

## Copper incorporation in foraminiferal calcite: results from culturing experiments

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**Abstract.** A partition coefficient for copper ( $D_{Cu}$ ) in foraminiferal calcite has been determined by culturing individuals of two benthic species under controlled laboratory conditions. The partition coefficient of a trace element (TE) is an empirically determined relation between the TE/Ca ratio in seawater and the TE/Ca ratio in foraminiferal calcite and has been established for many divalent cations. Despite its potential to act as a tracer of human-induced, heavy metal pollution, data is not yet available for copper. Since partition coefficients are usually a function of multiple factors (seawater temperature, pH, salinity, metabolic activity of the organism, etc.), we chose to analyze calcite from specimens cultured under controlled laboratory conditions. They were subjected to different concentrations of  $Cu^{2+}$  (0.1–20  $\mu\text{mol/l}$ ) and constant temperature (10 and 20°C), seawater salinity and pH. We monitored the growth of new calcite in specimens of the temperate, shallow-water foraminifer *Ammonia tepida* and in the tropical, symbiont-bearing *Heterostegina depressa*. Newly formed chambers were analyzed for Cu/Ca ratios by laser ablation-ICP-MS. The estimated partition coefficient (0.1–0.4) was constant to within experimental error over a large range of  $(Cu/Ca)_{\text{seawater}}$  ratios and was remarkably similar for both species. Neither did the presence or absence of symbionts affect the  $D_{Cu}$ , nor did we find a significant effect of temperature or salinity on Cu-uptake.

### 1 Introduction

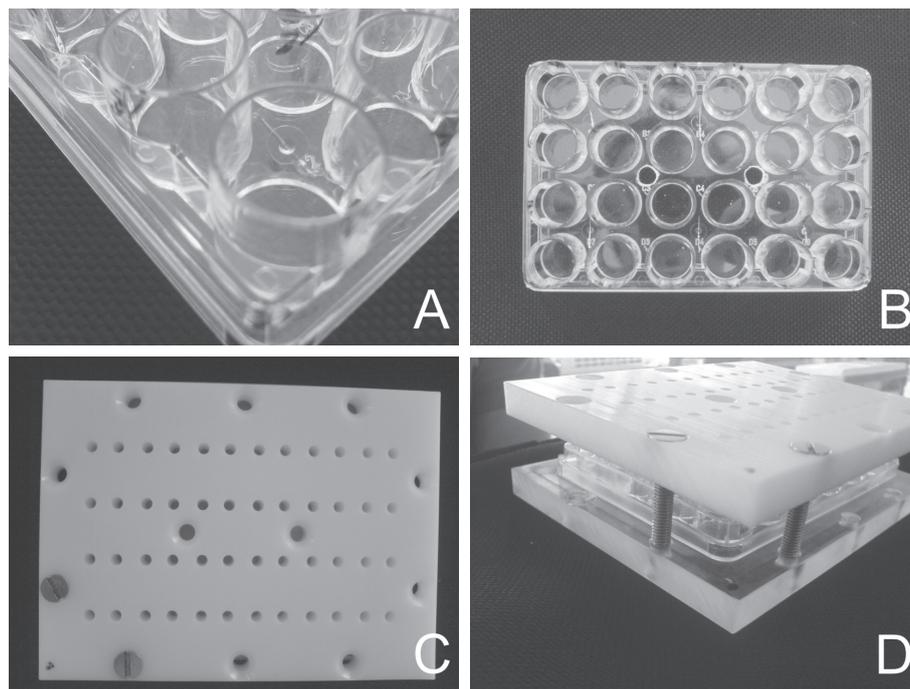
Trace elements incorporated in foraminiferal calcite tests are widely used in paleoceanography: Mg/Ca ratios are used to reconstruct sea surface (Nürnberg et al., 1996) and deep-sea

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temperatures (Rathburn and DeDecker, 1997), Cd and Ba are used to estimate past seawater nutrient levels and alkalinity, respectively (Boyle, 1988; Rosenthal et al., 1997; Lea and Boyle, 1991). These proxies rely on empirically derived partition coefficients ( $D_{TE}$ ) and the dependence of these coefficients on environmental variables. Temperature is the main controlling factor a  $D_{TE}$  in foraminiferal calcite, although salinity (Nürnberg et al., 1996) and TE/Ca ratios are also reported to affect the  $D_{TE}$  (Segev and Erez, 2006).

Although field experiments are useful to determine first order proxy relationships, reliable proxy calibrations should include the contribution of so-called vital effects and separate the effects of other possible contributing factors. The best way to unravel the contribution of separate variables is through culturing experiments, in which one variable is varied and all the others are kept constant. In the case of some divalent cations (e.g.  $Mg^{2+}$  and  $Sr^{2+}$ ; Nürnberg et al., 1996; Lea et al., 1999), culturing experiments also allow calibration of proxies out of the range of naturally occurring environmental conditions. This is important for trace elements that are associated with anthropogenic pollution with significantly raised concentrations above natural background levels.

Anthropogenic heavy metal pollution is often characterized by, amongst others, high Cu-concentrations (Borrego et al., 2004; Sáinz and Ruiz, 2006). Foraminifera have been used in several ways to investigate environmental pollution as high levels of Cu and other heavy metals potentially deform foraminiferal chamber alignment and influence foraminiferal community structure (Ellison et al., 1986; Samir and El-Din, 2001; Hallock et al., 2003; Armynot du Châtelet et al., 2004; Ruiz et al., 2004; Ferraro et al., 2006). However, a number of studies state that test deformations under high heavy metal concentrations occur less often than under



**Fig. 1.** Design of the experimental set-up. (A): close-up of a culture tray. (B): overview of a culture tray. (C) and (D): culture tray between plexiglass lids, with in- and outflow openings in upper lid.

medium pollution loads (Alve and Olsgard, 1999; Geslin et al., 2002; Le Cadre and Debenay, 2006). This suggests that reconstructions based on test deformations alone are not accurate.

