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Stable oxygen isotopes of *Thoracosphaera heimii* (Dinophyceae) in relationship to temperature; a culture experiment

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Abstract

To establish a relationship between temperature and the stable oxygen isotopic composition ($\delta^{18}O$) of vegetative cysts of the photosynthetic calcareous dinoflagellate cyst *Thoracosphaera heimii*, two unicellular cultures of *T. heimii* have been cultured under different temperatures by using a temperature gradient box.

There is a clear relationship between temperature variance and the isotopic composition of *T. heimii* cysts according to the relationship: T (°C)=−6.827 ($\delta^{18}O_c$–$\delta^{18}O_w$)–3.906 (R=0.921), with c = calcite and w = water.

Within this paper we are the first to discuss the possible vital effects that might cause an offset between the temperature–isotope relationship found for *T. heimii* calcite and that of equilibrium inorganic calcite precipitation. No indication for strong kinetic effects as result of fast calcite precipitation can be found. We observed a positive relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ and ambient mediumwater pH. We speculate that this might be the result of the presence of external carbonate anhydrase, which is common in photosynthetic dinoflagellates. The efficiency of this enzyme increases rapidly between pH 7.5 to 9, which could result in an increase in CO_2 uptake relative to HCO_3^- with increasing pH. We furthermore discuss the possibility of *T. heimii* using respirative carbon at least as part of its carbon source for calcite precipitation, which can be based on the light values of $\delta^{18}O_c$–$\delta^{18}O_w$ and $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ (DIC = dissolved inorganic carbon) found in this and previous studies on the isotopic composition of calcareous dinoflagellates.

The results of this study as well as the broad geographic distribution of *T. heimii*, its stable position within the water column, its presence in the geological record since the Late Cretaceous and its resistance against dissolution compared to other plankton groups underlines the potential for a wide usability of the oxygen isotope composition of *T. heimii* as palaeotemperature proxy for the deeper parts of the photic zone.

Keywords: oxygen isotopes; calcareous dinoflagellate cysts; temperature; culture

1. Introduction

The isotopic composition and elemental chemistry of calcareous microfossils form often the backbone of palaeoceanographic and palaeoclimatic studies. Foraminifera have been most widely used as a result of their

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abundance in the sediments and the ease in which monospecific samples can be isolated from the sediment. However, biological factors such as the migration of several planktonic species through different water masses, the influence of calcite shell isotopic composition by photosynthetic activity of symbionts and consumption of other organisms, the species specific seasonality of shell production combined with calcification at different water depths and the ontogeny of individual species can complicate the interpretation of these signals (see overview in e.g. Bemis et al., 1998; King and Howard, 2003). The study of the stable oxygen isotope signal of photosynthetic species that do not contain symbionts and that do form their calcite walls at a stable position within the water column, might overcome some of these problems. Recent studies for the use of primary producers such as coccolithophores give promising results despite the difficulties in extracting a monospecific assemblage from sediments due to their small sizes (Stoll et al., 2001, 2002; Stoll and Ziveri, 2002). This latter is a requirement for obtaining a clear isotopic signal since individual coccolithophorid species have large differences in so called "non-equilibrium" effects (Ziveri et al., 2003). The photosynthetic dinoflagellate cyst *T. heimii* (in former days thought to be a coccolithophore) can relatively easily be isolated from sediments. Calculated temperatures based on the palaeotemperature equation for inorganic calcite precipitation generally reflect mean annual temperatures at thermocline (Deep Chlorophyll Maximum) depths, which represent its preferred depth habitat (Zonneveld, 2004). However, to date only extremely little information is available about the relationship between the isotopic composition of its shell and temperature of the surface waters (Dudley et al., 1986; Friedrich and Meier, 2003). To enhance this information we have grown *T. heimii* under different temperatures at stable salinity conditions using a temperature gradient box. We compared the stable oxygen isotope composition to cyst production rates, pH and the stable carbon isotope composition of the shells to estimate the possible effects of population growth and metabolic and kinetic processes on the oxygen isotopic composition of the shell walls. This paper forms the basis of future studies on the cause and usability in palaeo-environmental studies of the vital effects that influence calcite precipitation in *T. heimii*.

2. Material and methods

Thoracosphaera heimii shells have been isolated from ocean surface water from the equatorial Atlantic (strain GeoB 92) and Eastern Mediterranean (strain GeoB 116) at 3°43.9′N – 42°45.4′W and 34°18.1′N – 19°53.9′E during Meteor cruises M38/2 and M40/4 respectively. Unicellular cultures have been established in 24-chambers microwells (Corning Inc., Corning, NY, USA; Costar 3524) containing ~1.0 mL of culture medium (K medium–Si). The basis of the medium consist of seawater collected at Station 227 at 0°00.00′N and 6°34.96′W with the RV Meteor during cruise M58-3. Salinity of this water is 37.1 psu. Microwells were incubated at 18 °C and a 12:12 h light and dark cycle. Light in the incubator was provided by cool white fluorescent tubes. The microwells were examined for germinating cysts once a week. Since survival of dinoflagellate motile cells in culture is strongly influenced by the volume of medium (Wall et al., 1967), the cells were transferred to a 6-chambers microwell (Corning Inc., Corning, NY, USA; Costar 3516) containing 50 mL medium when an individual microwell contains approximately 15 motile cells. When the culture was growing well, cells were transferred to 250 mL Erlenmayer flask containing 100 mL of medium to form the basis material for further experiments. To study the possible effects related to genetic differences between strains obtained from different cysts we have compared two strains for our experiments (GeoB 92 and GeoB 116).

