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1 **Title**

2 **Nitrogen isotopes in intra-crystal coralline aragonites**

3

4 **Authors**

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16

17 **Abstract** (192 words)

18 To assess the preservation of the nitrogen isotope composition in reef corals, nitrogen isotopes in a  
19 well-preserved Pliocene fossil coral (located in the Tartaro formation on Luzon Island, Philippines  
20 (14°N, 121°E)) and in a modern coral (Kochi, Japan (32°N, 132°E)) were analysed using stepwise  
21 heating methods. The thermal decomposition of aragonite triggered the largest release of nitrogen  
22 at 700 °C for the modern coral and 550 °C for the Pliocene coral. The highest rate of nitrogen gas  
23 emission occurred at the aragonite collapse temperature, indicating that organic nitrogen was  
24 bound within the intra-crystals of coralline aragonites in both corals. After the aragonite collapsed  
25 in both corals, the nitrogen isotope ratios increased due to fractionation and then decreased to  
26 values similar to those observed in bulk samples of the modern (+10.1%) and Pliocene (+4.4%)  
27 corals. These results suggested that fresh organic nitrogen was released due to the decomposition  
28 of the internal skeletal structure at higher temperatures (900-1000°C). Nitrogen isotopes in coral  
29 skeletons were preserved in intra-crystal aragonite, even in a Pliocene fossil, and stepwise heating  
30 methods were shown to be useful for determining the preservation of coralline nitrogen isotopes.

31 **KEYWORDS:** coral skeletons, nitrogen isotopes, Pliocene, stepwise heating methods

32

## 33 1. Introduction

34 The nutrient cycle in surface oceans regulates primary production, which influences the carbon  
35 dioxide concentration in the atmosphere. Global warming and cooling have occurred repeatedly  
36 over geological time scales and are often accompanied by nutrient concentration changes,  
37 especially in the polar and tropical oceans (Sigman and Boyle, 2000). Understanding nutrient  
38 circulation changes over geological time scales is useful for predicting future environmental  
39 changes. The nitrogen isotope proxy in coral skeletons ( $\delta^{15}\text{N}_{\text{coral}}$ ) may be applied to fossil corals  
40 for the reconstruction of palaeo-nitrogen cycles. Modern and fossil coral skeletons have been used  
41 as high-resolution recorders of surface ocean environments at low latitudes (e.g., Gagan et al.,  
42 2000, Correge, 2006, Watanabe et al., 2011). The modern  $\delta^{15}\text{N}_{\text{coral}}$  is a record of the  
43 high-resolution dynamics of nitrogenous nutrients in surface oceans (Yamazaki et al., 2011a, b).  
44 According to the fossil records, corals belonging to the Anthozoa class originated in the  
45 Palaeozoic era (Scrutton, 1997), and scleractinian corals appeared in the middle of the Triassic  
46 period (~237 Ma) (Stanley and Fautin, 2001). Muscatine et al. (2005) showed that the nitrogen  
47 isotope composition in Triassic coral skeletons is similar to that of modern symbiotic coral  
48 skeletons. The authors suggested that Triassic corals possessed symbiotic algae and preserved the  
49 nitrogen isotope composition in their intra-crystal skeletons. However, few studies have explored  
50 the application of nitrogen isotope proxy records in fossil corals over long periods of time because  
51 methods that can be used to confirm that organic nitrogen was preserved in intra-crystal coralline  
52 aragonites have not yet been developed, even in fossils. In the present study, the preservation of  
53 the nitrogen isotope composition in modern and fossil corals was examined using stepwise heating  
54 analysis. Stepwise heating methods have been used in the analysis of noble gases and nitrogen in  
55 rock samples (e.g., Reynolds et al., 1970; Sano and Pillinger, 1990). This technique has been

56 applied to nitrogen isotopes of modern coral by Uchida et al. (2008) to detect trace nitrogen in  
57 coral skeletons. We divided the heating process into seven steps (200°C to 1000°C) to determine  
58 the aragonite collapse temperature and the nitrogen isotope ratios preserved in extra- and  
59 intra-crystalline aragonite. We used well-preserved Pliocene fossil corals (3.5-3.8 Ma) collected  
60 from Luzon Island, Philippines, which is located in the western Pacific warm pool. In the middle  
61 of the Pliocene epoch, the annual mean temperature was 2-4°C warmer than that under  
62 preindustrial conditions (Haywood and Valdes, 2004, Haywood et al., 2009, Brierley et al., 2009).  
63 Pliocene coral specimens may contain nutrient circulation data in tropical warm pools under  
64 global-warming-like conditions.