In previous studies, Cu has been one of the most difficult elements to analyze in foraminiferal calcite (Boyle, 1981). However, recent advances in analytical methodology for trace element determination in foraminiferal calcite (Reichert et al., 2003) now enable the calibration of foraminiferal Cu to seawater chemistry for the first time, using cultured benthic foraminifera. Two different intertidal to neritic species (one temperate and one tropical) were cultured to establish possible interspecific differences in the partition coefficient of Cu in foraminiferal calcite.

## 2 Methods

### 2.1 Collecting and culturing foraminifera

Two similar culturing experiments were conducted in series. For the first experiment, sediment was collected at an intertidal flat in the Dutch Wadden Sea and was kept in the laboratory in the dark at 15°C. Large (>150 μm), living individuals of *Ammonia* cf. molecular type T6 (Hayward et al., 2004; further referred to as *A. tepida*) were transferred to our in-house designed flow-through culture vessels (Fig. 1). Vessels consist of a 24-well culture tray, sandwiched between two Plexiglas plates and cells were connected by silicon tubes,

attached with screws in the upper Plexiglas lid (Fig. 1). A small filter was placed between each cell and tube to prevent specimens from moving between cells. Trays were connected individually to a 2-liter reservoir with chemically altered seawater (see below) and a peristaltic pump was used to circulate seawater through the cells with a speed of 9 ml/h: in this way, six groups of 12 cells were formed, each connected to its own seawater reservoir. Four foraminiferal specimens were placed in each cell. Seawater was enriched with Cu from a stock solution at concentrations of 0, 0.10, 0.20, 0.50, 10 and 20 μmol/l. Calcein (C0875, Sigma-Aldrich, St Louis, USA) was added to the Cu-enriched seawater at a concentration of 5 mg/l.

Calcein is incorporated into biogenic calcite, while existing calcite (i.e. earlier formed chambers) is not affected. Since (incorporated) calcein is fluorescent, foraminiferal chambers that have been built during the time when individuals were incubated can be recognized (Bernhard et al., 2004). Cells with specimens of *Ammonia tepida*, contained a thin layer (<0.5 mm) of artificial sediment (Silica, 52–63 μm). Natural seawater from the eastern Mediterranean Sea was adjusted with MilliQ water to a salinity of 17 to mimic average Wadden Sea salinity. Salinity levels were regularly checked during the experiment with a WTW LF330 conductivity meter. All 6\*12 cells were kept at a constant temperature of 10°C for two months: before and after experiments, reservoirs were sub-sampled and seawater was analyzed by ICP-MS for Cu, Mg and Ca. At the start of the incubation period,

the individuals were fed  $\sim 0.5$  mg of autoclaved (20 min at 121°C) *Dunaliella* sp. During experiments, the set-up was subjected to the daily sunlight cycle (app. 14 h light/10 h dark).

For experiment 2, trays were replaced and lids rigorously cleaned with HCl, rinsed with MilliQ and re-used to incubate individuals of *Heterostegina depressa* in seawater with similar Cu-enrichments used in the first experiments. *H. depressa* is an epibenthic, tropical and symbiont-bearing foraminifer, that was kept in our laboratory under high light intensities (15 W tropical reef lamp; Arcadia, FO15) and after transferring them into the culturing set-up, similar light conditions were maintained in a daily rhythm (14 h light/10 h dark). No sediment was added to the cells, seawater salinity was kept at 35, with a constant temperature of 20°C. Because of their large size, only two specimens were placed in each cell.

## 2.2 Temperature and salinity of culture media

For culturing *Ammonia tepida*, we diluted 35PSU seawater with MilliQ water to mimic intertidal ambient conditions with seawater of 17PSU. The dilution decreased both  $[\text{Ca}^{2+}]$  and  $[\text{CO}_3^{2-}]$  and the alkalinity by approximately 50%, resulting in a pronounced reduction of the carbonate saturation state ( $\Omega$ ). The temperature maintained during these experiments was kept at 10°C, compared to 20°C for the *Heterostegina depressa* experiments, allowing gas exchange with the atmosphere (ambient pCO<sub>2</sub>) in both cases. The combined effect of these changes is a reduction in saturation state from about 5.5 for the *H. depressa* experiment to about 1.0 for the *A. tepida* experiment (calculations were performed in CO<sub>2</sub>sys; Lewis and Wallace, 1998). The lower seawater saturation state for the *A. tepida* cultures was most likely responsible for the fact that newly formed chambers were thinner than the pre-experiment chambers (see Results).

## 2.3 Laser ablation ICP-MS

Newly formed chambers were ablated using an Excimer laser (Lambda Physik) with GeoLas 200Q optics inside a helium atmosphere flushed ablation chamber. Pulse repetition rate was set at 6 Hz, with an energy density at the sample surface of 10 J/cm<sup>2</sup>. Ablation craters were 60 μm in diameter and ablated material was analyzed with respect to time (and hence depth) using a quadrupole ICP-MS instrument (Micromass Platform ICP).

Simultaneous monitoring of Al allowed us to discard the parts of the ablation profiles contaminated by clay minerals from further calculations of elemental concentrations. Since the analytical error increases with shorter ablation time we cleaned all specimens by an incubation of 24 h in 5% NaOCl (Gaffey and Brönniman, 1993) before analysis, maximizing the amount of data that could be used for calculating (Cu/Ca)<sub>calcite</sub> ratios.

