ^{13}C and ^{15}N as a reference. At the end of the experiments a precipitate of the algal powder was still visible in the crystallization dishes, which confirms the continuous availability of food during the experiments. The salinity was checked daily and corrected when necessary.

3.2. Sample preparation and processing

Before the start of the experiments all glassware was cleaned by combusting at 500 °C for 5 h in a muffle furnace. The „picking tools" and tin capsules were cleaned by rinsing with a 1:1 (v:v) mixture of dichloromethane (CH_2Cl_2) and methanol (CH_3OH). After the incubation period, foraminifera were removed from the crystallization dishes, cleaned and washed three times with distilled water. Then they were transferred into the tin capsules (Sn 99,9%, IVA Analysentechnik GmbH & Co. KG) and excess water was removed. The samples were air dried for three days (Enge et al. 2018) and then decarbonated with 4% HCl (3 x 5 µL for *A. tepida* and 2 x 5 µL for *H. germanica*). During the decarbonatization of foraminiferal tests, the samples were kept at 60 °C for 24 h. Finally, the samples were dried for three days at 60 °C, before being weighed to the nearest hundredth of a milligram.

3.3. Analyses

The measurements of C and N contents as well as the isotope ratios of the samples were carried out in the Stable Isotope Laboratory for Environmental Research (SILVER) laboratory of the University of Vienna. The ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ were measured by an isotope ratio mass spectrometry (IRMS, Delta^{PLUS}, coupled by a ConFlo III interface to an elemental analyzer EA 1110, Thermo Finnigan). In the following calculations, X stands for the heavy isotopes of C and N, i.e. ^{13}C and ^{15}N, respectively. The atomic percentage of heavy isotopes (at%^{13}C and at%^{15}N)

was calculated using the measured $\delta^{13}C$ and $\delta^{15}N$ values and the international standards for C (Vienna PeeDee Belemnite R_{VPDB} = 0.0112372) and N isotopes (atmospheric nitrogen R_{atmN} = 0.0036765) according to the following equations:

$$\delta X = (R_{sample}/R_{standard} - 1) \times 1000 \quad (1)$$

where R depicts the ratio of heavy isotope to light isotope i.e. $^{13}C:^{12}C$ or $^{15}N:^{14}N$ in samples and international standards, respectively.

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

Subsequently, the values needed to be corrected for the at%X present in the natural environment, i.e. in unlabeled foraminifera. The so-called isotope excess (*E*) was calculated according to Middelburg et al. (2000):

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

In the next step, the isotope incorporation was determined according to the following equation:

$$I_{iso}\ [\mu g\ mg^{-1}] \text{ or } [\mu g\ ind^{-1}] = E \times C\ (N)\ [\mu g\ mg^{-1}] \text{ or } [\mu g\ ind^{-1}] \quad (4)$$

Depending on the biomass units used, I_{iso} results in the unit µg mg^{-1} (based on dry matter of the cytoplasm) or µg ind^{-1} (based on the number of individuals).

Finally, the uptake of phytodetrital C (pC) and phytodetrital N (pN) was calculated for the cytoplasm of foraminifera:

$$pX = \frac{I_{\text{iso}}}{\frac{\text{at.}\%X_{\text{phyto}}}{100}} \quad (5)$$

where $at\%_{phyto}$ represents the isotopic enrichment in ^{13}C and ^{15}N of the labelled *D. tertiolecta* food. All results were additionally converted to time-based food uptake rates (µg mg^{-1} h^{-1}).

3.4. Statistics

Regression analysis was applied to statistically test for time effects on food uptake, and linear and curvilinear models were tested. The best models were selected based on the highest coefficient of determination (R^2). Three-way analysis of variance (ANOVA) was applied to test for main effects of species, salinity and time, and two-way ANOVA for salinity and time effects

on pC and pN within species, followed by Fisher's LSD post hoc tests. All statistical tests were performed using R (R development Core Team, 2008).

4. Results

4.1. Carbon uptake

The isotope measurements showed that the offered labeled food source was utilized by both, *A. tepida* and *H. germanica*. Three-way ANOVA showed a significant effect of species (*A. tepida* > *H. germanica*, $p<0,001$), time ($p<0,001$) and salinity ($p<0,001$) on pC. Moreover, two-way ANOVA highlighted a significant effect of time ($p<0,001$) and salinity ($p<0,001$) on pC in *A. tepida*, and of time ($p<0,001$) but not salinity ($p=0,0739$) on pC in *H. germanica*. Salinity had a major impact on food uptake (pC) only in *A. tepida*.

