

Figure 5 Model for the observed deformation on Sierra Negra during 1997–98. See Fig. 2b. **a**, The model has uniform opening of a horizontal sill (large rectangle) and slip on the normal faults that bound the elevated inner caldera floor. The four smaller rectangles show the surface projection of normal fault patches used in the modelling; a thick line marks the upper edge. The depth of the sill is not well constrained in this case; estimates range between 2.3 and 2.9 km. The difference between this and the depth obtained when trapdoor faulting was not present (2.2 km in 1992–97 and 1.9 km in 1998–99) is unlikely to be significant owing to imperfect resolution and model approximations. **b**, Residual between the data and the model prediction. Localized fringes near the fault are due to the simplified representation of the fault as four rectangular dislocation segments. **c**, Comparison between the data and the model showing the LOS displacement along the profile A–A' as well as the intra-caldera surface topography. The observed displacement follows the topography indicating repeated slip events.

maximum uplift in 1997–98 to over 100 m in the western caldera. In places the ridge actually extends above the caldera rim, indicating that trapdoor faulting has occurred numerous times since the caldera floor was resurfaced. The last four eruptions (and perhaps more) were not intra-caldera eruptions but occurred in the area north of the caldera. Our observations suggest that repeated slip on intra-caldera (or caldera bounding) faults may allow uplift without accumulating sufficient stress to permit vertical dyke propagation. Similar processes may explain large uplifts without eruptions at other rapidly deforming volcanoes³. □

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Correspondence and requests for materials should be addressed to S.J. (e-mail: jonsson@pangea.stanford.edu) or F.A. (e-mail: amelung@pgd.hawaii.edu).

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Unicellular C₄ photosynthesis in a marine diatom

John R. Reinfelder*, **Anne M. L. Kraepiel†** & **François M. M. Morel‡**

* Department of Environmental Sciences, Rutgers University, 14 College Farm Road, New Brunswick, New Jersey 08901, USA
 † Centre de Geochemie de la Surface, Université de Strasbourg, 1 rue Blessig, 67084 Strasbourg, France
 ‡ Department of Geosciences, Princeton University, Princeton, New Jersey 08544, USA

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 Nearly 50 years ago, inorganic carbon was shown to be fixed in microalgae as the C₃ compound phosphoglyceric acid¹. The enzyme responsible for C₃ carbon fixation, ribulose-1,5-bisphosphate carboxylase (Rubisco), however, requires inorganic carbon in the form of CO₂ (ref. 2), and Rubisco enzymes from diatoms have half-saturation constants for CO₂ of 30–60 μM (ref. 3). As a result, diatoms growing in seawater that contains about 10 μM CO₂ may be CO₂ limited⁴. Kinetic and growth studies have shown that diatoms can avoid CO₂ limitation^{5–7}, but the biochemistry of the underlying mechanisms remains unknown. Here we present evidence that C₄ photosynthesis supports carbon assimilation in

the marine diatom *Thalassiosira weissflogii*, thus providing a biochemical explanation for CO₂-insensitive photosynthesis in marine diatoms. If C₄ photosynthesis is common among marine diatoms, it may account for a significant portion of carbon fixation and export in the ocean, and would explain the greater enrichment of ¹³C in diatoms compared with other classes of phytoplankton. Unicellular C₄ carbon assimilation may have predated the appearance of multicellular C₄ plants.

There is a long-standing debate regarding the existence of a C₄ photosynthetic pathway in marine phytoplankton⁸⁻¹⁰. In 'classic' multicellular C₄ plants, carbon acquisition is facilitated by the enzyme phosphoenolpyruvate carboxylase (PEPCase), which catalyses the carboxylation of PEP (using HCO₃⁻ as the inorganic carbon substrate^{11,12} instead of CO₂) leading to the synthesis of a C₄ compound—malic or aspartic acid. Enzymatic decarboxylation of

the C₄ compound by malic enzyme or phosphoenolpyruvate carboxykinase (PEPCKase) then generates CO₂ for fixation by Rubisco in the first step of the Calvin cycle¹³. PEPCase and PEPCKase activities have been measured in marine diatoms^{8,10}