## 2.4 Calibration strategy

The low calcite saturation state used in the experiment with *Ammonia tepida* resulted in formation of new chambers with thin walls. A similar correlation between test wall thickness and carbonate saturation state has been observed earlier for tests of cultured planktonic foraminifera (Bijma et al., 2002). Unfortunately, these thin chambers break easily during ablation when high laser energies are used. Therefore, we ablated *Ammonia tepida* with a laser energy of 1 J/cm<sup>2</sup>, ten times less than the 10 J/cm<sup>2</sup> used to ablate newly formed chambers of *Heterostegina depressa*. Analyses were calibrated against NIST glasses 610 and 612, using concentration data of Pearce et al. (1997). Calibrating calcites against glasses is possible because of the relatively matrix independent ablation by the Excimer laser (Mason and Kraan, 2002). However, a fluence of <2 J/cm<sup>2</sup> was close to the ablation threshold for glass and calibration was performed instead against matrix matched in-house standards (i.e. pressed calcite powder tablets). Calcium was used as an internal standard because (1) the concentration is constant at 40 wt % in calcite and (2) it allows direct comparisons with trace metal to Ca ratios from wet-chemical studies. A collision and reaction cell was used to give improved results by reducing spectral interferences on the minor isotopes of Ca (<sup>42</sup>Ca, <sup>43</sup>Ca and <sup>44</sup>Ca; Mason and Kraan, 2002). Good agreement was observed when using both <sup>63</sup>Cu and <sup>65</sup>Cu isotopes to calculate Cu concentrations. Relative analytical precision for copper analyses was 15% on average, based on variability during the ablation calculated by GLITTER (New Wave Research, Fremont, CA, USA). This error includes both analytical uncertainties and internal, natural variability in test chemistry.

## 2.5 Seawater Cu-concentration

The concentration of Cu did not vary considerably in most of our experiments during the experimental period across the range of concentrations used (Table 1).

In the first experiment, all measured Cu-concentrations were lower than the target concentration and most total Cu-concentrations increased during the experiment, resulting in increased seawater Cu/Ca ratios (on average 17%). In experiment 2, most Cu-concentrations and all Cu/Ca ratios were higher at the start than at the end of the experiment. Identical procedures and techniques were used before and after subsampling the culture media, making it unlikely that sampling artifacts affected our measurements. Therefore, we used average solution Cu/Ca ratios to estimate the partition coefficient of Cu in foraminiferal calcite and incorporated differences between start and end concentrations for uncertainty calculations. Error bars plotted in the different graphs are based on these calculations and largely stem from these changes, which are an order of magnitude larger than the analytical uncertainties.

**Table 1.** Target concentrations of Cu in sea water and measured [Cu]<sub>seawater</sub> and (Cu/Ca)<sub>seawater</sub> at start and end of both experiments. n.a. = not available.

Experiment	Target [Cu] in $\mu\text{mol/l}$	Measured Cu			Cu/Ca	
		at the start of experiment in $\mu\text{mol/l} + 1\text{SD}$	at the end of experiment in $\mu\text{mol/l} + 1\text{SD}$	at start of experiment $\times 10^{-6}$ $+ 1\text{SD} \times 10^{-6}$	at end of experiment $\times 10^{-6}$ $+ 1\text{SD} \times 10^{-6}$	
1. <i>Ammonia tepida</i>	0	0.0843	0.100 $\pm$ 0.00584	6.32 $\pm$ 5.28	2.91 $\pm$ 0.221	
	0.10	0.0960 $\pm$ 0.0146	n.a.	2.95 $\pm$ 0.384	n.a.	
	0.20	0.197 $\pm$ 0.00173	0.256 $\pm$ 0.00206	6.00 $\pm$ 0.0326	7.34 $\pm$ 0.121	
	0.50	0.332 $\pm$ 0.00505	n.a.	10.1 $\pm$ 0.0143	n.a.	
	10	12.6 $\pm$ 0.293	14.0 $\pm$ 0.867	378 $\pm$ 4.62	408 $\pm$ 6.94	
	20	17.8 $\pm$ 0.489	20.0 $\pm$ 3.05	547 $\pm$ 13.5	473 $\pm$ 20.8	
2. <i>Heterostegina depressa</i>	0	0.125 $\pm$ 0.00554	0.208 $\pm$ 0.0119	1.69 $\pm$ 0.0236	3.62 $\pm$ 0.343	
	0.10	0.744 $\pm$ 0.00217	0.211 $\pm$ 0.0408	2.32 $\pm$ 0.0670	3.74 $\pm$ 0.500	
	0.20	0.242 $\pm$ 0.0228	0.368	4.06 $\pm$ 0.179	6.71	
	0.50	1.28 $\pm$ 0.0384	1.25 $\pm$ 0.0522	20.2 $\pm$ 0.0120	23.1 $\pm$ 0.711	
	10	11.2 $\pm$ 0.00672	10.4 $\pm$ 0.277	172 $\pm$ 0.844	212 $\pm$ 5.88	
	20	n.a.	n.a.	n.a.	n.a.	

**Table 2.** Thermodynamic data for calcein (Ueno et al., 1992). L = calceine. <sup>a</sup> reactions leads to negligible metal binding; <sup>b</sup> values extrapolated by assuming chemical behavior similar to Cu<sup>2+</sup> (see text for discussion).