65

## 66 **2. Methods**

### 67 **2.1. Modern coral specimen**

68 To decrypt the  $\delta^{15}\text{N}_{\text{coral}}$  pattern of the Pliocene coral, we compared it with modern  $\delta^{15}\text{N}_{\text{coral}}$  in a  
69 living *Porites* coral skeleton from Tatsukushi Bay, Kochi, Japan (32°N, 132°E). We measured  
70 modern coral skeletons using the chemical conversion methods described by Yamazaki et al.  
71 (2011a) to validate the bulk  $\delta^{15}\text{N}_{\text{coral}}$  values of modern coral skeletons. A rectangular coral  
72 skeleton (10×40×7 mm) containing 5 annual bands was cut from the coral colony and powdered  
73 using an agate mill to obtain a heterogeneous powder. To remove any organic materials attached  
74 to the outer crystals, the coral powder was placed in polypropylene tubes, soaked in NaOH (2 N)  
75 and placed inside a dry bath (60°C). Treatment times of 0, 0.5, 1, 2, 3, and 5 hours were tailored to  
76 capture intra-crystalline nitrogen. During the treatment process, NaOH and coral powder were  
77 mixed once every hour using the tube mixer. At each treatment step, the nitrogen isotope content  
78 was determined in 5 samples, and those that exhibited a poor recovery rate during the chemical

79 conversion process were excluded from further analysis. The  $\delta^{15}\text{N}_{\text{coral}}$  and total nitrogen content  
80 varied widely, especially when the samples were not cleaned (Fig. 1). After 5 hours of cleaning,  
81 the average  $\delta^{15}\text{N}_{\text{coral}}$  value in the modern coral was  $+9.1 \pm 1.5$  ( $2\sigma$ )%, and the average total nitrogen  
82 content was  $63 \pm 43$  ( $2\sigma$ ) ppm. The range ( $2\sigma$ ) of  $\delta^{15}\text{N}_{\text{coral}}$  and the total nitrogen values were  
83 generally in accordance after 1 hour and in subsequent steps. The heterogeneity of nitrogen  
84 released at every cleaning step was attributed to the spatial distribution of nitrogen components,  
85 the seasonal variability of  $\delta^{15}\text{N}_{\text{coral}}$  and of the total nitrogen (Yamazaki et al., 2011b) content and  
86 differences in the nitrogen recovery rates throughout the chemical conversion process.

## 87 **2.2. Fossil coral specimen**

88 *Porites* specimens were excavated from the Tartaro formation located on Luzon Island,  
89 Philippines ( $14^\circ\text{N}$ ,  $121^\circ\text{E}$ ). A previous study on the nanofossil assemblages showed that the age of  
90 the fossils was approximately 3.5-3.8 million years old (Watanabe et al., 2011). The  
91 well-preserved fossil coral was selected by conducting a diagenetic alteration test using X-ray  
92 radiography, X-ray diffraction analysis, microanalysis of thin sections via high-energy  
93 synchrotron X-ray diffraction, scanning electronic microscopy observations, and optical  
94 microscopy observations (Watanabe et al., 2011). Oxygen isotopes ( $\delta^{18}\text{O}$ ) were preserved without  
95 diagenetic alteration from aragonite to calcite. We used a fossil coral without diagenetic  
96 alterations to determine the intra-crystalline nitrogen content using stepwise heating methods.

## 97 **2.3. Stepwise heating methods**

98 Cubic samples of modern and fossil corals were obtained from each colony, cleaned using milli-Q  
99 water in an ultrasonic bath for 20 minutes and dried in an oven ( $40^\circ\text{C}$ ) for 2 days. Rectangular  
100 samples  $5 \times 3 \times 3$  mm in size, which captured approximately 1 year of calcification, were used to  
101 adjust the estimated quantity of nitrogen to the mass spectrometer. The sample weights of modern