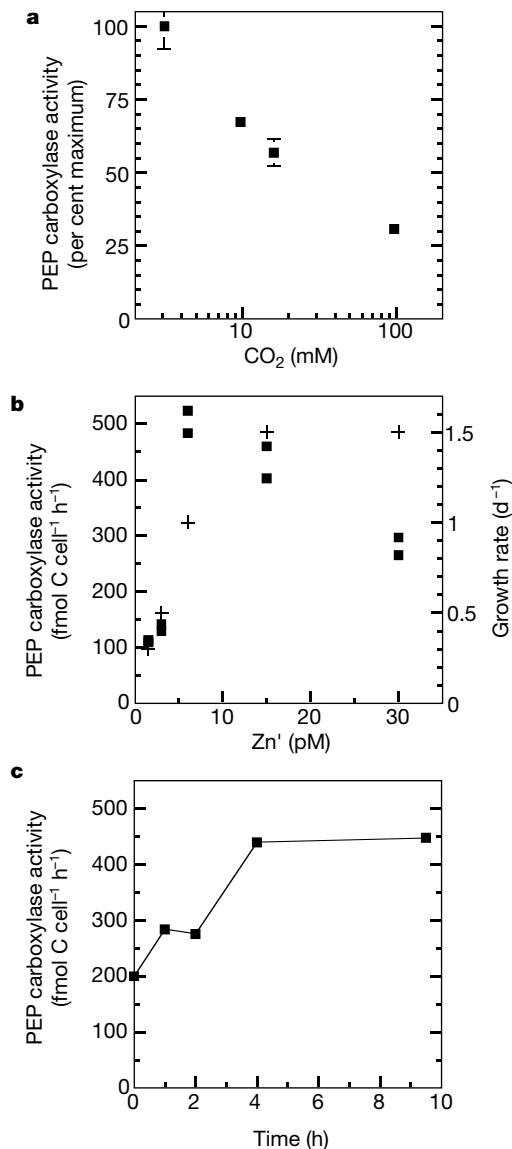


Figure 1 Phosphoenolpyruvate carboxylase (PEPCase) activities (squares) and growth rates (+) in the marine diatom *T. weissflogii*. **a**, *T. weissflogii* acclimated to a range of CO₂ concentrations. PEPCase activities are the means of triplicate analyses. **b**, *T. weissflogii* acclimated to a range of inorganic Zn (Zn²⁺) concentrations. PEPCase activities are the range of activities in replicate cultures. **c**, PEPCase activities in Zn-replete cells exposed to the carbonic anhydrase (CA) inhibitor ethoxzolamide (100 μM). These cells grew at a rate of 1 d⁻¹ and had a photosynthetic rate (measured by short-term ¹⁴C fixation) of 520 fmol C cell⁻¹ h⁻¹.