As shown in Fig. 1A, *A. tepida* had the highest pC value at a salinity level of 37 PSU for the most dates, followed by 24 PSU. At lowest salinity (11 PSU) pC further decreased. It should be noted that from day 1 to day 5 the uptake of C at 24 PSU and 37 PSU decreased considerably before it increased again towards day 14. This intermediate minimum was not recognizable at 11 PSU. At 11 PSU pC increased linearly with time (f(d) = 0.05163*d + 0.06530, R^2=0.9985, based of mean values of pC).

Time kinetics were different for *H. germanica*. After one feeding day the measured pC values did not differ between salinity levels and were lowest. Food C uptake peaked after five days and thereafter declined. However, salinity did not affect pC in this species.

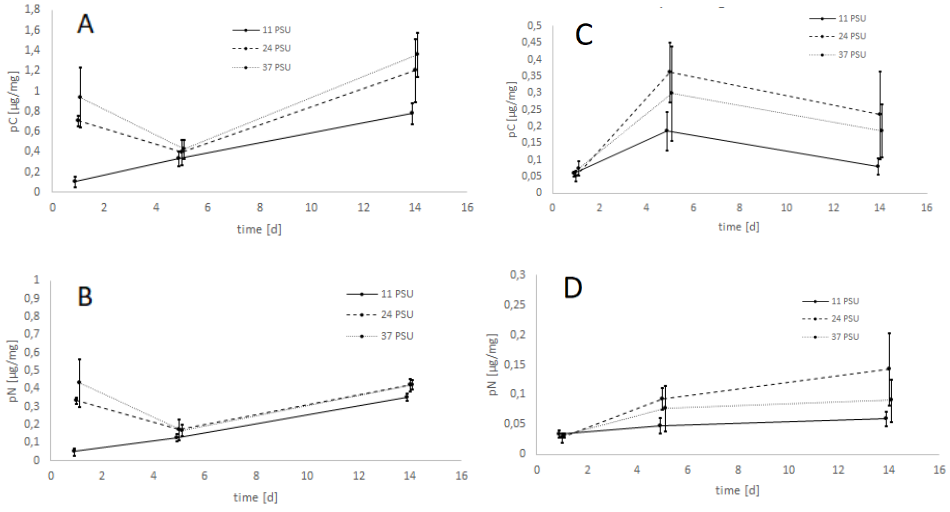


Figure 1: Time kinetics of algal C and N uptake (pC, pN) by (A, B) *A. tepida* and (C, D) *H. germanica*. pC and pN were measured at three salinity levels: 11, 24 and 37 PSU.

In addition to pC values, C uptake rates for the 1 day sample were also determined. *Ammonia tepida* showed highest uptake rates at 24 (0,029 µg/(mg*h)) and 37 PSU (0,036 µg/(mg*h)) after one day of food supply. For 11 PSU, C uptake rates were much lower (0,004 µg/(mg*h). For *H. germanica*, C uptake rates at salinities of 11 (0,002 µg/(mg*h)), 24 (0,003 µg/(mg*h))and 37 (0,002 µg/(mg*h)) PSU are in the same area.

4.2. Nitrogen uptake

Two-way ANOVA showed a significant effect of salinity ($p<0,001$) and time ($p<0,001$) on nitrogen uptake (pN) for *A. tepida*. For *H. germanica*, as with pC, pN was only affected by time ($p=0,0027$) but not by salinity ($p=0,0690$).

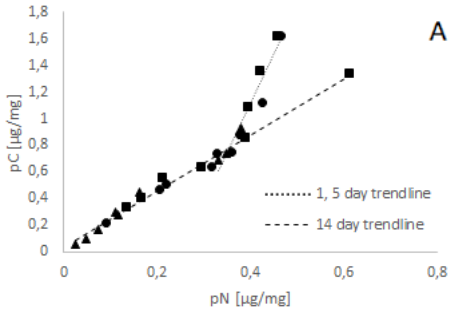
Nitrogen uptake of *A. tepida* showed a highly comparable pattern to C uptake (Figure 1). Minimum N uptake was always recorded at the lowest salinity level. However, the uptake of N after 5 days was approximately the same at 24 and 37 PSU, and reached here a minimum at both salinities. The development of pN at 11 PSU could be described by a straight line (f(d) = 0.02354*d + 0.02011) with a very high coefficient of determination ($R^2 = 0.9978$).

Haynesina germanica exhibited lower values of pN compared to *A. tepida* (Figure 1D). The highest N uptake after 5 and 14 days was at the moderate salinity level (24 PSU), though this was not significant. Again, food N uptake increased linearly with time (f(d) = 0.00185*d + 0.03522, $R^2 = 0.9317$) at the lowest salinity level, but showed a saturating behavior at 24 and 37 PSU.