To study cysts produced at different temperature regimes a Temperature Gradient Box (TGB) was used. The details of the equipment construction and parameters are given in (Karwath et al., 2000a). Based on experiments of Karwath (2000) the tube volume, medium type and experiment duration were chosen such that nutrients would not become limiting and cultures remain in the exponential growth phase during the experiment. Karwath (2000) showed that when cells are grown at 100 μE at 22 °C cutures are growing in an exponential phase between 14 and 50 days with about 0.2 div/day. In a complementary experiment Karwath et al. (2000a) showed that at different temperature ranges between 14 °C and 25.1 °C exponential growth rates lasted until about 80 days (14 °C and 16 °C) and 40–50 days (19 °C–25.1 °C) in f/2 and f/20 culture media. At 27 °C the exponential growth stopped after 25 days. However, a positive correlation between exponential growth rate duration and nutrient concentration of the culture medium is observed indicating that when the culture volume was enlarged and culture media contained higher nutrient and element concentrations the duration of exponential growth would be enhanced. This is confirmed by several growth experiments in our culturing facilities (Kirsch internal reports).

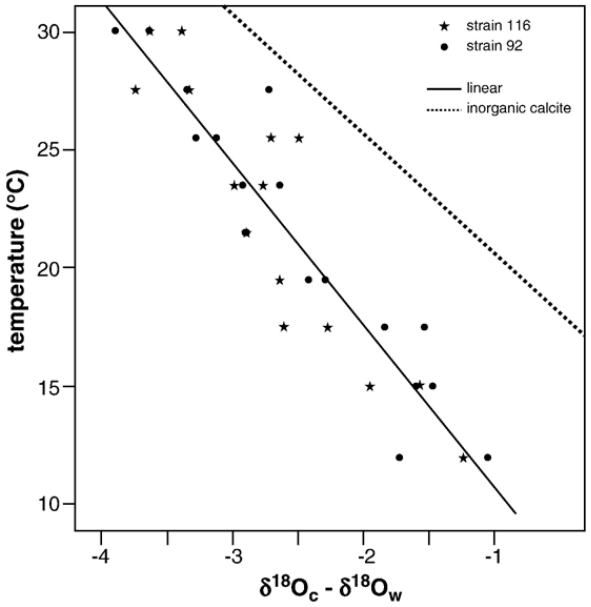

Fig. 1. Relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ of two stains of *Thoracosphaera heimii*, inorganic calcite and temperature. Inorganic calcite values are standardized for pH 8 (black stars and dots) according to Zeebe (2001) and uncorrected for standard pH (gray stars and dots).

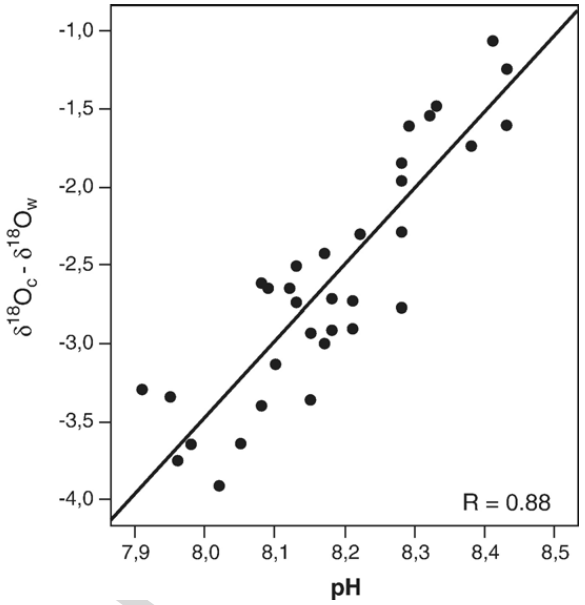

Fig. 2. Relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ of two stains of *Thoracosphaera heimii* and pH of the culture medium.

After 14 days of growth in the 250 mL Erlenmeyer flasks, the culture was homogenised and 1 mL of culture of the stains GeoB 92 and GeoB 116 were transferred to the glas tubes. The culture tubes contained 40 mL of K35 medium –Si (37.1 psu). The initial cell concentration in the culture tubes was 2000 cells/L. Successively duplo's of each strain were grown for 28 days at 12 °C, 15 °C, 17.5 °C, 19.5 °C, 21.5 °C, 23.5 °C, 25.5 °C, 27.5 °C, 30 °C and 32 °C. Culture media, volume and experiment duration were chosen such that the cultures were still in the exponential growth rate at the end of the experiment.