	Reaction	Functional groups	Log K
(1)	$\text{L}^{6-} + \text{H}^+ = \text{HL}^{5-}$	-COOH	11.7
(2)	$\text{HL}^{5-} + \text{H}^+ = \text{H}_2\text{L}^{4-}$	-COOH	10.8
(3)	$\text{H}_2\text{L}^{4-} + \text{H}^+ = \text{H}_3\text{L}^{3-}$	-COOH	5.5
(4)	$\text{H}_3\text{L}^{3-} + \text{H}^+ = \text{H}_4\text{L}^{2-}$	-OH	4.2
(5)	$\text{H}_4\text{L}^{2-} + \text{H}^+ = \text{H}_5\text{L}^-$	$\equiv\text{NH}^+$	2.9
(6)	$\text{H}_5\text{L}^- + \text{H}^+ = \text{H}_6\text{L}$	$\equiv\text{NH}^+$	2.1
(7)	$2\text{Cu}^{2+} + \text{L}^{6-} = \text{Cu}_2\text{L}^{2-}$	-COOH	28.9
(8)	$\text{Cu}^{2+} + \text{H}_2\text{L}^{4-} = \text{CuH}_2\text{L}^{2-}$	$\equiv\text{NH}^+$	8.3
(9)	$\text{Cu}^{2+} + \text{H}_4\text{L}^{2-} + \text{H}_3\text{L}^{3-} = \text{Cu}(\text{H}_4\text{L})(\text{H}_3\text{L})^{3-}$	-COOH + -OH	10.4 <sup>a</sup>
(10)	$2\text{Ca}^{2+} + \text{L}^{6-} = \text{Ca}_2\text{L}^{2-}$	-COOH	27.2 <sup>b</sup>
(11)	$\text{Ca}^{2+} + \text{H}_2\text{L}^{4-} = \text{CaH}_2\text{L}^{2-}$	$\equiv\text{NH}^+$	6.63
(12)	$\text{Ca}^{2+} + \text{H}_4\text{L}^{2-} + \text{H}_3\text{L}^{3-} = \text{Ca}(\text{H}_4\text{L})(\text{H}_3\text{L})^{3-}$	-COOH + -OH	8.73 <sup>a,b</sup>
(13)	$2\text{Mg}^{2+} + \text{L}^{6-} = \text{Mg}_2\text{L}^{2-}$	-COOH	28.5 <sup>b</sup>
(14)	$\text{Mg}^{2+} + \text{H}_2\text{L}^{4-} = \text{MgH}_2\text{L}^{2-}$	$\equiv\text{NH}^+$	7.9
(15)	$\text{Mg}^{2+} + \text{H}_4\text{L}^{2-} + \text{H}_3\text{L}^{3-} = \text{Mg}(\text{H}_4\text{L})(\text{H}_3\text{L})^{3-}$	-COOH + -OH	10.0 <sup>a,b</sup>

## 2.6 Cu speciation in seawater

In the absence of organic matter, Cu in seawater forms mainly Cu(OH)<sub>2</sub> and CuCO<sub>3</sub>, while small amounts of Cu<sup>2+</sup> and CuOH<sup>-</sup> are also present (Zirino and Yamamoto, 1972). In natural seawater, however, usually more than 99.9% of the

Cu is bound to organic compounds (Eriksen et al., 2001), mainly in the colloidal state (Mackey and Zirino, 1994). Foraminifera take up organic particles and seawater by endocytosis, likely ingesting both free Cu and Cu-ligand complexes. The internal routes that organic compounds follow are virtually uninvestigated in foraminifera and therefore, we

**Table 3.** Added and free copper concentrations and activities in the experiments. Suffix *t* = total copper concentration added to the experiment; suffix *c* = corrected ratios (total Cu or Ca minus calcein-complexed Cu or Ca).

Experiment	[Cu] <sub>t</sub> (μmol/l)	{Cu} <sub>t</sub> (μmol/l)	[Cu] <sub>t</sub> /[Ca] <sub>t</sub>	[Cu] <sub>c</sub> (μmol/l)	{Cu} <sub>c</sub> (μmol/l)	[Cu] <sub>c</sub> /[Ca] <sub>c</sub>
1. <i>Ammonia tepida</i>	0.156	0.133	$2.6 \times 10^{-5}$	0.156	0.133	$2.6 \times 10^{-5}$
	0.168	0.146	$2.9 \times 10^{-5}$	0.168	0.146	$2.9 \times 10^{-5}$
	0.384	0.333	$6.6 \times 10^{-5}$	0.384	0.333	$6.6 \times 10^{-5}$
	1.05	0.911	$18 \times 10^{-5}$	1.05	0.911	$18 \times 10^{-5}$
	18.6	16.1	$320 \times 10^{-5}$	18.6	16.1	$320 \times 10^{-5}$
	38.0	32.9	$660 \times 10^{-5}$	38.0	32.9	$660 \times 10^{-5}$
2. <i>Heterostegina depressa</i>	0.167	0.140	$1.6 \times 10^{-5}$	0.167	16	$1.6 \times 10^{-5}$
	0.190	0.163	$1.8 \times 10^{-5}$	0.190	18	$1.8 \times 10^{-5}$
	0.255	0.218	$2.4 \times 10^{-5}$	0.255	24	$2.4 \times 10^{-5}$
	1.36	1.16	$13 \times 10^{-5}$	1.36	130	$13 \times 10^{-5}$
	12.1	10.3	$110 \times 10^{-5}$	12.1	1100	$110 \times 10^{-5}$

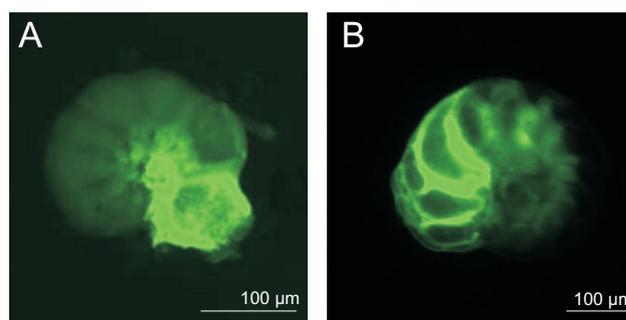
do not know which Cu species are present at the site of calcification.

## 2.7 Modelling Cu speciation

The calcein added in our experiments is a ligand that can bind TE's and could thus cause concentrations of free Cu to drop. Traditionally, total calcium and TE concentrations in solution are used to calculate partition coefficients for TE's in calcite. Ideally, activities or effective concentrations, of relevant metals are used to allow application of partition coefficients in solutions of different compositions (Morse and Bender, 1990).

