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Protein biomass quantification of unbroken individual foraminifers using nano-spectrophotometry

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Abstract. The ecological role of foraminifers has been largely unknown partly owing to difficulties in determining their individual biomass, although foraminifers are abundant in surface marine sediments. The present study provides a reliable and inexpensive method for the quantification of the protein content of hard-shelled foraminifers as a measure of biomass while preserving the tests for later analyses (e.g. morphometry, stable isotopes), using nano-spectrophotometry. The protein biomass, is significantly correlated with size, and shell weight of *Ammonia tepida* ($n = 102$, $p < 0.00001$, $R^2 = 0.462$, and $n = 181$, $p < 0.00001$, $R^2 = 0.855$). Variability in the relation between test size and weight, and cell biomass may result from natural variability in horizontal and vertical microenvironments encompassing metabolic state, as well as variability in test morphometry and calcite mass (i.e. test weight). In turn, knowing the size- and species-specific biomass of foraminifers adds valuable information on the trophic and ecologic conditions of modern and ancient marine environments, in particular on the reconstruction of the regional palaeoproductivity and flux of organic matter.

1 Introduction

Due to their abundance and the high fossilization potential, foraminifers are among the most utilized proxies in palaeoceanography and marine environmental reconstruction (Fischer and Wefer, 1999). The abundance of benthic foraminifers is attributed to their tolerance of a broad range of marine environmental conditions from mud-flats to the deepest parts of the world's oceans, constituting a significant part of the meiobenthos abundance and biomass (e.g. Moodley et al., 2000; Witte et al., 2003; Murray, 2006; Gooday et al., 2008). Food provides energy to heterotrophic organisms, including foraminifers, and organic carbon supply is considered the single most important factor limiting the growth of foraminifers in well-oxygenated normal marine environments (e.g. Lutze and Coulbourn, 1984; Jorissen et al., 1995). The export production and flux of particulate organic matter in the deep-water column and at the sea floor are known from empirical relations (e.g. Suess, 1980), and are better constrained since less variable in the deep sea than at shallow water depth (cf. Murray, 2001). Large ranges of primary production and organic carbon flux seem to be related to benthic foraminifer faunas, which are characterized by different species assemblages (Altenbach et al., 2003). While standing stocks seem to be controlled by the abundance of food (i.e. organic carbon flux), the distribution of foraminifers at the species level is much less well constrained (Altenbach et al., 1999; Murray, 2001). The relation of food supply and the distribution of foraminifers at the species level is anecdotal, in particular at shallow waters, and much better estimates of primary productivity and modern organic carbon flux are required to improve the reconstruction of past flux rates (Altenbach et al., 1988; Lutze and Altenbach, 1988; Murray, 2001). Consequently, the relation between food availability and the biomass of foraminifers needs to be calibrated at the species level and for a wide range of modern shallow- to deep-water environments to facilitate a much more accurate

reconstruction of regional and basin-scale palaeoproductivity using transfer functions.

The ecological role of foraminifers in modern and past oceans has been largely unknown, partly owing to methodological difficulties in determining their individual biomass (Murray, 2006). No attempt was taken to measure individual foraminifer biomass since the work of Altenbach (1985, 1987). Using the method of Altenbach (1985), the foraminifer test needs to be disintegrated to measure the organic carbon content though, impeding further analyses of the test, the latter being important for further palaeoceanographic interpretation of the biomass data. As such, it is important to preserve the tests for further analysis during the procedure of biomass analysis.

We have developed and calibrated a new, non-destructive technique for protein biomass analysis of foraminifers, applying nano-spectrophotometry. The method is inexpensive, reliable, and easy to apply at any land-based and sea-going laboratory. Experiments were designed both to quantitatively analyse foraminifers for their protein biomass and to preserve the foraminifer test for morphometric analyses and calcite weight. The new method is quick and easy to apply in the laboratory and even on small research vessels, and the data are reproducible. In addition, we present ~ 600 new data on cytoplasm and test mass of the neritic benthic foraminifer species *Ammonia tepida* from the French Atlantic coast and laboratory cultures. The species has been chosen because it has been intensely studied (Morvan et al., 2006), and growth and reproduction rates are known (Bradshaw, 1957; Goldstein and Moodley, 1993). In turn, the solid shell and small aperture of *Ammonia tepida* impedes easy access of the analytical chemicals to the cytoplasm, and we present alternative methods (osmotic shock, ultrasonication, and NaOH treatment) to quantitatively measure the foraminifer cytoplasm. As such, *Ammonia tepida* constitutes a worst-case study, and the methods presented here could possibly be successfully applied to most of the common foraminifer species for quantitative biomass analyses. The final aim of this study is to provide a new technique and data to facilitate calibration of the modern foraminifer biomass for the application to paleoecological and palaeoceanographic reconstruction.