Table 1
Regression table of the factors compared. *d.f.* regr. = degrees of freedom of regression, *d.f.* res. = degrees of freedom of residual

Regression variables	Model	*R*	*R* square	*d.f.* regre	*d.f.* resi	*F*
$\delta^{18}O_c$–$\delta^{18}O_w$–temperature	Linear	−0.921	0.849	1	31	174.1
$\delta^{18}O_c$–$\delta^{18}O_w$–pH	Linear	0.888	0.789	1	31	116.02
$\delta^{18}O_c$–$\delta^{18}O_w$–$\delta^{13}O_c$–$\delta^{13}O_w$	Linear	0.531	0.282	1	31	11.81
$\delta^{18}O_c$–$\delta^{18}O_w$–final yield	Linear	0.26	0.068	1	31	2.25
$\delta^{13}O_c$–$\delta^{13}O_w$–final yield	Linear	0.118	0.014	1	31	0.42
$\delta^{13}O_c$–$\delta^{13}O_w$–temperature	Linear	0.499	0.249	1	31	9.94
$\delta^{13}O_c$–$\delta^{13}O_w$–pH	Linear	−0.639	0.409	1	31	20.75
Final yield–pH	Linear	0.142	0.021	1	31	0.67
$\delta^{13}C_w$–pH	Linear	0.761	0.578	1	31	41.1

Salinity was kept constant by sealing of the culture tubes with parafilm, thus preventing evaporation. Culture tubes that showed ruptures in the parafilm at the end of the experiments and where salinity increased to values between 38.0 and 38.8 have not been included in the equations given below. Sealing with parafilm can inhibit CO_2 exchange with the atmosphere. CO_2 did not became depleted during the experiment as is shown by the presence of enough DIC in the culture medium at the end of the experiments to allow carbon isotopic measurements. Furthermore the stable carbon isotope ratio *T. heimii* shells were extremely light. In case CO_2 becomes depleted heavy stable carbon isotope values of *T. heimii* shells would be expected.

Stable oxygen and carbon isotope ratio's have been compared to pH of the seawater at the end of the experiment and to the final cyst yield as expressed by the total weight of *T. heimii* cysts at the end of the experiment. Since *T. heimii* shells are very small (10 μm–20 μm), their size is not known to vary with temperature (Karwath, 2000) with cyst wall thickness of 0.8–1 μm, and the total weight of cysts can be used as a qualitative estimate for the final cyst yield.

The stable isotopic composition of *T. heimii* cysts was determined with a Finnigan MAT 251 isotope ratio gas mass spectrometer directly coupled to an automated carbonate preparation device (Kiel II) and calibrated via NIST 19 international standard to the VPDB (Vienna Pee Dee Belemnite) scale. We used about 0.001 g dry weight of specimens with cell content per single measurement. Stable isotope values are given in δ-notation versus

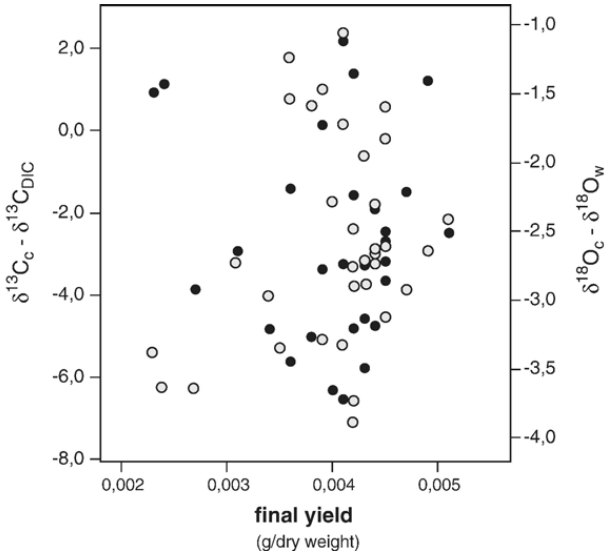

Fig. 3. Relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ (black dots) and $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ (gray dots) of two stains of *Thoracosphaera heimii* and final yield.

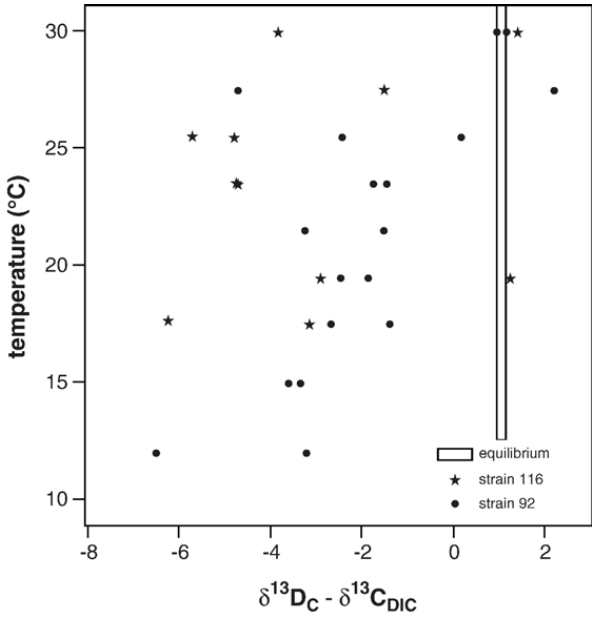

Fig. 5. Relationship between $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ of two stains of *Thoracosphaera heimii*, inorganic calcite and temperature. Equilibrium ^{13}C values of inorganic calcite are according to Romanek et al. (1992).

VPDB. The precision of the measurements at 1σ based on repeated analyses of an internal laboratory standard (Solnhofen limestone) over a one-year period was better than ±0.08 and ±0.06‰ for oxygen and carbon isotopes, respectively. Equilibrium $\delta^{13}C$ values of inorganic calcite are according to Romanek et al. (1992).

Dissolved inorganic carbon (DIC) was extracted from seawater with phosphoric acid in an automatic preparation line (Finnegan Gasbench I) coupled online with a Finnigan MAT 252 mass spectrometer to determine its 13C/12C ratio. All samples were run at least in duplicate. Results are reported in δ-notation relative to the VPDB-scale with an external reproducibility of ±0.1 ‰ at 2σ.