To correct for Cu binding to calcein, we calculated speciation of all abundant cations (Cu, Ca and Mg) in our solution. Speciation calculations were performed in PHREEQC 2.8.03 (Parkhurst and Appelo, 1999) with the Iln database and thermodynamic data for calcein listed in Table 2. For calcein complexation with calcium and magnesium, Reactions (11) and (14) are reported in the literature. These reactions will lead to competition between copper, calcium and magnesium in binding to an amine group on  $H_2L^{4-}$ , thus decreasing calcein-bound Cu. It is, therefore, likely that the behavior of Ca and Mg towards calcein is similar to Cu and similar competition between the three metals occurs in binding according to Reactions (7) and (9) via carboxyl groups (Lu and Allen, 2002). Composition of the solution in the model was either the Cu-enriched seawater with a salinity of 35 or of 17 for the experiment with *Ammonia tepida*, while both were open to atmospheric  $CO_2$ .

Cu and Ca-concentrations used to calculate the partition coefficients were those corrected for Cu and Ca complexed with calcein (Table 3).



**Fig. 2.** New chambers added by *Ammonia tepida* (A) and *Heterostegina depressa* (B), visible by fluorescence of incorporated calcein.

## 3 Results

### 3.1 New calcite and survival rates

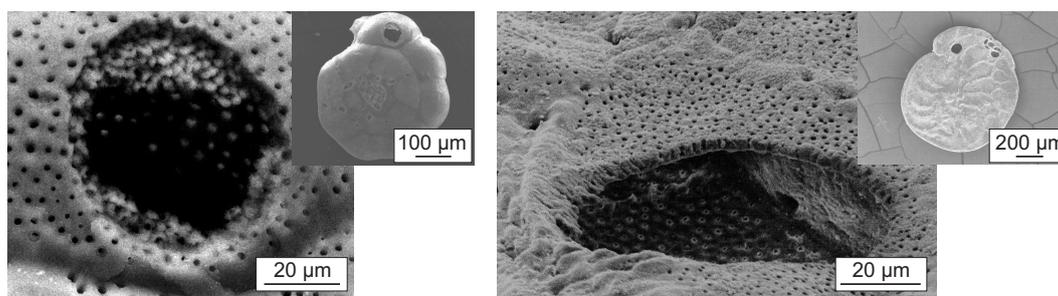
Specimens that grew new calcite were recognized by fluorescent, outer chambers (Fig. 2).

None of the individuals of *Heterostegina depressa* incubated at the target Cu-concentration of  $20 \mu\text{mol/l}$ , survived the experimental period. At  $10 \mu\text{mol/l}$  of added Cu, however, several survived of which 1 individual grew new calcite. At lower concentrations, generally more chambers were formed (Table 4). None of the added chambers ( $n=88$ ) showed abnormal alignments or deformations.

For *Ammonia tepida*, the number of successful laser-ablation analyses was significantly lower (3) than the number of added chambers (34). The limited size ( $<100 \mu\text{m}$ ) of newly added chambers did not allow multiple analyses of a single chamber. After an unsuccessful attempt to analyze a targeted chamber it was not possible to repeat this measurement as the largest part of the carbonate was consumed (Fig. 3).

**Table 4.** Number of individuals at the start of the experiments, number of specimens that formed new calcite and total number of added chambers.

Experiment	Target [Cu <sup>2+</sup> ] in $\mu\text{mol/l}$	Number of specimens at start of experiment	Number of individuals that grew new chambers	Number of new chambers added
1. <i>Ammonia tepida</i>	0	48	7	7
	0.10	48	11	12
	0.20	48	6	6
	0.50	48	3	3
	10	48	5	5
	20	48	1	1
2. <i>Heterostegina depressa</i>	0	24	5	8
	0.10	24	3	6
	0.20	24	0	0
	0.50	24	19	37
	10	24	1	3
	20	24	0	0

**Fig. 3.** Scanning electron microscope image of laser ablation craters in *Ammonia* (left) and *Heterostegina* (right). Insets depict the whole specimen. Scale bar is indicated in the lower right corner only for the magnified image.

### 3.2 Partition coefficient of Cu – *Ammonia tepida*

Two ablation profiles were obtained from two specimens of *Ammonia tepida* that grew new chambers at a low (0.20  $\mu\text{mol/l}$ ) Cu-concentration (Fig. 4). Measurements indicate that the partition coefficient lies between 0.1 and 0.4. In the right panel of Fig. 4, the same two measurements are depicted at the left end of the graph. Ratios for calcite formed at higher (Cu/Ca)<sub>seawater</sub>, indicated a partition coefficient between 0.1 and 0.4.

### 3.3 Partition coefficient of Cu – *Heterostegina depressa*

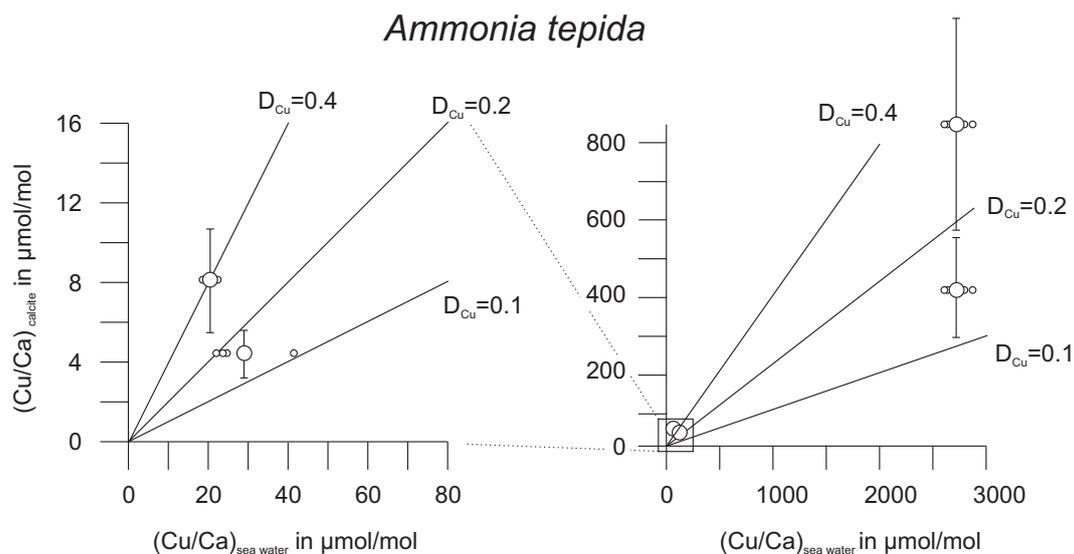
Although individuals of *Heterostegina* did not survive the highest Cu-levels, we obtained two ratios from specimens that added new chambers at a target concentration of 10  $\mu\text{mol/l}$ . From incubations with lower Cu-concentrations, more specimens were available that grew new chambers that could be analyzed for Cu-concentration (Fig. 5).