2 Materials and methods

A series of experiments was conducted with the aim to optimize a protocol of foraminifer cytoplasm measurement with a standard bicinchoninic method for protein quantification (Smith et al., 1985), in connection with analyses of the foraminifer test size and weight. The first experiments were designed to find a method alternative to crushing of the foraminifer shells to access the cytoplasm. *Ammonia tepida* are relatively thick-walled and have small apertures causing limited contact of analytical chemicals and cytoplasm. The easiest method to sidestep this problem is to crush the shell, but this would impede later analyses of the test morphology. Therefore, different methods were tested to break the cellular membrane for cytoplasm analyses, such as osmotic shock (with Milli-Q water and micro-filtered tap water), ultrasound treatment (for 2 and 5 s), and NaOH treatment. All experiments were designed to compare protein content of unbroken individuals with the protein content of crushed individuals, the latter of which served as a control group (see tables for a global overview of the different experiments). After the experiments, the presence of biases (1) linked to the small amount of proteins measured, and biases due to (2) variation in the duration of incubation time of foraminifer sample solution (FSS) between the first and the last measurements was checked. The preservation of tests was continuously assessed in all experiments. A detailed protocol of the preparation of foraminifers with the different treatments is given below. Finally, the relation between the foraminifer protein content and test size was assessed, as well as a potential impact of cultivation time (laboratory conditions and feeding combined) on protein content.

The chemical reagent used for the analyses of foraminifer protein content is a mixture of copper solution (Sigma-Aldrich) and bicinchoninic acid (BCA, Sigma-Aldrich) solution (Smith et al., 1985; Zubkov et al., 1999; Mojtahid et al., 2011). The Cu^{2+} contained in the copper solution is reduced to Cu^{+} by the proteins. The newly formed Cu^{+} reacts with the BCA and a strong purple colour is produced. The intensity of the colour increases proportionally with protein concentration, and the absorbance of the 562-nm wavelength was measured with a nano-spectrophotometer (NanoDrop 2000, Thermo Scientific). Analysis with a NanoDrop 2000 requires only 2 µl of solution, which allows several measurements of each standard and FSS (including BCA solution). Each standard and FSS was measured in triplicate.

Morphometric analyses of the foraminifer tests were carried out with an automated incident light microscope driven by analySIS® software (Bollmann et al., 2004; Clayton et al., 2009), installed at the University of Angers. A microbalance (Mettler Toledo XP2U) with a precision of 0.1 µg was employed to weigh the dry and empty individual foraminifer tests. The microbalance was used in an air-conditioned room at constant temperature and humidity. Each test was acclimatized in the weighing room for at least 12 h before weighing.

The software R (v.12.2.1) was used for analysis of the data and calculation of the regressions. All comparisons of the protein distributions were carried out using variance analyses. All regressions presented are exponential functions following the allometric development of *A. tepida*.

2.1 Sampling, cultivation, and preparation of the foraminifers

Ammonia tepida were sampled from the mud flats of the Baie de l'Aiguillon, 10 km north of La Rochelle, French Atlantic coast, 46°15′17″ N, 1°8′27″ W, on 13 May, 20 July,

and 23 November 2010 (Table 1). The sampling site was chosen because of the high abundance of the benthic foraminifer species *A. tepida*. The samples were transferred (~ 2 h drive) to the laboratory at Angers University in a cool box, and washed through a 63-µm sieve with 0.45-µm micro-filtered seawater. Microfiltration of the water ensured that the bacteria were removed and contamination was avoided. In May, July, and November, first batches of 45, 60, and 104 specimens, respectively, were immediately picked after sampling, cleaned, and measured for their protein biomass (Table 1). The spare individuals were cultivated for later analysis. For cultivation, specimens were placed in an incubator at 20 °C and repeatedly subsampled (Table 1). The seawater was changed twice a week to maintain good oxygenation. The foraminifers were first fed after 10 days, and from then on (1) once per week with 1 ml of dried *Chlorella* algae, and (2) twice per week with two strains of *Phaeodactylum* (1 ml of each).

Each analysed individual of *A. tepida* was carefully selected for its coloration indicating the presence of live cytoplasm or for the presence of a cyst indicating that they were feeding or reproducing and hence alive and healthy. Specimens that contained no measurable proteins were assumed dead and were not included in any of the following calculations relative to the protein content. Specimens were picked from all size classes (> 100 µm) to ensure that our data represent the in-situ assemblage of *A. tepida* and to enable identification of ontogenetic changes of the size-to-biomass relation. Each foraminifer was transferred into a bath of micro-filtered seawater and gently cleaned with a brush to remove all particles stuck to the specimen including organic matter. Subsequently, specimens were washed for one second in de-ionized water to remove the seawater. Each foraminifer was then stored individually in an Eppendorf cup and frozen at -80 °C to prevent disintegration of the proteins. For each of the different preparation methods, foraminifers were unfrozen and divided randomly between (1) the specimens that were crushed and served as control, and (2) the specimens used for testing one of the five preparation methods on the entire foraminifer tests. This insured that the two subsamples were representative of the original sample and that the protein content depended only on the preparation method and not on the order in which the foraminifers were processed.

To establish the relationship between protein content and test size, 104 foraminifer specimens from the November sampling campaign were analysed (Table 1). Each specimen was morphometrically analysed before deep-freezing. The foraminifers were then unfrozen and crushed, and the cytoplasm protein content was analysed.

Table 1. Dates of sampling and sample processing in 2010, cultivation periods (days), and number of specimens of *A. tepida* picked from each sample at different cultivation times.