Food N uptake rates are also calculated. For *A. tepida* the N uptake rates is similar to the C uptake rates and C uptake rates were approximately twice as high as N uptake rates. For *H. germanica* the average N uptake rates were very close at all three salinity levels, suggesting similar N uptake rates independent of salinity in *H. germanica*.

4.3. Relations between food C and N incorporation

All data of C and N uptake obtained in this study were plotted as pC to pN relationships in Figure 2.



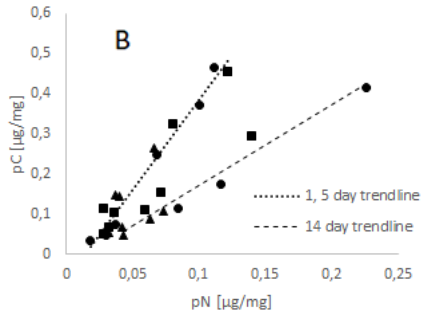


Fig. 2: Relationship between food N uptake (pN) and food C uptake (pC) for *A. tepida* (A) and *H. germanica* (B) for the time windows day 1 to 5, and day 14. Regressions were run separately for these time windows. The triangles correspond to the values at 11 PSU, the circles to those at 24 PSU, and the squares to the values at 37 PSU.

Ammonia tepida showed a continuous increase in pC with pN (Fig. 2). The 1-day and 5-day samples were all plotting on a straight line, with the lowest salinity samples having the lowest pC and pN values. At later stages (day 14) the slope and therefore the pC:pN ratios increased markedly (from 2.1 to 7.4). *Haynesina germanica* also showed a general increase in pC with pN. However, the slope between both of them decreased over time in contrast to *A. tepida*, indicating a decrease in pC:pN (from 4.5 at day 1 and 5 to 2.0 at day 14) and thereby an increased relative retention of food N compared to food C over time.

5. Discussion

5.1. Influence of salinity on food uptake

Both examined foraminifera species showed different responses to salinity variations in terms of food uptake and food uptake rates. The time course of pC and pN in *A tepida* showed a noticeably minimum after five days. This partial decrease in pC and pN was already reported in experiments testing the effects of temperature on food uptake in the same species (Wukovits et al. 2017). In the latter study food uptake was highest on day one and then decreased sharply (5 days) and remained nearly constant thereafter (14 days). These data suggest that *A. tepida* was „starved“ due to the 3-day acclimatization period and immediately responded with rapid food uptake, when food was added. The pseudopodia of *A. tepida* are particularly stimulated by the green algae *Dunaliella* (Lee et al 1961). Excessive food uptake in the short time (1 day) can lead to longer lasting saturation, which explains the significantly lower uptake rates at the intermediate time points.

There are some points, which could lead to the low (0,1%) uptake of food in comparison to the biomass of the foraminifera. An important aspect to consider is the method used when processing the samples. Foraminiferal tests are dissolved with hydrochloric acid and due to that carbonate is lost, but also new mineral phases are formed which influence the total weight of the sample. This step is needed to remove the ^{13}C, which may be bound in the test. Comparing with other studies like Wukovits et al. (2017), it can be seen, that the uptake values of our experiments lay in the same order of magitude.

Another aspect is, that foraminifera are stressed during experimental conditions and therefore may have a lower turnover. It should be noted, that the food uptake is determined by the isotope content in the cytoplasm, which can also vary over time.

Since these experiments were all carried out under laboratory conditions, we would not consider pC and pN as absolute values, due to seasonal and environmental fluctuations.

The time course of food uptake at the lowest salinity level was different in *A. tepida*, starting slow but then pC and pN increased continuously over time. This might be caused by lowest salinity levels being suboptimal in the short term and that therefore metabolic activation takes longer, causing the linear increase in pC and pN. This explanation supported

by the observation that after five days food uptake was similar across all three salinity levels. *Haynesina germanica* showed a different pattern than *A. tepida* in terms of time-dependency of pC and pN. In the former species the presence of kleptoplasts may have attenuated the „starvation effect“, with the result that only a small amount of ingested C and N can be measured after one day. This low initial C and N uptake can be related to the results of Cesborn (2017), which show that kleptoplasts are potential C or N sources for foraminifera in starvation periods. However, it should be considered that *H. germanica* less readily absorbed the offered food compared to *A. tepida*. Although there was a greater increase in pN between day 1 and 5 than between day 5 and 14, the C and N uptake rates were much lower than those of *A. tepida*.