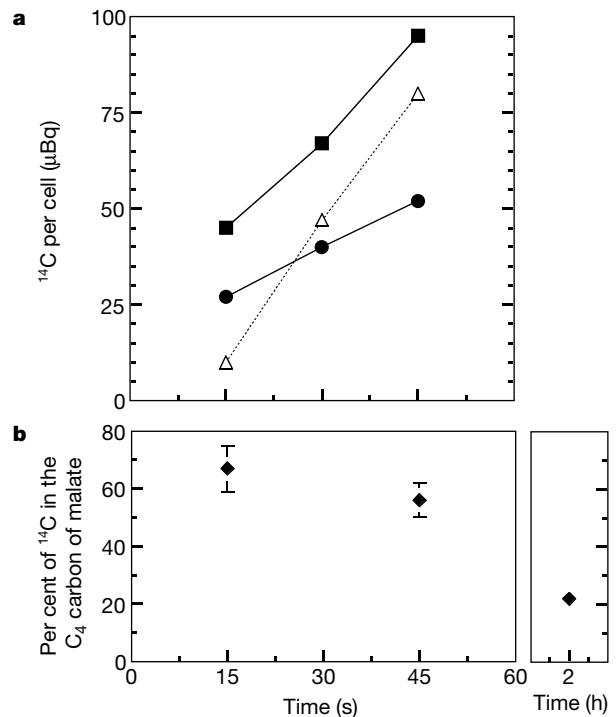


Figure 2 Short-term ¹⁴C assimilation in the marine diatom *T. weissflogii*. **a**, Radiocarbon contents (1 μBq = 10⁻⁶ disintegrations per second) of malate (squares), phosphoglyceric acid (circles) and sugars (triangles) in cells exposed to ¹⁴C for 45 s in pH-buffered medium containing 0.4 mM total inorganic carbon. Error bars for 2σ propagated counting errors are smaller than plot symbols. **b**, Per cent of ¹⁴C in the C₄ carbon of malate in diatom cells during short-term labelling and after 2 h exposure to ¹⁴C with a total inorganic carbon concentration of 1.8 mM. 2σ propagated counting errors were ≥ the difference between replicate analyses.

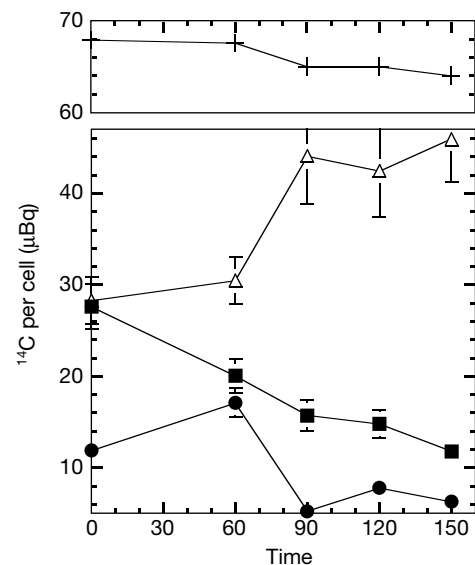


Figure 3 Transfer of ¹⁴C from malate to C₃ compounds (PGA and sugars) in the marine diatom *T. weissflogii*. Data are the radiocarbon contents (1 μBq = 10⁻⁶ disintegrations per second) of malate (squares), phosphoglyceric acid (circles), sugars (triangles) and total acid-stable ¹⁴C (+) in cells exposed to ¹⁴C for 60 s in pH-buffered medium containing 1.8 mM total inorganic carbon and then transferred to ¹²C medium containing 2 mM inorganic carbon.

but, on the basis of short-term ¹⁴C labelling studies that showed a lack of significant synthesis of the C₄ compounds malate or aspartate, it was concluded that PEP carboxylation may be involved in the synthesis of amino-acid precursors but it does not lead to carbon fixation through the Calvin cycle^{8,9}.

Previous studies might have missed the importance of PEPCase in diatom photosynthesis partly because culture conditions placed no burden on the carbon acquisition systems of the cells. We found that PEPCase activity in a coastal Atlantic Ocean clone (ACTIN) of the cosmopolitan marine diatom *T. weissflogii* increases in cells acclimated to low CO₂ (Fig. 1a), indicating a probable link between inorganic carbon uptake and PEPCase. If this is the case, we would also expect an increase in PEPCase activity in Zn-stressed cells. At low Zn, a decreased concentration and activity of the Zn enzyme carbonic anhydrase (CA, which reversibly catalyses the interconversion of CO₂ and HCO₃⁻) impairs inorganic carbon accumulation in *T. weissflogii*^{14,15}. We therefore examined PEPCase activity in *T. weissflogii* acclimated to a range of inorganic Zn concentrations (Zn') that are known to impose stress on the carbon acquisition system. The highest PEPCase activities are measured in *T. weissflogii* cells cultured with 6 pM Zn' (Fig. 1b), whose growth rate (1 d⁻¹) is slightly reduced relative to Zn-sufficient cells (1.5 d⁻¹). At very low Zn', PEPCase activity declines in proportion to the growth rate, which becomes Zn limited. Perhaps fortuitously, the measured PEPCase activity in low Zn cultures (110 fmol cell⁻¹ h⁻¹ at Zn' = 1.5 pM) is about 90% of the carbon fixation rate in cells growing at a rate of 0.3 d⁻¹ (0.3 d⁻¹ × 10 pmol cell⁻¹/24 h d⁻¹ = 125 fmol cell⁻¹ h⁻¹). In cells grown with 6, 15 and 30 pM Zn', measured PEPCase activity is about 120%, 70% and 45% of carbon fixation, respectively. Increased PEPCase activity in cells subjected to moderate Zn stress (Zn' = 6 pM) is probably a response to diminished intracellular and extracellular CA activity. Confirmation of this is seen in a similar increase in PEPCase activity measured in cells exposed to the CA inhibitor ethoxzolamide (Fig. 1c).