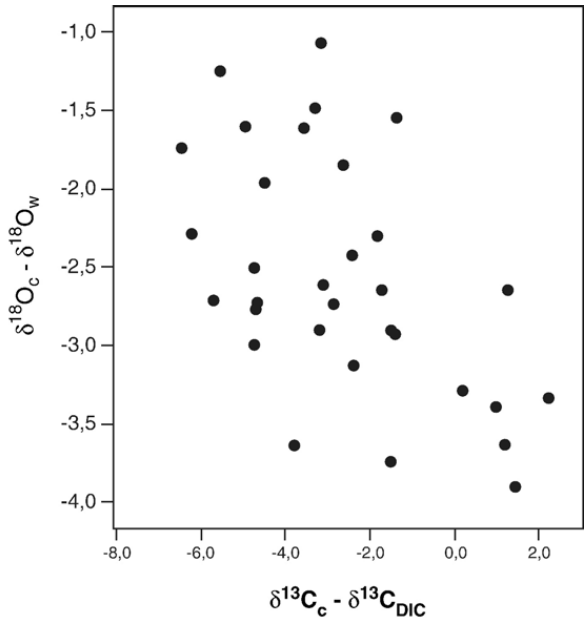

Fig. 4. Relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ and $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ of two stains of *Thoracosphaera heimii*.

For the oxygen isotope determination of water, 7 mL of water were equilibrated in 13 mL headspace with CO_2 gas using an automated Finnigan equilibration device, online connected with a Finnigan MAT Delta-S mass spectrometer. At least two replicates (including preparation and measurement) were run for each oxygen isotope determination. Results are reported in δ-notation relative to the VSMOW scale with an external reproducibility of ±0.03 ‰ at 1σ.

Results have been compared to the precipitation of inorganic calcite according to the palaeotemperature equation for inorganic calcite of Kim and O'Neil (1997) converted from $10^3 \ln\alpha$ notation to quadratic approximation by Bemis et al. (1998). Since we have grown our dinoflagellate at a pH 7.9 (beginning of experiment) to 7.9–8.4 (end of the experiment) which correspond to pH values that assure optimal growth conditions for *T. heimii* cultures (Karwath, 2000, Kirsch personal communication), we adjusted the equation for inorganic calcite for pH 8.0 according to (Zeebe, 2001). Conversion of VSMOW to VPDB is according to Hut (1987): δ_w (VPDB)= 0.99973 $\delta^{18}O_w$ (VSMOW) − 0.27. Relationships have been calculated using the statistical software package SPSS 12.

3. Results

Both strains of *T. heimii* that originate from the equatorial Atlantic Ocean and eastern Mediterranean, were growing between 12 °C and 30 °C. Cultures did not produce cell amounts high enough to enable isotopic measurements at 32 °C. The two studied strains showed a similar relationship between temperature and the $\delta^{18}O$ and $\delta^{13}C$ signals of their cysts (Fig. 1).

Significant relationships between the stable oxygen isotope signal of *T. heimii* and temperature can be observed (Fig. 1). The relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ and temperature can be described as linear at pH 8.0 according to the equation (Fig. 1, Table 1)

$$T(°C) = -6.827(\delta^{18}O_c - \delta^{18}O_w) - 3.906 \quad R = 0.921$$

with c = calcite and w = water.

The $\delta^{18}O$ values are lighter than that of the equilibrium calcite, precipitated at low temperatures (Kim and O'Neil, 1997) adapted for pH 8.0 according to the relationship given by (Zeebe, 2001) (Fig. 1).

The PH did not remain completely constant during the experiments and varied from pH 7.91 at the beginning of the experiment to 7.91–8.43 at the end of the experiment. A positive linear relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ and pH can be found. No relationship between pH and temperature changes can be observed (Fig. 2, Table 1).

No significant relationship can be observed between stable oxygen and carbon isotopes and total cyst weight at the end of the experiment (Fig. 3, Table 1). Furthermore, no significant relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ and $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ can be observed (Fig. 4, Table 1). The temperature gradient does not show a significant relationship with variation within $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ (Fig. 5, Table 1). pH and $^{13}C_c$–$\delta^{13}C_{DIC}$ have a weak negative relationship (Fig. 6, Table 1).

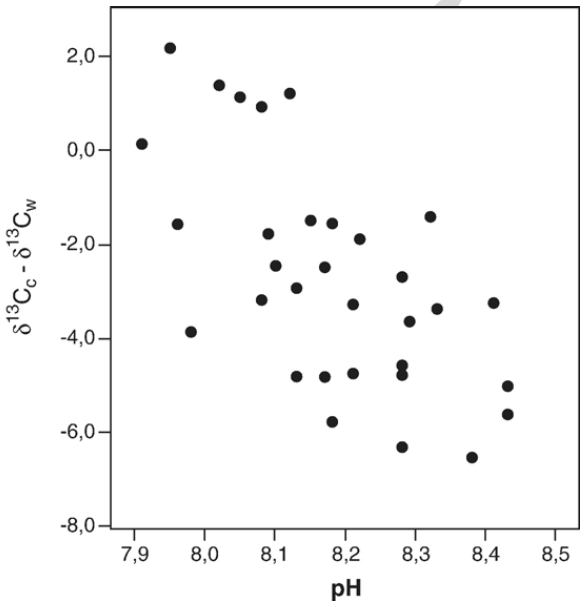

Fig. 6. Relationship between $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ of two stains of *Thoracosphaera heimii* and pH of the culture medium.