## 4 Discussion

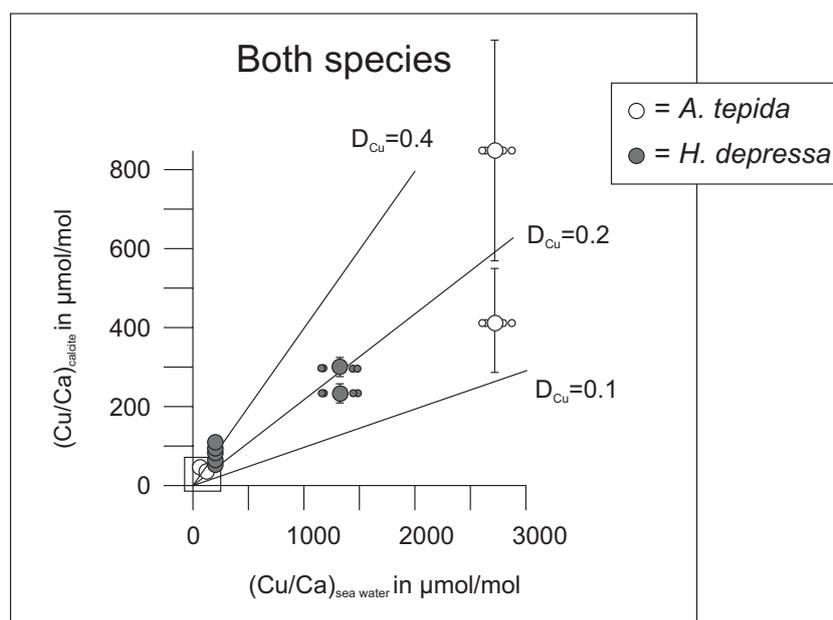
Within the experimental and analytical error both species show a similar (Cu/Ca)<sub>seawater</sub> to (Cu/Ca)<sub>calcite</sub> relation, indicating a partition coefficient ( $D_{\text{Cu}}$ ) of  $0.25 \pm 0.15$ . No significant difference was observed in copper incorporation between *Ammonia tepida* and *Heterostegina depressa*, despite large differences in ecology and habitat. Some interspecimen variation in Cu/Ca<sub>calcite</sub> was observed in *H. depressa* grown at low Cu-concentrations, in which rather large uncertainties in culture water Cu-concentration (Fig. 5) resulted from changes in [Cu<sup>2+</sup>] over time. Moreover, the alternative calibration method used for the thin-walled chambers of *A. tepida* (i.e. using a pressed calcite pellet and a lower ablation energy) increased the analytical uncertainty in the laser ablation-ICP-MS analyses (Fig. 5). Despite these errors, the calculated  $D_{\text{Cu}}$  was not significantly dependent on either temperature or salinity.

### 4.1 Experimental uncertainties

The seawater Cu-concentrations in most culture media increased during the incubation period (Table 1), however,



**Fig. 4.** Cu/Ca ratios in *Ammonia tepida* test carbonate versus Cu/Ca in sea water. Left graph is an enlargement of right one: small circles indicate the separate  $[\text{Cu}]_{\text{seawater}}$  measurements (replicate samples before and after the incubation period), large circles represent the average Cu/Ca<sub>seawater</sub> values. The two measurements in the right panel are from chambers of the same specimen. Lines represent partition coefficients of 0.1, 0.2 and 0.4.



**Fig. 5.** Plot of Cu/Ca ratios in foraminiferal calcite of the added chamber versus the Cu/Ca of the sea water in which they were incubated. The two *Heterostegina*-measurements in the center of the graph represent two chambers of the same specimen. Lines indicate partition coefficients of 0.1, 0.2 and 0.4.

there is no systematical difference in Cu/Ca<sub>seawater</sub> ratios before and after the two experiments. Therefore, analytical errors during ICP-MS or during subsampling of the culture vessels are unlikely to play an important role in this offset. Sorption of Cu onto organic matter in the culture media or onto the calcite of the foraminifera would have low-

ered the Cu-concentrations in the media, which happened in some cases, and therefore could have contributed to the differences in Cu/Ca ratios. Alternatively, release of Cu by the culture vessels or trays could have increased seawater Cu-concentrations, although this is unlikely since the equipment used was new or cleaned prior to the experiment. Moreover,

the control media (no Cu added) did not contain considerable amounts of Cu, indicating that contamination by the materials used played a minor role. The foraminifera themselves are unlikely to have contained high concentrations of Cu at the start of the experiments since concentrations of this metal are low in natural seawater. Cu-pollution of seawater during subsampling for seawater Cu-analyses may have caused an increase in Cu-concentrations, although care was taken to avoid such contamination. Differences in seawater Cu-concentrations before and after the incubation period are likely to be the result of a combination of the processes mentioned above.

The use of calcein in our experiments may have affected the uptake of Cu, although it is suggested that the incorporation of trace elements do not seem to be affected by the presence of calcein (Hintz et al., 2004). Measured Mg/Ca ratios in chambers that incorporated calcein were of the same order of magnitude as pre-existing chambers of the same specimens (data not published). This too suggests that the presence of calcein does not influence the uptake of trace elements.