If PEPCase supports carbon fixation by Rubisco in cells whose carbon acquisition system is compromised by either low CO₂ or Zn', we would expect to find significant short-term fixation of carbon as the C₄ product of PEPCase in CO₂ or Zn-stressed *T. weissflogii*. In short-term photosynthesis experiments, after 10–15 s, the C₄ compound malate was the principal ¹⁴C-labelled intracellular pool in low Zn (Zn' = 4 pM) diatoms (Fig. 2a) and almost twice as large as the labelled C₃ pool (phosphoglyceric acid; PGA) (malate:PGA = 1.8 ± 0.09, n = 3). In diatoms grown with

higher Zn (Zn' = 15 pM), the short-term ¹⁴C-labelled malate:PGA ratio was ≤ 1 and decreased from 1.0 ± 0.08 to 0.73 ± 0.06 as the concentration of aqueous CO₂ in the cultures was increased from 3 to 16 μM. All these results are consistent with major primary carbon fixation by PEPCase in cells that are stressed by either low concentrations of CO₂ (< 3 μM) or low Zn (Zn' < 15 pM). The observed β-carboxylation should lead to the short-term labelling of carbon in the C₄ position of malate. Indeed, *in vitro* decarboxylation of pulse-labelled malate from low Zn cells showed that nearly 70% of the ¹⁴C accumulated in 15 s in malate was in the C₄ carbon (Fig. 2b). After 2 h this proportion decreased to about 25%, as expected for uniform label incorporation in malate (Fig. 2b).

We followed the fate of malate carbon in ¹⁴C-labelled cells transferred to ¹²C medium. During the first 60 s of this 'chase', there was a simultaneous decline in the ¹⁴C activity of malate and an increase in the ¹⁴C content of PGA and sugars (Fig. 3). Mass balance of ¹⁴C during this period requires the transfer of carbon from malate to PGA; ¹⁴C content of malate decreased by 8 μBq while that of PGA increased by 5 μBq and that of sugars increased by 2 μBq. Thus malate is being decarboxylated and the released CO₂ is being fixed by Rubisco to produce PGA and sugars. We have observed this pattern of ¹⁴C transfer from malate to PGA in four experiments with different batches of cells (Table 1).

The operation of C₄ photosynthesis requires a decarboxylase in close proximity to Rubisco to insure efficient transfer of carbon. As PEPCKase activity has been measured in marine diatoms³, we reasoned that malate is decarboxylated by PEPCKase (after oxidation to oxaloacetate) in the chloroplast of *T. weissflogii*. We found 75% of PEPCKase activity in *T. weissflogii* located in chloroplasts (Table 2). As this is the same percentage as measured for the chloroplastic enzyme Rubisco (which is less than 100% because of chloroplast breakage during preparation), all the PEPCKase is probably in the chloroplast. In very low Zn cells (Zn' = 1.5 pM), we measured a PEPCKase activity (170 ± 10 fmol C cell⁻¹ h⁻¹) that is of the order of carbon fixation rates (125 fmol C cell⁻¹ h⁻¹) expected at that Zn-limited growth rate.

In the cytoplasm of the mesophyll cells of multicellular C₄ plants, CA provides PEPCase with HCO₃⁻ through the hydration of CO₂ and the cytoplasmic localization of CA is characteristic of such cells¹⁶. In *T. weissflogii*, all of the observed intracellular CA activity is also in the cytoplasm, the remainder being extracellular CA (Table 2; A. M. L. K. *et al.*, manuscript in preparation). This suggests that CA provides HCO₃⁻ to PEPCase which should also be located in the

Table 1 Transfer of ¹⁴C from malate to C₃ compounds (PGA and sugars) in the marine diatom *T. weissflogii*

	Zn' = 4 pM		Zn' = 4 pM		Zn' = 15 pM		Zn' = 15 pM	
	Pulse	Chase	Pulse	Chase	Pulse	Chase	Pulse	Chase
Duration	10 s	25 s	60 s	60 s	5 s	12 s	10 s	12 s
Malate	60	39	41	30	43	20	40	19
PGA	31	43	17	25	51	72	52	73
Sugars	9	18	42	45	6	8	8	8

Cells acclimated to either 4 or 15 pM inorganic Zn (Zn') were labelled with ¹⁴C for 5–60 s (pulse) and then transferred to ¹²C medium for 12–60 s (chase). Values are percent total organic ¹⁴C present as malate, phosphoglyceric acid (PGA) or sugars.