4. Discussion

The present results show that there is a clear relationship between the oxygen isotopic composition of *T. heimii* cysts and temperature. Both strains showed similar relationships indicating that interspecific differences did not affect the results of this study. The oxygen isotopic ratio's of *T. heimii* are lower (isotopically "lighter") than the equilibrium temperature relation predicted for inorganic calcite (Fig. 1). It differs slightly from the relationship found for *T. heimii* by Dudley et al. (1980). At the time this latter publication appeared, *T. heimii* was still thought to be a coccolithophore rather than being a dinoflagellate. Dudley et al. combined the isotopic values of two coccolithophorid species *Calcidiscus leptoporus* and *Cricosphaera cartera*, with those of *T. heimii* as result of the low amount of data. The two original isotopic values of *T. heimii* given in their paper correspond, to the values found in the present study. Consequently we assume that the slight discrepancy of our findings with the study of Dudley et al. results from this compilation of data and might be subscribed to the different relationships between oxygen isotope ratio's of *C. leptoporus* and *C. cartera* compared to that of *T. heimii*. Further support that the light values found in our study are not an "artefact" of our experiment, comes from a study in the early nineties of the last century by Paull and Balch (1994). They compared the oxygen isotope ratios of the fine fraction of particulate carbonate with that of the ambient seawater during a coccolith–dinoflagellate bloom in the Gulf of Maine. They found extreme negative values within three samples that were characterised by a dominance of *Thoracosphaera* cysts rather than by coccolithophores. Light isotopic values were also found in a study of the isotopic composition of *T. heimii* cysts from surface sediments from the equatorial and South Atlantic Ocean (Zonneveld, 2004). The observation that Late Cretaceous calcareous dinoflagellate cysts are also characterized by light values suggests that this might be a common characteristic of calcareous dinoflagellates (Friedrich and Meier, 2003).

The relationship found in this study falls within the range of equations known from coccolithophores that until now form the only calcite producing primary

producer group for which the relationship between oxygen isotopic composition and temperature has been investigated (Fig. 7, e.g. Dudley et al., 1980, 1986; Ziveri et al., 2003; Stoll and Ziveri, 2005). However, there are indications that dinoflagellates and coccoliths have completely different calcification mechanisms. Within coccolithophores, heterococcolith calcification takes place within a membrane delimited space, completely isolated from the cytosol, the coccolith vesicle (e.g. Young et al., 1999; Langer et al., 2006). The newly formed coccoliths are then transported to the cell surface. Thus seawater is separated from the site of calcification by at least two membranes. For calcareous cyst forming dinoflagellates the biomineralisation process is practically unknown (Inouye and Pienaar, 1982; Gao et al., 1989). According to Inouye and Pienaar (1982) calcification of *T. heimii* takes place between the outer two membranes that surrounds the cell. Between those membranes "newly formed crystals are irregularly grouped over the surface of the cells". The calcification process of a single cell takes one to three days. This suggests that the calcification site of *T. heimii* might be separated by only one membrane from ambient seawater. More detailed studies are required to confirm this.

The species specific difference in relationship between coccolith species is thought to be the result of species specific vital effects. Recent studies by Ziveri et al. (2003) and Stoll and Ziveri (2005) show that a large range of interspecific oxygen isotope and, related to that, carbon isotope vital effects that are present in coccolith species, can be correlated to differences in maximal growth rates between individual species. Within this study we observed no significant relationship between oxygen and carbon isotope ratio's and final yield of the dinoflagellate (Fig. 3., Table 1). This confirms our assumption of a different calcification process in coccolithophores and *T. heimii*.

There are three potential effects that are known to lead to the difference in isotopic composition of biogenic- and inorganic produced calcite; 1. kinetic fractionation during calcite precipitation, 2. pH dependent fractionation, and 3. fractionation of the intracellular carbon pool during photosynthesis.

1. If the precipitation of carbon is faster than the hydration and hydroxylation of carbon dioxide ($H_2O+CO_2 \rightarrow HCO_3^- + H^+ \rightarrow CO_3^{=} + 2H^+$), then the faster hydration of ^{12}C and ^{16}O bearing CO_2, results in co-varying depletions in both ^{18}O and ^{13}C of the carbonate compared to equilibrium (e.g. McConnaughey, 1989, 2003; Adkins et al., 2003). The most rapidly precipitated carbonate is isotopically lighter for both oxygen and carbon. In this case we would expect to find a linear relationship between $\delta^{18}O_c$–$\delta^{18}O_w$ and $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ in calcite of *T. heimii*. However, we do not observe such a relationship for *T. heimii* in this experiment (Fig. 4, Table 1). This is confirm earlier findings in natural environments where no relationship was observed between $\delta^{18}O_c$–$\delta^{18}O_w$ and $\delta^{13}C_c$–$\delta^{13}C_{DIC}$ in *T. heimii* cysts of surface sediments from the equatorial and South Atlantic Oceans (Zonneveld, 2004). We therefore assume that kinetic effects as described above do not influence the isotopic fractionation during the calcification process in *T. heimii*.
2. Since the culture experiments of living foraminifera (e.g. Spero et al., 1997) it has become clear that the seawater carbonate chemistry can largely affect the oxygen and carbon isotopic signal from biogenic carbonates, which calcify extracellularly. Different equilibrium fractionations among different carbonate species with respect to water result in different isotopic compositions of the precipitated carbonates, assumed that these carbonates are formed from a mixture of carbonate species in proportion to their relative contribution to the dissolved carbonate species $S=[H_2CO_3]+[HCO_3^-]+[CO_3^{=}]$ (Spero et al., 1997; Bijma et al., 1999; Zeebe, 1999, 2001). With increasing pH, the solution would consist of relatively higher concentrations of $CO_3^{=}$. Since HCO_3^- is isotopically heavier than $CO_3^{=}$, the oxygen and carbon isotope composition of the dissolved carbonate species decreases with increasing pH. Within our experiment pH of our culture medium did not remain completely constant. To our surprise we found an "opposite pH effect" with increasing $\delta^{18}O_c$–$\delta^{18}O_w$ values instead of the expected decreasing values at increasing pH (Fig. 2). Final yield did not show a relationship with pH.