Sampling Date	Processing Date	Cultivation Period	Number of Specimens
13/05	14/05	1	45
13/05	17/05	4	52
13/05	19/05	6	49
13/05	25/05	12	42
13/05	21/06	39	60
13/05	24/06	42	31
20/07	21/07	1	60
20/07	04/08	15	29
20/07	05/08	16	30
23/11	24/11	1	104
23/11	02/12	9	30
23/11	08/12	15	29
23/11	15/12	22	29

2.2 Analytical protocol

2.2.1 Preparation of the "working reagent" (WR)

The WR was produced by mixing 50 parts of BCA with 1 part of copper solution. The BCA solution was composed of bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N sodium hydroxide. The copper solution was composed of 4 % (w/v) copper (II) sulphate pentahydrate (kit number BCA-1, Sigma). Mixing the two reagents produces a solution of light green colour. The WR is stable over 24 h at ambient temperature, and could thus be prepared long before the addition of the proteins.

2.2.2 Preparation of the protein standards set (StS)

A protein StS was prepared using a solution of bovine serum albumin (BSA; Sigma-Aldrich) at a protein concentration of $1.0\,\mathrm{mg\,ml^{-1}}$. Different concentrations of protein standards were produced by dilution with de-ionized (Milli-Q) water or micro-filtered tap water to a certain volume of protein-BSA solution (Table 2). For each concentration, three replicates were produced by adding 20 µl of the protein solution to 18 Eppendorf cups. 400 µl of WR were added to each cup containing protein solution. Protein standards and WR were mixed for 3 s using a vortex (Lab Dancer, LaboTech).

The protein standards were incubated to obtain a coloration of the solution resulting from the protein concentration. The chemical reaction and resulting coloration depend on incubation time and temperature, which needed to be adjusted to the application to foraminifer protein contents and to produce a solution of measurable differences in colour intensity. Different incubation times and temperatures were tested on several protein StSs. An optimum colour spectrum was obtained at an incubation time of 24 h at room

Table 2. Volumes of BSA and MilliQ- or tap water used to prepare the StS. For analyses of the specimens treated with an osmotic shock and ultrasonication, StSs of 0–20 µg protein concentration were used. For NaOH treatment and for specimens sampled in November, standards of 0–10 µg of protein concentration were used.

Final protein content (µg)	Protein standard BSA (µl)	Milli-Q or micro-filtered tap water (µl)	Total volume (µl)	StS from 0 to 20 µg of proteins	StS from 0 to 10 µg of proteins
0	0	80	80	yes	yes
2	8	72	80	yes	yes
4	16	64	80	no	yes
5	20	60	80	yes	no
6	24	56	80	no	yes
8	32	48	80	yes	yes
10	40	40	80	yes	yes
15	60	20	80	yes	no
20	80	0	80	yes	no

Table 3. Equations of the regressions of the StS of each experiment (Exp.), number of standards (# std.) analysed, standard deviation (R^2), probability (p), and standard error of the residuals (SE res.) associated with the regression.

Exp.	# std.	Equation of the Regression	R^2	p	SE res.
(1)	63	$y = -5.103 \times 10^{-5}x^2 + 1.001 \times 10^{-2}x + 3.706 \times 10^{-3}$	0.9965	< 0.001	0.003559
(2) and (3)	63	$y = -5.124 \times 10^{-5}x^2 + 1.081 \times 10^{-2}x + 2.664 \times 10^{-3}$	0.9935	< 0.001	0.005281
(4)	63	$y = -1.012 \times 10^{-4}x^2 + 1.260 \times 10^{-2}x + 3.517 \times 10^{-3}$	0.9901	< 0.001	0.00704
(5)	54	$y = 1.508 \times 10^{-4}x^2 + 3.495 \times 10^{-3}x - 4.127 \times 10^{-4}$	0.9638	< 0.001	0.003351
Nov. 1	54	$y = 9.276 \times 10^{-5}x^2 + 9.663 \times 10^{-3}x - 3.456 \times 10^{-3}$	0.9944	< 0.001	0.002753
Nov. 9, 15, 22	54	$y = 9.970 \times 10^{-5}x^2 + 9.059 \times 10^{-3}x - 4.560 \times 10^{-3}$	0.9893	< 0.001	0.003607

temperature (20 ± 2 °C). After incubation, each cup was centrifuged for 3 s at 5000 rpm, and the absorbance was measured.

The WR turns very dark at high concentrations of proteins, and its absorbance is affected both by colour and brightness. Polynomial regressions have been used to account for these two factors. Table 3 shows the regression results for the StSs absorbance data ($R^2 > 0.95$; Table 3).

It is important to note that the WR needs to be added to the protein StS and FSS at exactly the same time to make sure that the incubation time and temperature are identical.

2.2.3 Five different preparation methods for the measurement of the foraminifer cytoplasm protein content

Five different preparation methods, an osmotic shock with Milli-Q water or micro-filtered tap water, ultrasonication for 2 or 5 s, and NaOH treatment were applied to the foraminifers in different experiments (Table 4). All the specimens were frozen at −80 °C before protein measurement. In each of the different methods, crushed foraminifers served as control group, assuming that the proteins contained in the cytoplasm were quantitatively released to the chemical reagent.