Table 2 Distribution of enzyme activities among cellular fractions of the marine diatom *T. weissflogii*

Fraction	PEPCase	PEPCKase	Rubisco	CA (×10 ⁻⁵)
Whole cell	78	97	175	1.49
Chloroplasts	41 (53)*	73 (75)*	136 (78)*	~0 (0)‡
Cytoplasm	37 (47)†	24 (25)†	39 (22)†	0.81 (54)
Extracellular	n.d.	n.d.	n.d.	0.67 (45)

T. weissflogii were grown with 3 pM Zn' (PEPCase, PEPCKase, Rubisco) or 15 pM Zn' (CA). Values are activities in fmol C per cell per hour for PEPCase, PEPCKase and Rubisco, and enzyme units per cell as defined²⁶ for CA. Percentages of whole-cell activities are given in parentheses. n.d., not determined. * Calculated as the product of measured chloroplast activity per mg Chla and mg Chla per whole cell. † Calculated as the difference between measured whole-cell and chloroplast activities. ‡ Calculated as the difference between measured whole-cell activity and cytoplasmic plus extracellular activities.

cytoplasm to prevent futile competition with Rubisco. We found nearly 50% of PEPCase in the cytoplasm of *T. weissflogii* (Table 2). The PEPCase activity we measured in the chloroplast fraction of *T. weissflogii* is probably cytoplasmic enzyme that partitioned with the denser material of the chloroplasts¹⁷.

Although the exact mechanisms of inorganic carbon uptake and assimilation in diatoms, particularly the relative importance of the C₄ and C₃ pathways under various conditions, need to be further elucidated, our results show that C₄ photosynthesis accounts for a major proportion of carbon fixation in CO₂ or Zn-stressed cells of *T. weissflogii*. As in the aquatic monocot *Hydrilla verticillata*¹⁸, carboxylation and decarboxylation occur simultaneously in a single cell, but in separate intracellular compartments. Because marine diatoms are especially important in the biological removal of CO₂ from surface waters to the deep sea¹⁹, a significant portion of export production in the ocean may be fixed through a C₄ assimilation pathway. The use of C₄ photosynthesis may explain the enrichment of ¹³C in diatoms relative to other classes of marine phytoplankton²⁰, and the increase in the ¹³C content of sediment organic matter from 35 Myr (early Oligocene) to the present²¹. Evolutionarily, the appearance of C₄ photosynthesis in diatoms is probably an adaptation to carbon acquisition stress caused by low CO₂ and/or Zn concentrations in surface waters. Diatoms radiated during the Mesozoic²²—an era of low concentrations of atmospheric CO₂ relative to earlier eras (Precambrian, Paleozoic)²³ when most photosynthetic microorganisms evolved. In this case, C₄ photosynthesis in aquatic microalgae would be much more ancient than in terrestrial C₄ and Crassulacean acid metabolism plants where it arose only about 7 Myr ago (late Miocene)²⁴. □

Methods

Enzyme activities

We grew cultures of *T. weissflogii* (clone ACTIN) axenically in trace-metal-buffered, artificial seawater (Aquil²⁵) at each concentration of CO₂ or Zn²⁺ for at least two transfers (9–10 generations) and collected them during mid-exponential growth. Phosphoenolpyruvate carboxylase activities were measured in cells homogenized by sonication in 50 mM bicine buffer (0 °C, pH 7.5) containing 10 mM NaHCO₃, 1.5 M glycerol, 1 mM EDTA, 10 mM MgCl₂, 5 mM dithiothreitol (DTT) and 5 mg ml⁻¹ bovine serum albumin (BSA) by PEP-dependent ¹⁴C-fixation at 25 °C. Phosphoenolpyruvate carboxylase activities were determined by PEP-dependent ¹⁴C fixation in the presence of ADP and Mn in cells homogenized in 25 mM HEPES buffer (pH 7.1), containing 10 mM NaHCO₃, 1.5 M glycerol, 0.2 mM EDTA, 2 mM MnCl₂, 2 mM ADP, 0.1 M KCl, 5 mM DTT and 5 mg ml⁻¹ BSA. Carbonic anhydrase was measured in homogenized whole cells (total activity), intact whole cells, (extracellular activity) and in cell-fractionation (see below) supernatants (cytoplasmic activity) by a pH drift assay²⁶.