102 and fossil corals were 17.89 and 17.93 mg, respectively. Each cubic sample was loaded into a  
103 double-walled quartz glass tube and placed overnight in a furnace equipped with a resistance wire  
104 under vacuum. A solid cube sample was used to reduce blank effects. Each sample was heated  
105 stepwise to temperatures of 200, 450, 550, 700, 800, 900, and 1000 °C over 4 days (2 steps/day).  
106 After the sample was heated for 90 minutes at each step, the nitrogen and argon gases released  
107 from the samples were directed to a purification vacuum line designed to measure molecular  
108 nitrogen at the sub-nanomole level (Takahata et al., 1998). The procedure used for the purification  
109 of nitrogen gas is shown in Fig. 2. Carbon dioxide (CO<sub>2</sub>) and water (H<sub>2</sub>O) were trapped using  
110 liquid nitrogen (cold trap 1). Carbon monoxide, hydrocarbons, and hydrogen were then oxidised to  
111 CO<sub>2</sub> and H<sub>2</sub>O using pure oxygen produced by a copper oxide finger heated to 850°C and a  
112 platinum foil catalyst heated to 1000°C. To resorb excess oxygen, the copper oxide furnace was  
113 cooled to 600°C and finally to 450°C. In this deoxidised state and in the presence of the platinum  
114 foil catalyst, nitrogen oxide gas was deoxidised to nitrogen gas (N<sub>2</sub>). CO<sub>2</sub> and H<sub>2</sub>O were adsorbed  
115 in cold trap 2 during the cooling of the copper oxide finger. A quadrupole mass spectrometer  
116 (QMS; HAL201, Hiden Analytical) was used to determine the sample size introduced to the mass  
117 spectrometer. The dilution process was repeated to obtain the proper sample volume. To detect the  
118 isotopes of trace nitrogen gas at the sub-nanomole level, we used the high-sensitivity static  
119 vacuum mass spectrometer (a modified VG3600, VG Micromass Ltd.) at the Atmosphere and  
120 Ocean Research Institute at the University of Tokyo. Nitrogen gas calibrated to nitrogen in air  
121 ( $\delta^{15}\text{N}=0\%$ ) was analysed before and after each sample. Repeated analysis of the standard over the  
122 course of 20 days showed that the overall reproducibility was 0.25% (Takahata et al., 1998). After  
123 the most recent sample was evaluated at 1000°C, blank analyses were performed at 450°C and  
124 1000°C to estimate the background noise.

125

### 126 3. Results and Discussion

127 During the stepwise heating of the modern coral (Fig. 3a), the nitrogen concentration released  
128 from 200 °C to 550 °C was negligible (less than 1 ppm, where ppm is the parts per million of  
129 calcium carbonate). The nitrogen concentration was 41 ppm at 700 °C, 27 ppm at 800 °C, 26 ppm  
130 at 900 °C, and 5 ppm at 1000 °C. The total nitrogen concentration in the modern coral skeleton  
131 was 98 ppm. The  $\delta^{15}\text{N}_{\text{coral}}$  ratios in the modern coral were +5.3% at 700 °C, +11.4% at 800 °C,  
132 +15.9% at 900 °C, and +11.4% at 1000 °C. The nitrogen/argon ratios ( $\text{N}_2/\text{Ar}$ ) in the isotope  
133 analysis of the modern coral were 6980 at 700 °C, 6771 at 800 °C, 7563 at 900 °C, and 1385 at  
134 1000 °C, indicating that the effects of air contamination ( $\text{N}_2/\text{Ar}$ : 83) were negligible. The bulk  
135  $\delta^{15}\text{N}_{\text{coral}}$  ratio in the modern coral, which was calculated using the expression shown below, was  
136  $+10.1 \pm 0.9$  ( $2\sigma$ )%.

137

$$138 \delta^{15}\text{N}_{\text{bulk}} = \sum(f_i \cdot \delta^{15}\text{N}_i)$$

139 where  $f$  is the fraction and  $i$  is the heating temperature step.

140

141 The modern bulk  $\delta^{15}\text{N}_{\text{coral}}$  and total nitrogen content analysed by stepwise heating corresponded to  
142 the values measured by chemical conversion methods ( $+9.1 \pm 1.5\%$ ,  $63 \pm 43$  ppm).

143 The nitrogen released from the Pliocene coral skeleton (Fig. 3b) was less than 1 ppm at 200 °C. At  
144 the 450 °C step, only a small amount of nitrogen was released (5 ppm). Subsequently, the  
145 concentration of nitrogen was 21 ppm at 550 °C, 14 ppm at 700 °C, 9 ppm at 800 °C, 7 ppm at  
146 900 °C, and 18 ppm at 1000 °C. The  $\delta^{15}\text{N}_{\text{coral}}$  ratios of the Pliocene coral were -0.5% at 450 °C,  
147 -0.7% at 550 °C, +2.9% at 700 °C, +14% at 800 °C, +6.9% at 900 °C, and +5.9% at 1000 °C. The