The number of new chambers formed by the cultured foraminifera was generally low, especially at high Cu-concentrations (Table 4). The limited production of new calcite may indicate that the conditions during the experiments were not optimal for the foraminifera. The accumulation of waste products in the culture vessels, for example, was not monitored and may have changed the seawater chemistry. Increasing the seawater reservoir size may reduce the effect of such accumulations (see Hintz et al., 2004, 2006a, b). In addition, the micro-environments in which the specimens were kept and calcified, may have differed from natural conditions in other ways. The environmental stability in our set-up is not found in intertidal flats, nor was it possible for specimens of *Ammonia tepida* in these experiments to occupy dysoxic sediment layers, as regularly observed in field studies. Therefore, the results obtained in these experiments, may not be applicable for all environments in which the two species potentially calcify. Additional experiments, including anoxia, different pH's, different light/dark regimes may thus be necessary to investigate the full array of environmental conditions under which foraminiferal calcite may be produced. Improving culturing setups and optimizing laboratory conditions is important and comparison of our results with those from optimized, future experiments is necessary to establish a more precise  $D_{Cu}$  for foraminiferal calcite.

#### 4.2 Cu in the calcite lattice

Crystalline  $CuCO_3$  does not exist, because the most common coordination of Cu-carbonate complexes is distorted tetragonal pyramids or distorted octahedrons (Wells, 1984). These shapes do not allow precipitation of pure  $CuCO_3$  crystals and rather  $Cu_2CO_3(OH)_2$  (malachite) will form. However, sorption studies have shown that at the calcite-water interface

these so-called Jahn-Teller distortions can be overcome and a solid solution  $Cu_xCa_{(1-x)}CO_3$  forms (Schlosseler et al., 1999). It has been proposed that copper in calcite is present in clusters, based on studying the transformation of vaterite to calcite (Nassrallah-Aboukais et al., 1996, 1998). Recent XAFS work, however, has shown that this mechanism is not applicable to calcite surfaces (Elzinga and Reeder, 2002). This rather unusual complexation behavior would suggest that sorption and subsequent incorporation into the crystal lattice for copper is limited to part of the crystal surface only. This, in turn, would result in a lower partition coefficient for copper than expected based on its ionic radius only.

Contrary to these results, it has been shown that during inorganic coprecipitation experiments Cu is incorporated in calcite with a distribution coefficient ( $K_{Cu}$ ) of 23 and constant under a range of Cu-concentrations (Kitano et al., 1980). In the initial stage of calcification, the  $K_{Cu}$  can be even higher (40) probably due to the strong affinity of  $Cu(OH)_2$  for calcite surfaces (Franklin and Morse, 1982; Pickering, 1983; Papadopoulos and Rowell, 1989). This indicates that the Jahn-Teller distortions are easily overcome during calcification.

Generally, divalent cations with an ionic radius close to Ca (=1.0 Å) have a partition coefficient in calcite close to 1. Cd has an ionic radius of 0.95 Å (Shannon, 1976) and is incorporated in both planktonic and benthic species with a D between 1 and 4 (Boyle, 1981, 1988; Havach et al., 2001; Maréchal-Abram et al., 2004), independent of temperature (Marchitto, 2004). Sr (ionic radius = 1.31 Å) is incorporated in foraminiferal calcite with a D of 0.11–0.19, measured in several planktonic genera (Bender et al., 1975) and 0.05–0.25 in *Cibicidoides* (Elderfield et al., 1996). Coretop studies on *Cibicides* and *Uvigerina* show that Ba (1.47 Å) is incorporated with a partition coefficient of 0.3–0.4 at 3°C (Lea and Boyle, 1989). In the planktonic genera *Globorotalia* and *Globoquadrina*, Ba is incorporated with a D of 0.19 (Lea and Boyle, 1991). Cu has an ionic radius close to Mg (0.73 and 0.72 Å, respectively), but the partition coefficient of Mg is much lower ( $0.1-1 \times 10^{-3}$ ; Bender et al., 1975; Delaney et al., 1985) than the measured 0.1–0.4 for Cu (Fig. 7). The large difference between the foraminifer-mediated Cu-incorporation and the inorganic incorporation of Cu in calcite indicates that much energy is spent on removal of Cu at the site of calcification.

#### 4.3 Biological control on $D_{Cu}$

Since magnesium inhibits calcite growth (Berner, 1975; Mucci and Morse, 1983) and high levels of Mg are likely to be present in foraminiferal calcifying reservoirs, it is necessary for foraminifera to remove Mg before calcification. It has been suggested that foraminifera actively pump Mg from their calcifying reservoir in order to stimulate  $CaCO_3$  precipitation (Duckworth, 1977; Bentov and Erez, 2006). Usually, Cu is present only in very low concentrations in seawater

and therefore no need exists to actively remove Cu from calcifying reservoirs, despite its ability to modify the crystalline structure of calcite. Although under high Cu-concentrations it may be beneficial to remove Cu from calcifying reservoirs, apparently the foraminifera does not do so, as the  $D_{Cu}$  is similar for high and low Cu-concentrations. Alternatively, the concentrations used in our experiments may still be too low to seriously impede  $CaCO_3$  precipitation.

Another reason for active removal of trace elements from calcifying reservoirs is that these elements are necessary for cellular processes. Since Cu is known to play only minor roles in eukaryotic metabolic processes (Bruland et al., 1991; Sunda and Huntsman, 1995; Chang and Reinfelder, 2000), it is unlikely that the  $D_{Cu}$  is affected by cellular needs. Organic compounds may increase Mg contents in foraminiferal calcite (Bentov and Erez, 2006). High concentrations of Mg at the primary organic membrane (Hemleben et al., 1986) may explain the observed intra-test variability of Mg/Ca (e.g. Toyofuku and Kitazato, 2005). Cu also has a strong affinity for organic compounds (see below), so that the  $D_{Cu}$  may be partly determined by the presence of organic compounds in the calcite.