It must be noted that while food C and N uptake are related through the C:N of the food source, internal foraminiferal metabolism and release processes can cause a decoupling of C and N metabolism and of isotope labeling patterns. Carbon is incorporated into organic molecules as well as into the calcareous shells or simply released during cellular respiration (e.g. Hannah et al 1994). The latter leads to a release of carbon into the environment, whereby the measured values of C isotope incorporation are influenced. Nitrogen is also utilized for the production of organic molecules such as DNA or proteins (DeLaca 1982, Nomaki et al 2014). Again the release of nitrogen-rich excretion products into the environment has an impact on the nitrogen isotope incorporation patterns.

It should be mentioned, that the processing method could result in loss of the cytoplasm. After the experiment, foraminifera were washed with distilled water to remove any salts around their tests, which could influence the mass. This washing process should be carried out carefully, because the tests could burst. In general, all samples were always treated the same way, which means they were all washed with the same volume of distilled water. This way any impact that may have arisen from using the distilled water has the same effect on all samples.

According to Stouff et al. (1999) *A. tepida* shows hardly any shell deformations at normal marine conditions of 37 PSU. This observation is consistent with the results of this study, as *A. tepida* had a higher uptake and turnover of organic matter at higher salinities (24 – 37 PSU) and therefore its optimal living conditions at higher salinity levels. Yet, in the hypersaline environment (50 PSU) this species generates a high number of deformed juvenile individuals (Stouff et al. 1999). The German Wadden Sea is subject to seasonal salinity fluctuations and has a mean salinity of 30.7 – 32.5 PSU (Postma 1983). Depending on the supply of fresh water and evaporation rates, the water in this region can drop to salinities of 25 and reach up to 37 PSU (Maywald 1991). Our experiments showed that the change in salinity from 24 to 33 PSU had a smaller impact on food uptake than that between 11 and 24 PSU. This shows once again that the two commonly occurring species, *A. tepida* and *H. germanica*, have adapted very well to these fluctuations. The lowest salinity (11 PSU) in our experiments represents the transition from brackish to a marine milieu. It turned out that at this salinity level the food uptake tended to be the lowest for both species. From the literature it is known that such brackish marshes are mainly inhabited by agglutinated foraminifera (Sen Gupta 1999). Considering the uptake of C and N by *A. tepida* and *H. germanica* in our experiments

(Fig. 1), it can be seen that the low salinities do not correspond to the optimum conditions of these foraminifera.

5.2. Effect of salinity on cytoplasmic C:N ratios and $\delta^{13}C$ values

Foraminiferal C:N ratios and $\delta^{13}C$ signatures in the cytoplasm have been applied as a salinity proxy for marine systems for some time (e.g. Scott and Medioli 1986. Chmura and Aharon 1995. Mackie et al. 2005). According to Mackie et al. (2005) $\delta^{13}C$ values in the range of -16 to -22‰ represent organic matter and organisms of marine origin. Brackish and freshwater organisms have lighter $\delta^{13}C$ values (-22 to -25‰ and -25 to -30‰ respectively) (Mackie et al. 2005). The foraminiferal species studied here showed background $\delta^{13}C$ values of -13.9‰ (*H. germanica*) and -15.9‰ (*A. tepida*). These values clearly point towards marine isotope signatures, concordant with a salinity of 24.2 PSU measured during the sampling of the foraminifera.

A change in cytoplasmic C:N ratio of foraminifera in intertidal habitats is fundamentally influenced by two factors: on the one hand by the composition of the local fauna and flora (food) (Stelzer and Lamberti 2001, Bowman et al 2005, LeKieffre 2018) and on the other hand by changes in the physiological processes in the organisms themselves (Frost and Elser 2002, Cross et al 2005). Both benthic foraminifera species showed divergent changes in C versus N metabolism of ingested food over time. *Ammonia tepida* showed an increase in pC:pN with feeding time, resulting from a combination of altered N metabolism (storage of N in form of proteins or DNA versus N excretions) and/or changes in C metabolism (investment of C into cellular components versus losses by cellular respiration). The observed increase in pC:pN may therefore represent either an increase in C incorporation relative to N incorporation due to lower stress (less cellular respiration) or a decrease in N retention (increased N excretion) in the foraminifera after a prolonged feeding time. *Haynesina germanica* also showed a general increase in pC with pN. However, the slope between pC and pN decreased over time, indicating a decrease in pC:pN and thereby an increased relative retention of food N compared to food C. In our experiments the change in salinity did not affect the pC:pN ratios. In other words the salinity did not cause a change in relative C versus N metabolism in both species. Investigating the behavior of other nutrients such as P or Mg alongside C and N might provide further interesting insights into the intake and metabolism of food and its biochemical constituents. Phosphorus serves as an important building block in nucleic acids and phospholipids and might be an indicator for cellular energy status because it is used for the formation of energy storage molecules such as ATP. The behavior of P at changing environmental conditions may therefore indirectly indicate the stress behavior of foraminifera. Magnesium is an important component of chlorophyll. Based on the Mg content of foraminifera it is possible to reconstruct the amount of chlorophyll and therefore the presence of chloroplasts. It is also possible to quantify chlorophyll directly, for example via spectroscopy. However, this is only possible if the pure cytoplasm is examined without the residues of the shells.