148 nitrogen/argon ratios ( $N_2/Ar$ ) in the isotope analysis of the Pliocene coral were 3389 at 550 °C,  
149 4554 at 700 °C, 3407 at 800 °C, 7461 at 900 °C, and 4880 at 1000 °C, indicating that the effects of  
150 air contamination were negligible. The total nitrogen content in the Pliocene coral (69 ppm) was in  
151 agreement with the nitrogen content of modern corals (50-150 ppm: Marion et al., 2005;  
152 Yamazaki et al., 2011a, b; this study). The bulk  $\delta^{15}N_{coral}$  ratio in the Pliocene coral was  $+4.4 \pm 0.8$   
153 ( $2\sigma$ )%. The Pliocene  $\delta^{15}N_{coral}$  was similar to the modern  $\delta^{15}N_{nitrate}$  in the surface water of the  
154 tropical western Pacific ( $+3.6$ - $+4.4\%$ : Liu et al., 1996) and to the modern tropical  $\delta^{15}N_{coral}$   
155 ( $+2.5$ - $+6.5\%$ : Muscatine et al., 2005; Marion et al., 2005; Uchida et al., 2008; Yamazaki et al.,  
156 2011b). In the present study, the modern  $\delta^{15}N_{coral}$  in Tatsukushi Bay was  $+5\%$  higher than that of  
157 the Pliocene corals due to the latitudinal distribution of  $\delta^{15}N_{nitrate}$ . Yamazaki et al. (2011b)  
158 suggested that  $\delta^{15}N_{coral}$  values in low latitudes (tropical to subtropical) decreased due to  $N_2$   
159 fixation ( $\delta^{15}N$ ;  $-2$ - $0\%$ ).

160 Cuif et al. (2004) reported that the thermal decomposition of coral aragonite begins to accelerate at  
161 550 °C, which is supported by the fact that aragonite collapse triggered the maximum nitrogen  
162 release from the modern coral at 700 °C and from the Pliocene coral at 550 °C. The fossil coral  
163 was more brittle than the modern coral. After the collapse of aragonite, the  $\delta^{15}N_{coral}$  values of the  
164 modern and Pliocene corals increased during the first three heating steps; however, at higher  
165 temperatures (900-1000 °C), the  $\delta^{15}N_{coral}$  values suddenly decreased and became similar to the  
166 bulk nitrogen isotope values. To examine kinetic fractionation effects during the release of  
167 nitrogen, a simple test can be performed. Namely, a plot of  $\delta^{15}N_{coral}$  ratios remaining in the sample  
168 versus the amount of nitrogen remaining in the sample can be created using Rayleigh's law (Fig.  
169 4., e.g., Boyd et al., 1993).

170



171  $\ln R = \ln R_0 + (1/\alpha - 1) \ln f,$

172

173 where  $R$  is the isotopic ratio ( $^{14}\text{N}/^{15}\text{N}$ ) of nitrogen remaining in the sample ( $\ln [^{14}\text{N}/^{15}\text{N}]_{\text{AIR}} =$   
174 5.609),  $R_0$  is the original isotopic composition of nitrogen in the sample,  $f$  is the fraction of  
175 nitrogen remaining in the sample, and  $\alpha$  is the fractionation factor. The relationship between the  
176  $\delta^{15}\text{N}_{\text{coral}}$  values and the corresponding quantities of released nitrogen was linear. At higher  
177 temperatures, the  $\delta^{15}\text{N}_{\text{coral}}$  results departed from the theoretical values expected for a Rayleigh  
178 distillation. At the three lower temperatures of aragonite collapse, the isotope fractionation factors  
179 ( $\alpha$ ) were 0.996 ( $R^2=0.974$ ) for the modern coral and 0.995 ( $R^2=0.996$ ) for the Pliocene coral. The  
180 internal skeletal structure collapsed during the high-temperature steps, which may have released  
181 fresh organic nitrogen, thus yielding lower nitrogen isotope ratios.