Although no details are known about the process of the *T. heimii* biomineralisation, there is some information present about the uptake of inorganic carbon by other photosynthetic dinoflagellate species. Dinoflagellates are known to be unique among marine phytoplankton groups in their type of ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco). Rubisco catalyses the initial assimilation reaction of CO_2 during photosynthesis but is not optimal efficient since it is also sensitive for O_2, which accumulation initiates a photorespiration pathway. In contrast to other phytoplankton groups, dinoflagellates are known to have a type of Rubisco (type II) that is much more sensitive for oxygenation compared to the common available Rubisco type I.

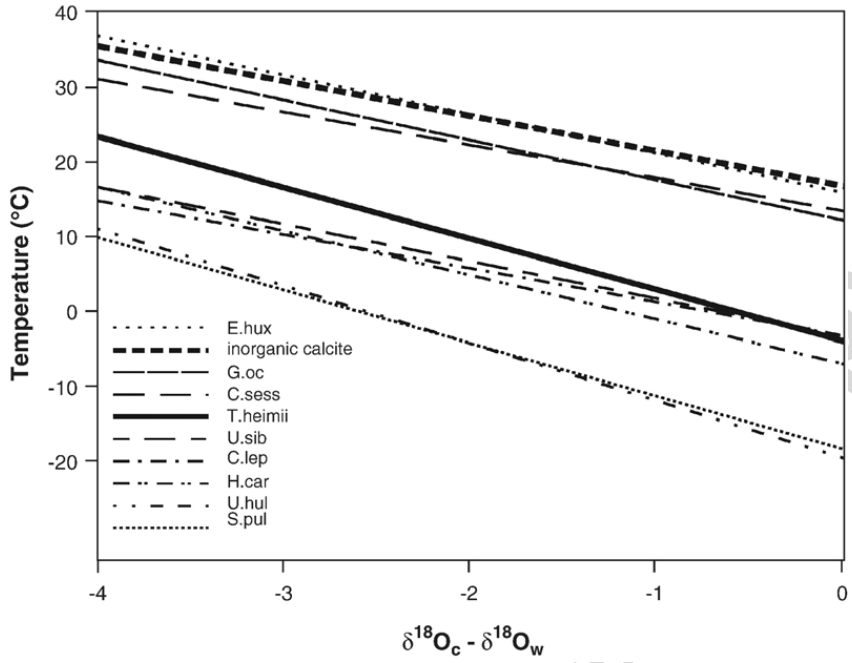

Fig. 7. Relationship between the isotopic composition of several coccolithophores, *T. heimii* and temperature (coccolithophore relationships according to Dudley et al., 1980, 1986; Ziveri et al., 2003; Stoll and Ziveri, 2005. E.hux = *Emiliania huxleyi*, C. sess = *Crenolithus sessilis*, C. lep = *Calcidiscus leptoporus*, H. Car = *Helicoshaera carteri*, S. Pul = *Syracosphaera pulchra*, U. Hul = *Umbilicosphaera hulburtiana*, U. Sib = *Umbilicosphaera sibogae*.

It is therefore important for dinoflagellates to accumulate CO_2 near the cell surface or internally at the sites where Rubisco is located, to minimise the photorespiration pathway. Laboratory experiments have shown that dinoflagellates can overcome this problem by accumulating inorganic carbon relative to the ambient concentrations with accumulation factors varying between a 5 and 70-fold increase, depending on the species (e.g. Giordano et al., 2005; Rost et al., 2006, and references therein). The mechanisms include active uptake of CO_2 or HCO_3^- or the presence of internal or external carbonic anhydrase (iCA and eCA respectively). CA's catalyse the dehydration of HCO_3^- ($HCO_3^- + H^+ \rightarrow H_2O + CO_2$), which results in a maintaining of the intercellular [CO_2] (iCA) or the [CO_2] near the cell surface (eCA).