For each of the foraminifer preparation methods tested, the same amount of crushed foraminifers was produced from the same sampling period and cultivation time. For measurement of the protein biomass of crushed specimens, Eppendorf cups with single foraminifers were unfrozen. 20 µl of either deionized water or micro-filtered tap water (depending on what was used for the StS and the FSS) and 400 µl of WR were added to each Eppendorf cup. Foraminifers were then individually crushed with a clean needle to ensure that all the proteins would be released into the solution.

1. Osmotic shock with Milli-Q water

Out of 187 specimens of *A. tepida* sampled in May 2011, 93 specimens were exposed to Milli-Q water to break the membrane of the cytoplasm by osmotic shock (Table 4). Eppendorf cups with single foraminifers were unfrozen, and 20 µl of Milli-Q water were added to each cup. We then allowed 30 minutes for the osmotic shock to take place. Then, 400 µl of WR were added. Each FSS was mixed for 3 s with a vortex and incubated for 24 h at room temperature. Before spectrophotometric measurement, each cup was centrifuged for 3 s at 5000 rpm to remove any particles from the liquid. The remaining 94 specimens were crushed and analysed for

Table 4. Overview of the five processing methods for protein quantification plus control group of crushed tests. Specimens submitted to ultrasonication for 5 s were severely damaged, and the original test weight could not be determined. All specimens that were not crushed were weighed and analysed for their morphometry.

Sampling period + cultivation time (days)	Treatment	Number of foram.	Foram. preparation	Protein content	Weight	Size	Comments
May 1, 4, 6, 12	Crush	92	Wash, freeze, crush after adding WR	Yes	No	No	–
	(1) Osmotic shock	96	Wash, freeze, add 20 µl of Milli-Q water 30 min before adding WR	Yes	Yes	Yes	Signs of dissolution and/or first chamber damaged, probably due to low pH = 6.21 of Milli-Q water
May 39, 42	Crush	30	Wash, freeze, crush after adding WR	Yes	No	No	–
	(2) Osmotic shock	31	Wash, freeze, add 20 µl of micro-filtered tap water 30 min before adding WR	Yes	Yes	Yes	–
	(3) Ultra-sonication 2 s	30	Wash, freeze, ultrason. for 2 s after adding WR	Yes	No	Yes	Time of ultrason. too short to break cell membrane
July 1	Crush	30	Wash, freeze, crush after adding WR	Yes	Yes	Yes	–
	(4) Ultra-sonication 5 s	30	Wash, freeze, ultrason. for 5 s after adding WR	Yes	No	Yes	Time of ultrason. too long, and tests broken
July 15, 16	Crush	29	Wash, freeze, crush after adding WR	Yes	Yes	Yes	–
	(5) 10 % NaOH	30	10 µl of Milli-Q water plus 10 µl of 10 % NaOH in the StS and FSS	Yes	Yes	Yes	NaOH seemed to have interfered with WR
November 1	Crush	104	Wash, freeze, crush after adding WR	Yes	No	Yes	–
November 9, 15, 21	Crush	88	Wash, freeze, crush after adding WR	Yes	No	Yes	–

their protein content (Table 4). The individuals submitted to an osmotic shock were analysed for their protein content, cleaned, dried, photographed and weighed (Table 4).

2. Osmotic shock with micro-filtered tap water

This method is similar to the previous one, but micro-filtered tap water was used instead of Milli-Q water. The osmotic shock was applied in the same way, and the FSS preparation was the same. We analysed 46 specimens from the May sample (Table 4). 28 specimens were crushed, and 18 specimens were submitted to an osmotic shock from micro-filtered tap water (0.2-µm polycarbonate membrane filter, Whatman). The tap water had a higher pH (pH = 8.18) than Milli-Q water (pH = 6.21) and was thus less corrosive to the calcareous foraminifer shells. In this experiment, micro-filtered tap water was also used for the preparation of the StS. The protein content from the two batches of entire and crushed individuals was measured (Table 5). The individuals submitted to an osmotic shock were weighed and analysed for their morphometry.

Table 5. Comparison of protein concentrations analysed with different preparation methods. Protein concentrations are compared within each experiment, and not between experiments. Specimens with no measurable protein content were interpreted as dead and data were not further used. The mean protein content of all individuals is given along with the standard deviation. The probability (p) is associated with the variance analysis comparing each treatment to the control (crushed individuals). Values marked with an asterisk (*) are significantly different from the control group of crushed individuals.

Sampling period and cultivation time (days)	Number of specimens	Treatment	Mean protein content	Standard deviation	p Comp. control
May 1, 4, 6, 12	90	Crush	2.040	1.084	Control
	93	(1) Osmotic shock (Milli-Q water)	1.491	1.140	< 0.001*
May 39, 42	28	Crush	1.020	0.715	Control
	18	(2) Osmotic shock (micro-filtered tap water)	0.428	0.282	< 0.01*
	28	(3) Ultra-sonication (2 s)	0.886	0.812	0.348
July 1	23	Crush	0.682	0.641	Control
	21	(4) Ultra-sonication (5 s)	0.410	0.259	0.122
July 15, 16	29	Crush	1.588	0.835	Control
	9	(5) 10 % NaOH	2.589	2.604	0.075

3. Ultrasonication for 2 s

Ultrasonication for 2 s was applied to 31 specimens from the May sample (Table 4). This experiment was conducted in parallel to the previous experiment (2), and the same control group of crushed specimens was used (Table 4). Foraminifers were unfrozen, 20 µl of micro-filtered tap water were added to each Eppendorf cup, WR was added, and the FSSs were submitted to ultrasonication for 2 s. They were vortexed several times over a 24-h incubation period at room temperature. The FSSs were then centrifuged. The protein content of ultrasonicated individuals was measured, and the tests were weighed and analysed for their morphometry.