Bresler and Yanko (1995) showed that some benthic, epiphytic foraminifera have tryptofan-containing proteins that can bind  $Cu^{2+}$  and prevent intracellular Cu-concentrations from becoming harmful. When a significant part of the  $Cu^{2+}$  would have been immobilized by these Cu-binding proteins this would also have lowered the Cu activity in the solution and thus  $D_{Cu}$ . Since we have not observed such a decrease, it is unlikely that such molecules play a major role in decreasing intracellular Cu-concentrations.

Seawater pH is a potentially important modulator of trace metal uptake (Lea et al., 1999; Zeebe and Sanyal, 2002). To investigate the potential effect we compared species with and without symbionts. In the symbiont-bearing *H. depressa* the photosynthetic activity of the symbionts changes the local carbonate chemistry because  $CO_2$  is taken up and pH lowered during light conditions. However, the lack of any systematic offset in Cu/Ca between the *H. depressa* and *A. tepida* suggests no significant effect of pH on Cu incorporation. It may be, however, that difference in pH at the site of calcification between and within the species is partly responsible for the observed variation in  $D_{Cu}$ .

#### 4.4 Test deformation and mortality

A number of studies over the last 20 years have attempted to correlate the number of deformed tests to environmental pollution (Alve, 1991; Elberling et al., 2003; Armynot du Châtelet et al., 2004). The empirical correlation between number of deformed tests and for instance heavy metal or hydrocarbon concentration levels was interpreted to signify a causal relationship. Results from such investigations are difficult to apply widely, since deformations in foraminifera take place easily in some groups (e.g. Mil-

liolids and *Discorinopsis*), while in other taxa (e.g. within the family Glaberatellidae) deformities are rarely found (Arnold, 1954). Furthermore, in foraminiferal cultures that were exposed to oil pollution no increased test abnormalities were observed (Ernst et al., 2006). In addition, not a single deformed chamber alignment was observed in our experiments, despite the fact that Cu-concentrations were occasionally well above levels found at even the most polluted sites. Although the limited number of observations does not allow a statistical evaluation, our results strongly suggest that high levels of Cu do not cause test deformities. This is also shown by Alve and Olgard (1999), who found no test deformities in foraminifera living in seawater with high Cu-concentrations.

Therefore, we think that relative abundances of deformed tests in fossil samples are not suitable to reconstruct past copper concentrations. Most likely other environmental factors, co-varying with environmental trace metal levels must have been responsible for the observed increase in test deformities. The complete absence of deformations in our experiments is in high contrast to the low but still detectable levels of natural occurring test deformities under environmental pristine conditions. This suggests that the protected environment of the culture trays may actually have shielded our foraminifera by providing them with a constant temperature, salinity and seawater chemistry in general.

In seawater with the highest concentration of Cu ( $20 \mu\text{mol/l}$ ), none of the *Heterostegina's* survived and only one specimen grew new chambers when cultured at  $10 \mu\text{mol/l}$ . Since the growth or survival of *Ammonia tepida* did not appear to be hampered by high concentrations of Cu, we hypothesize that either the symbionts of the tropical foraminifera are vulnerable to high Cu-concentrations (Brandt et al., 1986), or that individuals of *A. tepida* are adapted to cope with (occasional) high levels of heavy metals.

#### 4.5 Application of Cu/Ca ratios in foraminiferal calcite

In order to quantify pollution levels, heavy metal concentrations are often analyzed using strong acid extractions and subsequent ICP-MS analyses of bulk sediment. Since heavily polluted sites are frequently characterized by high concentrations of (labile) organic matter, polluted sediments are often anoxic with high levels of sulphate reduction and associated production of free sulfide. These high sulfide-levels result in immobilization of heavy metals such as Zn, Cu, Cd and Pb, which are precipitated as the highly insoluble minerals  $PbS$ ,  $CuS$  and  $ZnS$ , or as co-precipitates in pyrite (Rashid and Leonard, 1973; Saxby, 1973; Huerta-Diaz and Morse, 1992). Because these metals are no longer bioavailable they do not reflect toxicity of the overlying water to, for instance, benthic biota. Actual analyses of the overlying water itself or organisms living in these waters would give a much more applicable concentration to assess pollution (Nelson and Donkin, 1985; Bryan and Langston, 1992). This

becomes even more important when at a later stage organic loads decrease and/or the oxygen level increases, (e.g. after improved wastewater treatment). Under these conditions lower sedimentary trace metal levels could result in higher actual toxicity as these metals are remobilized by progressive re-oxidation of the sediment and escape to the overlying water (Petersen et al., 1997). Monitoring foraminiferal test Cu/Ca ratios could be used to establish the bioavailable fraction of Cu and potentially also could record relatively short episodes with elevated bottom water Cu-levels.

Sludge dump sites and associated elevated concentration levels of heavy metals are mostly limited to coastal and estuarine environments. These settings experience considerably varying seasonal and even daily temperatures and salinity levels. A significant impact of either temperature or salinity on partition coefficients would, therefore, render foraminiferal trace metal records useless for any reliable reconstruction and/or monitoring of such dump sites. Since the obtained  $D_{Cu}$  is not markedly dependent on either temperature or salinity, foraminiferal Cu/Ca ratios may be a powerful proxy for the quantitative reconstruction of past heavy metal pollution, even in highly variable environments.

## 5 Conclusions

Copper is incorporated into foraminiferal calcite with an estimated partition coefficient of  $0.25 \pm 0.15$  with respect to seawater Cu/Ca values. No effects on the  $D_{Cu}$  of species-specific control or of temperature and salinity could be observed with the experimental setup used here. Additional experiments are needed to better constrain  $D_{Cu}$  and unravel the effects of other likely important environmental factors such as temperature, salinity and seawater carbonate chemistry.

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