From several studies it is known that some dinoflagellate species primarily and/or selectively take up CO_2 as inorganic carbon source (e.g. Nimer et al., 1999; Dason et al., 2004). Recently it has been shown, however, that in other species the uptake of HCO_3^- contributes to more than 80% of the photosynthetic carbon fixation in a few species (Rost et al., 2006). The affinity of HCO_3^- uptake even increases when pH increases. If this latter would be the case for *T. heimii* we would expect to find an extremely strong decrease of $\delta^{18}O_c$ in relationship to increasing pH values. Since this is not the case, we assume that HCO_3^- is not preferably taken up by *T. heimii*. We also assume however, that CO_2 is not the only carbonate species that is taken up by *T. heimii* (see following paragraph). The presence of internal carbon anhydrase (iCA) and external carbon anhydrase (eCA) has been demonstrated by several dinoflagellate species (e.g. Berman-Frank et al., 1995; Nimer et al., 1997, 1999; Dason et al., 2004). The type of CA and its efficiency, expressed by enzyme unit per cell (E.U./cell number) appears to be species specific. It has been shown that the CA efficiency increases from about 30 E.U./10^6 cells to 200 E.U./10^6 cells when pH increases from 7.25 to 9. At pH values higher than 9 (9–11) the efficiency of the enzyme decreased again to 150 E.U./10^6 cells (Berman-Frank et al., 1995; Nimer et al., 1999). This observation lead us to the suggestion of a possible mechanism to explain the "opposite pH effect" found in this study. If we assume that *T. heimii* uses eCA to accumulate CO_2 and when *T. heimii* takes up both CO_2 and HCO_3^- from ambient seawater, the large increase in efficiency of the enzyme with increasing pH would cause in a relative increase in [CO_2] on the cell surface rather than a decrease that is found in the ambient seawater. As a result, more CO_2 relative to HCO_3^- would be accumulated by the cell resulting in heavier oxygen isotopic values in the

internal *T. heimii* cell. If this mechanism takes place an "opposite pH effect" should occur only at intermediate pH levels between 7.5 and 9 when the enzyme shows its increase in efficiency (Berman-Frank et al., 1995). If *T. heimii* uses an internal carbon source rather than an external source (see next paragraph) only the internal pH at the calcification site would influence the isotopic composition of the *T. heimii* calcite.
Note that we suggest a pH change induced physiological "overreaction" resulting in an artificial concentration change in different carbonate species towards a dominance of CO_2 at the cell surface. The effect found in the isotopic concentration of *T. heimii* shells is thus disconnected from the "isotope effect" in the medium water. As result we can't compare the $\delta^{18}O_c$–$\delta^{18}O_w$ values of *T. heimii* with the oxygen isotope fractionation factors of carbonate species with respect to water in relations to medium pH as given in Zeebe (1999) since we don't know the cell internal pH at the calcification site.

3. If there is a significant exchange between the carbon pools used for photosynthesis and calcification, the strong kinetic isotope fractionation of carbon by Rubisco during photosynthesis could influence the carbon isotopic composition of *T. heimii* (McConnaughey et al., 1997). The respired CO_2 would be isotopically lighter than the CO_2 diffused through the cell membrane with respect to carbon. For *T. heimii* we find carbon values that are lighter than equilibrium (Fig. 3, Romanek et al., 1992). This might suggest that *T. heimii* uses at least partly, respired carbon as source for calcite precipitation. Negative carbon isotopic values compared to equilibrium have been observed in *T. heimii* cysts from equatorial and South Atlantic surface sediments (Zonneveld) whereas extreme negative values compared to equilibrium and surface-dwelling planktic foraminifera species are also found in two cretaceous calcareous dinoflagellate species (Friedrich and Meier, 2003). This suggests that the use of respiratory carbon for calcite precipitation could be a common feature in calcareous dinoflagellates. More detailed studies on the biomineralisation process of calcareous dinoflagellates are required to investigate if this is really the case. Since we do not find a significant change in carbon isotopes with changing temperatures, nor a significant relationship between changes in carbon and oxygen isotope composition, the observed relationship between oxygen isotope composition of *T. heimii* and temperature change, are probably not the result of different temperature related respiration rates.

5. Concluding remarks

This study documents a clear relationship between the oxygen isotope composition of *T. heimii* cysts and temperature that subscribes its potential to be a useful tool to reconstruct palaeotemperatures of the upper water column, notably the deep chlorophyll maximum (Karwath, 2000; Karwath et al., 2000b). Although there exist already a considerable amount of palaeotemperature proxies based on isotopic, elemental or biomarker composition, the usability of these proxies can be hampered by the production of their calcareous remains at different depths in the water column, ontogenic induced variability, sensitivity to (selective) dissolution and or geographically determined seasonal production (e.g. Spero and Lea, 1996; Grice et al., 1998; Fisher and Wefer, 1999; Stoll and Ziveri, 2005). Studies on the biology of *T. heimii* indicate that the immotile vegetative cyst stage forms the major stage within its life cycle whereas the motile phase exist only for a few minutes to a few hours (Inouye and Pienaar, 1982). This suggests that the ability of *T. heimii* to move vertically within the photic zone is rather limited and confirms observations that migration ability of dinoflagellates is limited to several meters only, as result of their small size (e.g. Anderson and Stolzenbach, 1985; Lieberman et al., 1994; Kamykowski et al., 1998).

In comparison to other calcareous fossil groups reflecting surface water conditions, calcareous dinoflagellates seem to be more robust against dissolution (Baumann et al., 2003; Vink, 2004). This together with the fact that *T. heimii* is known in the fossil record from the Late Cretaceous onward (Hildebrand-Habel and Willems, 2000; Streng et al., 2004) suggest that this proxy might be widely used in temperate to tropical regions.