4. Ultrasonication for 5 s

Foraminifers were unfrozen and prepared in the same way as for the previous experiment, but were ultrasonicated for 5 s. 60 individuals from July were analysed. Half of the specimens were crushed, and the remaining 30 specimens were ultrasonicated. The protein content of the ultrasonicated foraminifers was measured and compared with the crushed specimens. The uncrushed tests were morphometrically analysed.

5. NaOH treatment

A solution of 10 % sodium hydroxide (NaOH) was applied to break the cellular membranes of *A. tepida* and to release the cytoplasm content to the WR (Piña-Ochoa et al., 2010). Due to a known interaction with the BCA solution, the final concentration of sodium hydroxide had to be maintained below 100 mM (BCA technical bulletin, Sigma-Aldrich). For this purpose, the Eppendorf cups containing foraminifers were unfrozen, and 10 µl of 10 % NaOH and 10 µl of Milli-Q water were added. The FSSs were vortexed and 400 µl of WR were added. After a 24-h incubation period at room temperature, the FSSs were centrifuged and the absorbance of the 562-nm wavelength was measured. Note that for this treatment, 10 µl of the Milli-Q water of each standard of the protein standard set had to be replaced by the same volume of 10 % NaOH to take the interaction between the NaOH and the BCA solution into account.

The NaOH method was applied to 59 specimens from the July sampling campaign. About half of the specimens were crushed (29), and 30 specimens were analysed with complete tests (Table 4). Both the crushed and complete specimens were treated with a solution of 10 µl of Milli-Q water plus 10 µl of a 10 % NaOH solution, and the protein content was analysed. The foraminifers submitted to 10 % NaOH were weighed and morphometrically analysed.

2.3 Effect of cultivation on protein content

Large *A. tepida* (88 specimens > 250 µm) from the November campaign were picked at different cultivation times (30 specimens after 9 days, 29 sp. after 15 days, and 29 sp. after 21 days; see Table 1) to analyse the effect of cultivation on the protein content. Each specimen was photographed and measured before deep-freezing. The foraminifers were then unfrozen and crushed for the protein measurement, and compared to the specimens sampled at different cultivation intervals (May and July batches; Table 4).

3 Results

3.1 Protein data produced with the different preparation methods

1. The mean protein contents of crushed specimens (2.040 µg) and specimens submitted to an osmotic shock using Milli-Q water (1.491 µg) were significantly different ($p < 0.001$; Table 5). Some of the foraminifer tests were damaged by the Milli-Q water, i.e. tests were partly dissolved, and the last chamber was broken in several specimens, probably because of test dissolution at the low pH of the Milli-Q water (pH = 6.21).

2. The mean protein content of the crushed foraminifers was 1.020 µg, and 0.428 µg for foraminifers submitted to an osmotic shock using micro-filtered tap water, being significantly different between the two batches ($p < 0.01$; Table 5). In contrast to the treatment with Milli-Q water (see above) the tests of *A. tepida* were not damaged by the use of micro-filtered tap water.

3. The mean protein content was 0.886 µg for individuals ultrasonicated for 2 s, and not significantly different from the crushed foraminifers (1.020 µg, $p = 0.348$; Table 5). Some of the ultrasonicated individuals were partly broken, though, which would explain why their protein content was not significantly different from the crushed individuals. In turn, some of the individuals submitted to ultrasonication were still filled with cytoplasm after treatment, indicating that the proteins were partly not measured.

4. Most of the individuals showed damaged tests after 5 s of ultrasonication. The last chamber was missing in many specimens, and in some individuals several chambers were broken (see Fig. 1). The morphometry of the broken individuals could hence not be compared with that of the other experiments and was not used in the following discussion about protein-to-size relation. The mean protein content was 0.682 µg for the crushed foraminifers and 0.410 µg for the ultrasonicated foraminifers, being not significantly different ($p = 0.122$; Table 5).

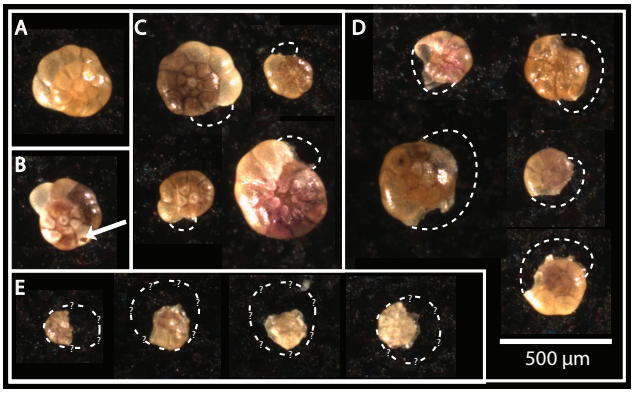


Fig. 1. Examples of the different levels of damaged *A. tepida* submitted to ultrasonication for 5 s. Dashed lines give a reconstruction of the damaged parts of the test. **(A)** Test of *A. tepida* with an intact test. **(B)** Test with a hole in the middle of a chamber. **(C)** Individuals with the last chamber missing. **(D)** Individuals with several external chambers missing. **(E)** Individuals with only the inner chambers intact, making it difficult to reconstruct the original shape and size of the test.