Short-term ¹⁴C assimilation

Mid-exponential phase diatoms were concentrated 50–100 times, resuspended in 1 or 2 ml buffer (350 mM sorbitol, 10 mM bicine, pH 8) and incubated in a stirred cell in the light (400 μmol photons m⁻² s⁻¹). For short-term uptake studies cells were incubated in the light in a sealed oxygen electrode cell (Fig. 2a) until no net O₂ production was observed (10 min) indicating that all inorganic carbon had been depleted from the buffer, or in previously prepared low dissolved inorganic carbon buffer (Table 1, Zn²⁺ = 4 pM, 10 s pulse and Zn²⁺ = 15 pM). We began experiments by adding 5 μCi (0.5 ml) of ¹⁴C as aqueous NaH¹⁴CO₃ resulting in a total inorganic carbon concentration of 0.4 mM. For the malate labelling (Fig. 2b) and ¹⁴C chase (Fig. 3) experiments, cells were incubated in the light in an unsealed vial for about 2 min before the addition of 5 μCi of ¹⁴C, resulting in a total inorganic carbon concentration of 1.8 mM. For chase experiments, ¹⁴C-labelled cells were collected on a 3-μm polycarbonate filter (Fig. 3) or centrifuged through a silicone oil layer (Table 1, Zn²⁺ = 4 pM, 10 s pulse and Zn²⁺ = 15 pM), resuspended into buffer containing 2 mM total inorganic ¹²C (no ¹⁴C), and returned to the light. Cell samples (0.25 ml) were transferred to 2 ml CHCl₃:MeOH (2:1) for kill and extraction. The upper phase of each extract (H₂O:MeOH mixture) was diluted 1:1.25 with 0.3 M KH₂PO₄ (pH 2.8) and the MeOH evaporated. Radiolabelled organic compounds were separated by anion exchange chromatography using high performance liquid chromatography²⁷ and ¹⁴C was quantified by liquid scintillation counting.

Malate decarboxylation

We determined the radiocarbon content of the C₄ carbon of malate by enzymatic digestion²⁸ of malate collected in the short-term labelling experiment described above. Radiolabelled malate was incubated at 30 °C with 0.25 units of malic enzyme and 20 units of glutathione reductase (Sigma) in 0.1 M HEPES (pH 7.5) containing 40 μM NAPD, 20 mM oxidized glutathione and 4 mM MnCl₂. Decarboxylation was carried out for 48 h

to ensure completeness at which time the reaction was stopped by the addition of 80 μl 6 M HCl. Samples were evaporated and remaining ¹⁴C quantified by liquid scintillation counting. The percentage of ¹⁴C in the C₄ carbon was calculated as 100 × [1 - (malate ¹⁴C post-decarboxylation / malate ¹⁴C pre-decarboxylation)].

Cell fractionation

Diatom chloroplasts were isolated from *T. weissflogii* cells homogenized with a Polytron blender (3 × 30 s, 15,000 r.p.m.) in 50 mM HEPES buffer (0 °C, pH 8) containing 626 mM sorbitol, 1 mM EDTA and 10 mg ml⁻¹ bovine serum albumin. Lysed cells were overlain on top of a 70% Percoll solution (626 mM sorbitol, 1 mg ml⁻¹ bovine serum albumin) and centrifuged for 4 min at 4,000 r.p.m. and 4 °C. Chloroplasts were collected from above the Percoll cushion. Cytoplasm for CA analysis was collected as the supernatant of the Percoll centrifugation.

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Correspondence and requests for materials should be addressed to J.R.R. (e-mail: reinfelder@envsci.rutgers.edu).