182 The organic matrix in the coral skeletons may play a major role in the crystal nucleation process  
183 and in the micro- and macro-regulation of the crystal morphology in the biomineralisation process  
184 (reviewed by Cohen et al., 2003; Allemand et al., 2004; and Tambutte et al., 2011). Early in the  
185 calcification process, corals form centres of calcification (COCs). Subsequently, fibre-like  
186 aragonite surrounds the COCs (Pratz, 1882, Cuif and Dauphin, 1998). Geochemical analysis of the  
187 COCs indicated high concentrations of sulphur-bearing organic molecules (amino acids and  
188 sugars) and magnesium, which are more likely to fractionate in COCs than in fibres. Therefore,  
189 the organic matrix should be preserved in the inner skeletal structures that collapsed at higher  
190 temperature steps (900°C-1000°C). In the Pliocene coral, the nitrogen fraction at the 1000°C step  
191 was greater than that at the (900°C) previous step. This result suggests that nitrogen had been  
192 firmly preserved in the aragonite skeleton of the *Porites* coral since the Pliocene epoch.

193 Although more data are required, these results suggest that nitrogen in coral skeletons can  
194 withstand diagenesis over long geological time scales. In future work, the time series of  $\delta^{15}\text{N}_{\text{coral}}$   
195 can be used as a proxy for nitrogen dynamics in palaeo-oceans. Our study showed that stepwise  
196 heating methods can effectively demonstrate the preservation of  $\delta^{15}\text{N}_{\text{coral}}$  in modern and fossil  
197 corals.

198

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205 Tokyo.

206

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275

## 276 **Figure captions**

277 Fig. 1 The (a)  $\delta^{15}\text{N}_{\text{coral}}$  and (b) total nitrogen content in a modern coral analysed by the chemical  
278 conversion methods reported by Yamazaki et al. (2011a). To analyse the nitrogen released by  
279 intra-crystalline organic matter, the modern coral powder was soaked in hot NaOH (60°C, 2 N)  
280 before cleaning (for 0, 0.5, 1, 2, 3, and 5 hours). The number in parentheses is the number of  
281 samples at each step. The error bar shows the uncertainty ( $2\sigma$ ) at each cleaning time.

282

283 Fig. 2 Diagram of the nitrogen purification procedure used for nitrogen isotope analysis, which  
284 was performed using a modified noble gas mass spectrometer (VG3600)

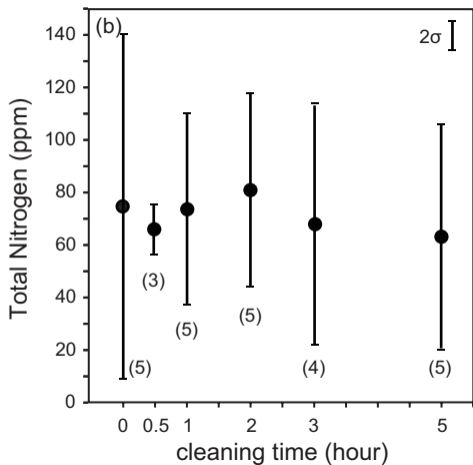
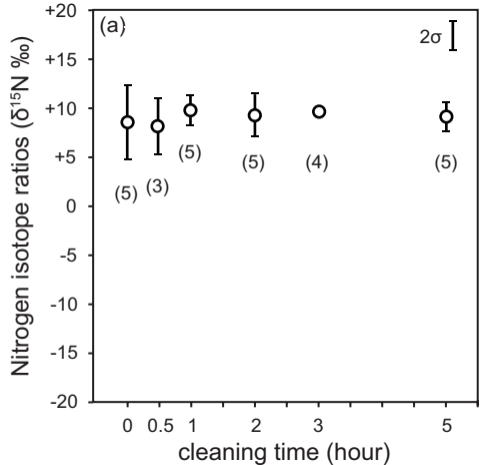
285