5. The mean protein content of the crushed foraminifers was 1.588 µg, and 2.589 µg for the specimens submitted to NaOH treatment, being significantly different ($p = 0.075$; Table 5). The strong interaction between the WR and 10 % NaOH seems to increase the variability of the intensity of the colour between the different replicates of the StS, and this might be also true for the FSS. When measuring small protein quantities, a strong variability between the replicates indicated that the quality of the data must be considered less good than in the other methods.

3.2 Effect of cultivation on protein content

The mean protein contents of *A. tepida* from each sampling campaign in May, July, and November, and for each cultivation interval between 1 and 42 days are shown in Fig. 2a. Only the protein contents of the crushed specimens are compared, to avoid any effect due to differences between analytical methods. The test size distribution for each sampling and cultivation time for all uncrushed specimens submitted to an osmotic shock (both Milli-Q and micro-filtered tap water), ultrasonication for 2 s, and 10 % NaOH treatment is shown in Fig. 2b. Given that these two groups of crushed and uncrushed foraminifers belong to the same population, we assume that the size distribution was similar for the crushed and uncrushed foraminifers and that differences in protein content can be related to test size.

Protein content and minimum test diameter for each sampling batch vary over the duration of cultivation (Table 1, Fig. 2, and Table 6). The minimum test diameter is used as a measure of size to allow comparability of our data

Table 6. Development of protein content and size (minimum diameter) of *A. tepida* from the three sampling campaigns in May, July, and November 2010, and for all cultivation intervals between 1 and 42 days. Specimens with no measurable proteins were interpreted as dead and were not used for the variance analysis. The probability (p) associated with the variance analysis between the subsample of the cultivation time and the previous subsample. Values marked with an asterisk (*) are significantly different from the previous subsample.

		Protein content (µg)				Size (µm)			
Sampling Date	Time of Culturing	Number of Specimens	Mean	Standard Deviation	p Comp. Previous	Number of Specimens	Mean	Standard Deviation	p Comp. Previous
13/05	1	29	1.909	1.129	-	14	352.34	73.14	–
	4	51	2.130	1.110	0.397	0	–	–	–
	6	0	–	–	–	48	348.44	70.44	0.857
	12	10	1.960	0.843	0.647	31	361.25	49.95	0.382
	39	18	0.898	0.562	< 0.001*	39	370.55	42.52	0.403
	42	10	1.238	0.924	0.235	20	340.11	42.71	0.012*
20/07	1	23	0.682	0.641	–	0	–	–	–
	15	14	1.361	0.689	< 0.005*	15	372.62	62.13	–
	16	15	1.800	0.925	0.161	15	354.74	61.91	0.437
23/11	1	102	0.723	0.513	–	104	243.5	52.89	–
	9	25	0.414	0.282	0.004*	30	283.31	36.17	< 0.001*
	15	25	0.622	0.415	0.043*	29	289.42	30.32	0.486
	22	24	0.477	0.416	0.228	29	293.91	26.42	0.550

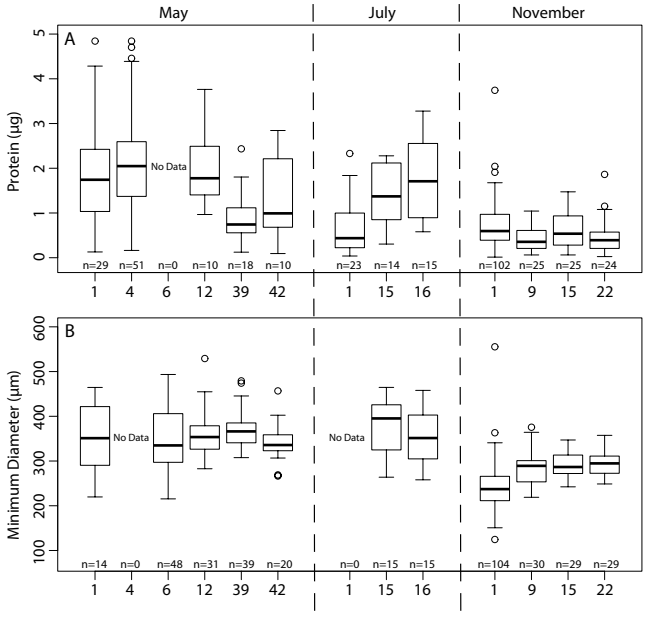


Fig. 2. (A) Protein content of crushed *A. tepida* of the three sampling campaigns in May, July, and November 2010, and for all cultivation intervals of 1 to 42 days ($n = 373$). **(B)** Development of the minimum diameter of non-crushed *A. tepida* of the different sampling campaigns and cultivation intervals. Individuals represented here are all the non-crushed individuals, i.e. the specimens treated with an osmotic shock, ultrasonication for 2 s, and 10 % NaOH ($n = 374$).

to size fractions of sieved foraminifers. Freshly sampled foraminifers (incubation interval = 1 day) from the three different sampling campaigns were significantly different regarding their mean protein content ($p < 0.001$) and test size ($p < 0.001$), and can hence not be directly compared. In the May samples, the only significant decrease in protein content occurred between 12 and 39 days of incubation (Table 6), and the only significant change in size occurred between 39 and 42 days of incubation (Fig. 2). In the July samples, a significant increase in protein content occurred between 1 and 15 days of incubation. In the November samples, a significant decrease in protein content occurred between 1 and 9 days, and 9 and 15 days of incubation, and a significant increase in size occurred between 1 and 9 days of incubation (Fig. 2 and Table 6).