An important point is the different affinity of foraminifera to food. As *H. germanica* possesses kleptoplasts, which are absent in *A. tepida*, the two species have different metabolisms and food dependencies. *Ammonia tepida* showed an approximately 10-fold higher food uptake

as *H. germanica*, partially explained by the preference of *A. tepida* for the green algae *Dunaliella sp*. (Lee et al 1961) which served as the food source here while *H. germanica* prefers to eat diatoms due to kleptoplastidy.

Furthermore, the alteration and aging of food sources can play an important role affecting feeding and food metabolism, as indicated by the preference for „fresh“ or „younger“ phytodetritus (Lee et al 1966). In the experiments here food from the same lyophilized algal batch was always used to avoid this effect. Moreover, selective food uptake of different species of foraminifera needs to be considered, and this was clearly demonstrated in a study where a total of 28 different diatom and chlorophyte species were fed to three littoral benthic foraminifera species but only 4-5 of these food sources were consumed at significant rates (Lee and Müller 1973). Ultimately one needs to be aware that contamination by bacteria or other microbes cannot be ruled out, particularly in longer-term experiments, as these organisms also use the food offered as a C or N source (Murray et al 1986. Dobbs et al 1989. Middelburg et al. 2000. Gihring et al 2009).

5.3. Effects of salinity on the foraminiferal community

The foraminifera of the mudflats of Friedrichskoog have been investigated for their responses to environmental parameters such as temperature and organic matter flux (Llobret-Brossa et al. 1998. Brasse et al. 1999. Tillmann et al. 2000). In this study we could show that *A. tepida* and *H. germanica* reacts with a lower food uptake compared to a decreasing salinity. At low tide the benthic organisms are strongly exposed to the ambient weather conditions such as wind, rain or sunlight. Due to the geographic location the growth of organisms is strongly linked to the spring and summer months. Past data from Tillmann et al. (2000) showed that growth of phytoplankton in winter is limited or almost zero. During spring local phytoplankton blooms may occur with a daily water column particulate gross production up to 2200 mg C m^{-2} day^{-1} (Tillmann et al. 2000). Over this period food availability is not a limiting factor for foraminifera and this situation corresponds to the conditions in our experiments.

The composition of the foraminiferal community in the German Wadden Sea changes within small areas (subzones) (Müller-Navarra et al. 2016). The specific microhabitats are formed by natural parameters such as sediment grain size, pH or food source availability but also by anthropogenic influences such as diking, ditching or sheep grazing (Müller-Navarra et al. 2016). This leads to changes in the hydrological situation, and in combination with natural factors such as precipitation or seepage of ground water, the salinity in mudflats varies significantly in relation to the open ocean (De Rijk 1995). It seems that the assemblage of foraminifera in such human-influenced salt marshes is controlled mainly by changes in salinity (De Rijk 1995). De Rijk (1995) showed that in areas with widely varying salinity only few different types of foraminifera occur. Moreover, it was shown that in years with high precipitation the salinity in areas such as the Wadden Sea or in salt marshes is reduced, causing the density of foraminifera to decrease sharply. (Murray 1968). So the tidal habitats in the region around Friedrichskoog are characterized by multiple environmental factors. This leads to the formation of subzones, where particularly physical influences such as pH, salinity, temperature or tides play an important role. This area is also of particular interest for the future as the

anthropogenic impact on fluctuating ecosystems can be monitored very well here. Changes in salinity therefore are a major factor shaping the composition and activity of foraminiferal communities. In this study we could show that the two tested foraminiferal species, *A. tepida* and *H. germanica*, responded very differently to salinity in terms of food intake and C and N metabolism. Moreover, a former study demonstrated that the temperature response and temperature optima also differ between these two most abundant foraminifera species of the German Wadden Sea (Wukovits et al. 2017). Therefore environmental and climate change can strongly affect the composition of the foraminiferal community, thereby causing changes in the feeding rates and in the C-N metabolism of the foraminiferal community, and ultimately altering the C-N cycling of these intertidal ecosystems.

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