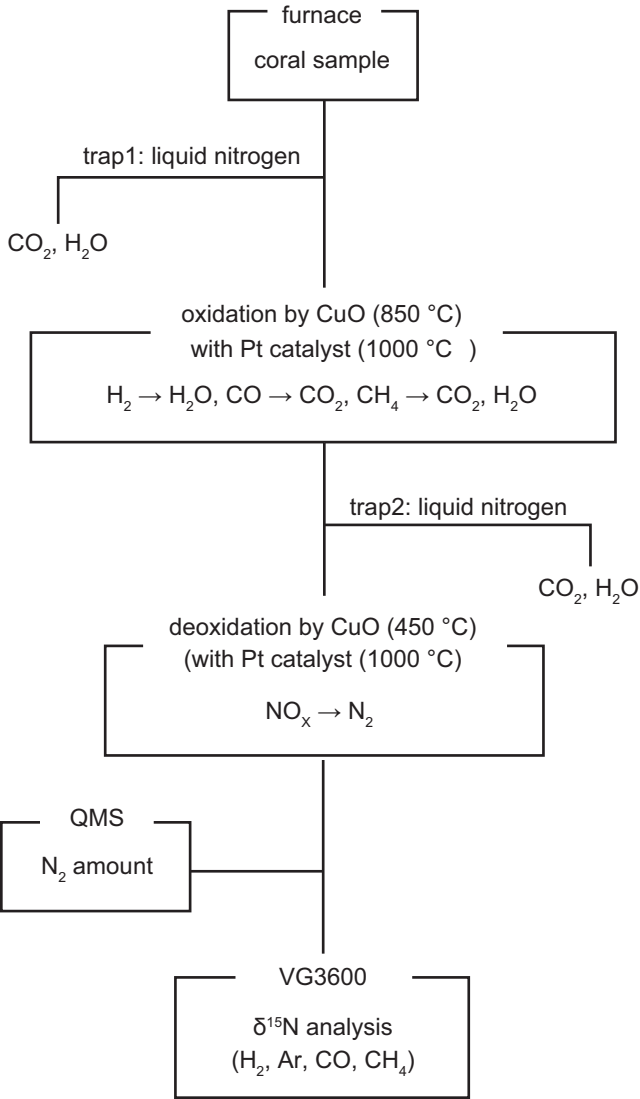
286 Fig. 3 Nitrogen isotopes (line, right axis) and released nitrogen gas (bar, left axis) at each heating  
287 step. The nitrogen isotope values ( $\bullet$ ) and corresponding uncertainty ( $\sigma = \sqrt{(\sigma_{\text{standard}}^2 + \sigma_{\text{sample}}^2)}$ ) are

288 plotted. (a) Living modern coral collected from Tatsukushi Bay, Kochi, Japan. (b) Pliocene fossil  
289 coral excavated from the Tartaro formation (3.5 Ma) located on Luzon Island, Philippines.  
290 Aragonite collapse temperatures (inside grey shading) were determined based on the maximum  
291 total nitrogen concentration. The dotted lines indicate the bulk nitrogen isotope values, which were  
292 calculated as  $\delta^{15}\text{N}_{\text{bulk}} = \Sigma(f_i \cdot \delta^{15}\text{N}_i)$  [f: fraction, i: heating temperature step]

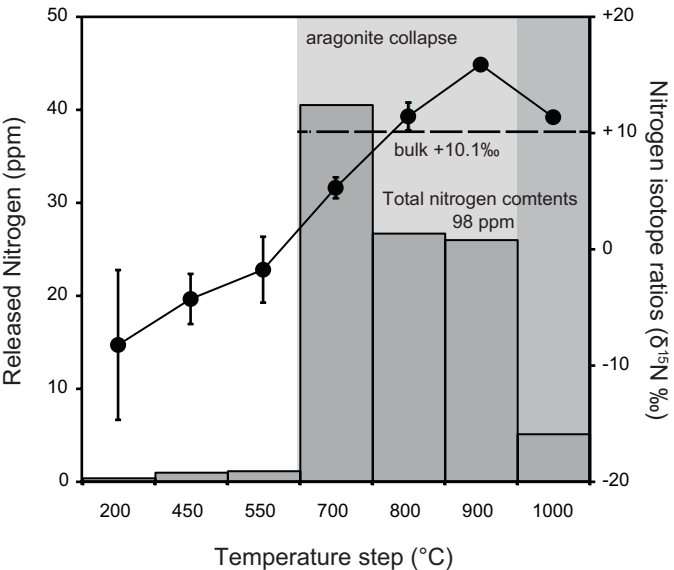
293

294 Fig. 4 Calibration lines for the kinetic fractionation of nitrogen isotopes during sample heating.  $R$   
295 is the isotopic ratio ( $^{14}\text{N}/^{15}\text{N}$ ) of nitrogen remaining in the sample ( $\ln [^{14}\text{N}/^{15}\text{N}]_{\text{AIR}} = 5.609$ ), and  $f$   
296 is the fraction of nitrogen remaining in the sample. The fractionation factors ( $\alpha$ ) were 0.996  
297 ( $R^2=0.974$ ) for the modern coral ( $\blacktriangle$ ) and 0.995 ( $R^2=0.996$ ) for the Pliocene coral ( $\bullet$ ) and were  
298 calculated from values obtained in the lower three temperatures of aragonite collapse. The  
299 fractionation lines of nitrogen isotopes for the modern ( $\Delta$ ) and fossil ( $\circ$ ) corals diverged at high  
300 temperatures.





(a) Modern Coral



(b) Pliocene Coral