3.3 Relation between foraminifer protein content, test size, and test weight

The protein content of the 104 specimens of *A. tepida* sampled in November 2010 (Tables 1 and 4) was correlated to a minimum test diameter with an $R^2 = 0.462$ ($p < 0.00001$; residual standard error = 0.626; Fig. 3). The correlation between protein content and minimum test diameter is described by the equation

$$y = 5.537 \times 10^{-8} x^{2.941}, \quad (1)$$

with y being the protein content (µg) per individual and x being the diameter (µm) of the test.

The size of *A. tepida* ranged from 124 to 555 µm. The mean test weight of *A. tepida* with a minimum diameter of

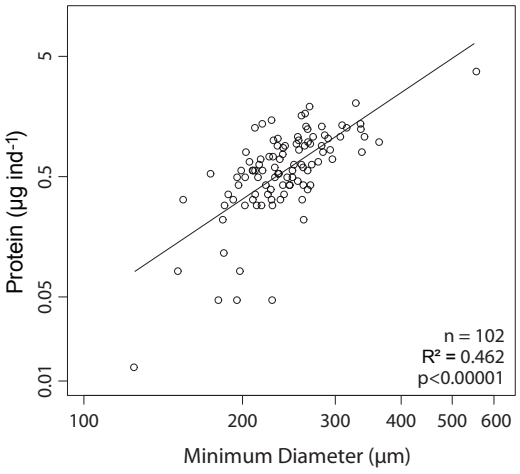


Fig. 3. Correlation between protein content and minimum test diameter of *A. tepida*. Residual standard error = 0.626. Without the single large specimen > 500 m, the correlation is still significant ($R^2 = 0.439$, $p < 0.00001$, residual standard error = 0.626, exponential fit).

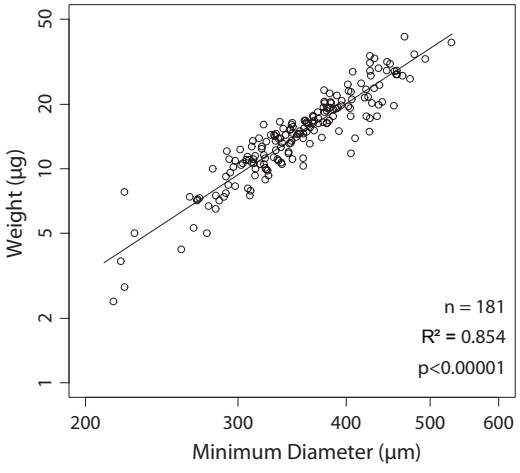


Fig. 4. Correlation between minimum test diameter of *A. tepida* and shell weight. All individuals measured and weighed during this study are shown (residual standard error = 0.181, exponential fit).

124–250 µm was 4.3 µg, 9.13 µg in the > 250–315 µm size class, 18.1 µg in the > 315–500 µm size class, and 38.9 µg in the > 500 µm size class (one specimen only).

The ultrasonicated individuals were not weighed, because a majority of the tests were partly broken. Similarly, foraminifers that were crushed were not weighed. In the other experiments, 181 tests were both measured for their size and weighed. The relation between minimum test diameter and weight is shown in Fig. 4. Both parameters, minimum test diameter and test weight, are correlated with an $R^2 = 0.855$ ($p < 0.00001$; residual standard error = 0.181). The correlation between test weight and minimum diameter is described

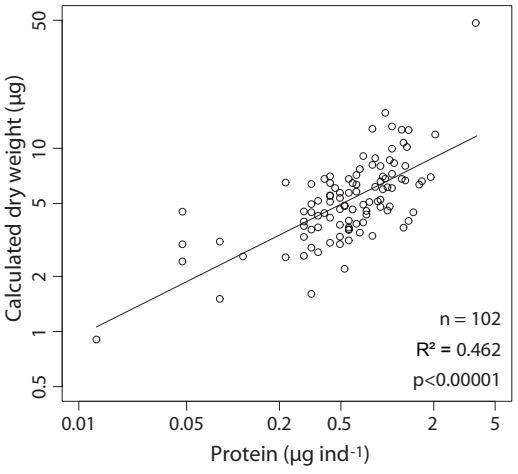


Fig. 5. Calculated dry weight (Eq. 3) calculated for individuals of *A. tepida* sampled in November 2010 (residual standard error = 0.387, exponential fit).

by the equation

$$y = 2.424 \times 10^{-6} x^{2.661}, \quad (2)$$

with y being the dry weight (µg) of the empty test and x being the diameter (µm) of the test.