Our experiment is the first that provides detailed information on the relationship between temperature and stable oxygen isotopes of *T. heimii* and the first that discusses the presence of vital effects affecting the isotopic composition of calcareous dinoflagellate cysts. During this study several fascinating aspects and questions came up that require future studies on the biomineralisation process of *T. heimii*. For instance, does *T. heimii* uses eCA and what is the relationship between its efficiency and changing pH values. What is the carbon source for calcite precipitation and where in the cell does the biomineralisation process takes place. Furthermore, information is required about the relationship between the carbon and oxygen stable isotopic concentrations of *T. heimii* shells are when pH

Table 2
Environmental and stable isotopic values (measured and calculated) that form the basis of the statistical analyses presented in this study

Culture	Temperature (°C)	Salinity (psu)	pH	Final yield (g)	$\delta^{13}C_{DIC}$ vs PDB	$\delta^{13}C_c$ vs PDB	$\delta^{13}C_c - \delta^{13}C_{DIC}$	$\delta^{18}O_w$ vs SMOW	$\delta^{18}O_c$ vs PDB	$\delta^{18}O_c - \delta^{18}O_w$	$\delta^{18}O_c - \delta^{18}O_w$ stand. pH
91-a	30	37.5	8.05	0.0024	−3.97	−2.82	1.15	1.78	−2.39	−3.71	−3.64
91-a	27.5	37.9	7.95	0.0041	−4.66	−2.47	2.19	1.58	−2.02	−3.27	−3.34
91-a	25.5	38.1	7.91	0.0039	−2.70	−2.54	0.16	1.52	−1.94	−3.16	−3.29
91-a	23.5	37.4	8.09	0.0044	1.47	−0.29	−1.76	1.38	−1.88	−2.77	−2.64
91-a	21.5	37.3	8.18	0.0042	1.30	−0.24	−1.54	1.37	−2.14	−3.17	−2.91
91-a	19.5	37.4	8.22	0.0044	1.83	−0.04	−1.87	1.35	−1.47	−2.61	−2.30
91-a	17.5	37.5	8.32	0.0036	1.46	0.05	−1.41	1.40	−0.86	−1.99	−1.54
91-a	15	37.5	8.33	0.0039	3.06	−0.30	−3.36	1.41	−0.82	−1.94	−1.48
91-a	12	37.4	8.41	0.0041	2.57	−0.65	−3.22	1.41	−0.53	−1.64	−1.06
91-b	30	38.5	8.02	0.0042	−4.45	−3.05	1.40	1.93	−2.46	−3.93	−3.90
91-b	27.5	37.8	8.21	0.0044	3.74	−0.99	−4.73	1.40	−1.79	−3.02	−2.73
91-b	25.5	37.7	8.1	0.0045	0.59	−1.85	−2.44	1.38	−2.13	−3.27	−3.13
91-b	23.5	37.9	8.15	0.0047	−0.10	−1.57	−1.47	1.38	−2.01	−3.14	−2.93
91-b	21.5	37.4	8.21	0.0043	2.70	−0.56	−3.26	1.34	−2.07	−3.20	−2.90
91-b	19.5	37.4	8.17	0.0051	1.22	−1.26	−2.48	1.42	−1.52	−2.66	−2.42
91-b	17.5	37.3	8.28	0.0045	2.34	−0.34	−2.68	1.36	−1.16	−2.24	−1.84
91-b	15	37.4	8.29	0.0045	3.16	−0.46	−3.62	1.38	−0.89	−2.02	−1.61
91-b	12	36.4	8.38	0.0041	5.55	−0.98	−6.53	1.37	−1.18	−2.27	−1.73
116-a	30	36.5	7.98	0.0027	1.75	−2.10	−3.85	1.31	−2.45	−3.62	−3.64
116-a	27.5	38.2	7.96	0.0042	−2.38	−3.94	−1.56	1.57	−2.41	−3.69	−3.75
116-a	25.5	37.1	8.13	0.0042	3.05	−1.76	−4.81	1.31	−1.65	−2.68	−2.50
116-a	23.5	37.2	8.17	0.0034	2.66	−2.15	−4.81	1.31	−2.43	−3.23	−2.99
116-a	19.5	37.6	8.13	0.0031	−0.67	−3.59	−2.92	1.47	−1.70	−2.92	−2.73
116-a	17.5	37.4	8.08	0.0045	0.26	−2.90	−3.16	1.38	−1.62	−2.72	−2.61
116-a	15	36.9	8.28	0.0043	3.29	−1.27	−4.56	1.39	−1.23	−2.35	−1.96
116-a	12	37	8.43	0.0036	4.33	−1.28	−5.61	1.36	−0.75	−1.85	−1.24
116-b	30	38.5	8.08	0.0023	−4.74	−3.80	0.94	1.41	−2.04	−3.51	−3.39
116-b	27.5	36.4	8.15	0.0035	–	−2.45	–	1.35	−2.50	−3.57	−3.36
116-b	25.5	37.2	8.18	0.0043	3.48	−2.29	−5.77	1.36	−1.91	−2.97	−2.71
116-b	23.5	37.2	8.28	0.0042	3.46	−1.31	−4.77	0.77	−2.37	−3.17	−2.77
116-b	19.5	37.5	8.12	0.0049	−3.80	−2.56	1.24	1.36	−1.64	−2.82	−2.65
116-b	17.5	37.3	8.28	0.004	4.88	−1.41	−6.29	1.33	−1.60	−2.68	−2.28
116-b	15	37.1	8.43	0.0038	3.93	−1.08	−5.01	1.40	−1.08	−2.20	−1.59

(alkalinity) remains constant over the course of the experiment. This study forms therefore the basis for further investigations on the "vital effect" on the isotopic composition of *T. heimii* shells and we would like to invite biologists and geologists to help us to unravel some of our questions (Table 2).

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