Equation (2) was used to calculate the weight of the individuals analysed using size and protein content of *A. tepida*. Test weight and protein content are correlated (Fig. 5) with an $R^2 = 0.462$ ($p < 0.00001$; residual standard error = 0.387), described by the equation

$$y = 6.638 x^{0.423}, \quad (3)$$

with y being the calculated dry weight (µg) of the test and x being the protein content (µg) per individual.

4 Discussion

4.1 Foraminifer preparation

Different methods – (1) osmotic shock, (2) ultrasonication, and (3) NaOH treatment – were applied for protein quantification of hard-shelled foraminifers while maintaining the foraminifer tests for further analyses of, for example, test morphometry and allometric development, and chemical composition. Since all specimens were frozen at −80 °C before protein measurement, formation of ice crystals could have damaged the cellular membrane and contributed to cytoplasm exposure to the analytical reagents.

1. An osmotic shock with Milli-Q water or micro-filtered tap water did not heavily damage the tests of *A. tepida* but did also not produce complete release of the proteins to the analytical chemicals (WR). The low efficiency of the method might be caused by restricted

penetration of the Milli-Q water and WR through the small apertures of *A. tepida*. The method might work better on foraminifer species with larger apertures, for example, most planktic foraminifer species. However, we recommend the use of micro-filtered tap water instead of Milli-Q water to avoid any dissolution of the test.

2. Ultrasonication appeared to break the tests. Time and intensity of ultrasonication could not be sufficiently adjusted to the shell thickness of each specimen analysed, though. The use of the method should be restricted to well-defined samples, and would need to be calibrated to each foraminifer species and robustness of its test to ultrasonication. Some species with very thin tests would probably be destroyed even at lower intensities and shorter time intervals of ultrasonication, for example, planktic foraminifers.

3. 10 % NaOH appears to interact with the working reagent (WR). This experiment shows a regression curve that is less good than in the other experiments (see Table 3). The replicates differ in absorbance values, and the standards for 4 and 6 µg of protein overlap for a small range of absorbance values between 0.017 and 0.019. This could be due to the interference of 10 % NaOH with the WR. The different replicates bear a larger variability and lower accuracy than the other StS.

4.2 Protein content, morphometric parameters, and seasonal variations

Quantification of individual foraminifer cytoplasm protein content is presented for the first time after Altenbach (1985), and relations between protein content, test morphometry, and test weight are established (Figs. 3, 4 and 5). Based on the specimens analysed here, the average protein content of *A. tepida* from the mudflats of the Baie de l'Aiguillon ranges at $\sim$ 1.1 µg. The mean protein content obtained for *A. tepida* in this study is consistent with that obtained with the same BCA method for *Haynesina germanica* and *Ammonia beccarii* (0.6 to 1.4 µg) from the Hamble estuary at Warsash, Hampshire, UK (Mojtahid et al., 2011).

Significant variations in test size between the sampling batches analysed here are related to variations in protein content. In May, the average protein content and test size were slightly higher than in November (Fig. 2 and Table 6). Since *A. tepida* is directly exposed to a wide range of environmental changes in the temperate intertidal oceans, such as changes in water temperature, salinity, and abundance of prey, which affect its growth (Bradshaw, 1957, 1961, 1968), changes in test size and volume of cytoplasm are interpreted as seasonal signals. Variation in protein content observed for similar test sizes of *A. tepida* from similar sampling batches could be explained by the fact that the last chamber was filled with cytoplasm in some individuals and empty in others, and the relation of test size to cytoplasm volume would diverge.

4.3 Effect of cultivation on protein content

Considering the different sampling times (i.e. seasons) and possible differences in the metabolic state and timing within the reproduction cycle of *A. tepida* (Bradshaw, 1957, 1961), we can not unequivocally conclude on a systematic increase or decrease in protein content with increasing incubation time (Table 6, Fig. 2). Further analyses would be needed to unravel the waxing and waning of cytoplasm biomass of *A. tepida* in laboratory cultures with controlled environmental parameters to assess the effect of different parameters on the test growth and changes in cytoplasm volume and protein concentration of *A. tepida*. In addition, feeding experiments would need to be carried out with different types and quantities of food.

5 Summary and conclusion

Quantification of the protein biomass of individual *Ammonia tepida* (benthic foraminifera) using bicinchoninic acid and nano-spectrophotometry has been tested and improved. Different methods, i.e. osmotic shock and ultrasonication, are now ready to be applied to protein quantification of benthic and planktic foraminifers. Successful quantification of protein content crucially depends on the complete exposure of cytoplasm to the analytical chemicals, while preserving the foraminifer test for later biogeochemical and morphometric analyses. We suggest exposing foraminifer species with large apertures and foramens (easy penetration of chemicals into the test and complete cytoplasm exposure; i.e. most globigerinids) to an osmotic shock sufficient for quantitative protein analysis. Species with small apertures and robust shells should be processed using ultrasonication for cytoplasm exposure. Specimens with fragile shells should be ultrasonicated with care. NaOH treatment is not recommended, because it reacts with the analytical chemicals when using the bicinchoninic acid method.

A systematic increase of protein biomass with test size and shell weight of the benthic foraminifer *Ammonia tepida* is shown. Additional data of different species from a wide variety of ecological conditions would be needed, though, to assess the biogeochemical role of foraminifers in benthic and planktic ecosystems at a regional scale.

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