Symbiosis, 6 (1988) 225–236
Balaban Publishers, Philadelphia/Rehovot

Uptake of Inorganic Carbon by *Fragilaria Shiloi*, the Symbiont of the Foraminifer *Amphistegina lobifera*

B.H. TER KUILE[1], A. KAPLAN[2] and J.J. LEE[2]

[1] *The Hebrew University of Jerusalem, The Interuniversity Institute of Eilat, P.O. Box. 469, Eilat 88103, Israel*

[2] *Department of Botany, The Hebrew University of Jerusalem, Givat Ram, Jerusalem 91904, Israel*

[3] *Department of Biology, City College of City University of New York Convent Avenue at 138th St., New York, NY 10031, USA*

Received January 5, 1988; Accepted July 4, 1988

Abstract

Fragilaria shiloi, an algal symbiont of the foraminifer *Amphistegina lobifera*, does not change the $K_{1/2}$ for inorganic carbon (Ci) during incubation at low Ci levels. Internal accumulation of Ci could not be demonstrated, suggesting it does not possess active uptake mechanisms for Ci. The optimal pH for photosynthesis is around the normal pH of seawater and the calcium concentration in the medium hardly affects photosynthetic rates. Calculations suggest that the generation of CO_2 from HCO_3^- was sufficient under all experimental conditions to support the photosynthetic rates measured. Our findings do not confirm that with respect to its photosynthetic Ci response *F. shiloi* is uniquely adapted to a symbiotic life.

Keywords: Symbiont, inorganic carbon uptake, ecological status

0334-5114/88/$03.00

1. Introduction

Algae that live as symbionts in benthonic foraminifera are usually also found as free living species in the same environment, albeit in very low frequencies (Lee, 1983), suggesting ecological disadvantages over other species. One of these disadvantages may be the high nutrient requirement of many symbiotic species (Lee, 1983). In earlier studies it was shown that foraminiferal hosts may supply their symbionts with nutrients obtained from feeding (Jorgensen et al., 1985; ter Kuile et al., 1987). Another aspect of the physiology of algae that may affect their ecological status is the ability to accumulate inorganic carbon (Ci) at the carboxylating site for photosynthesis (Lucas, 1983). In this study we determined photosynthesis and Ci uptake of *Fragilaria shiloi* as affected by the external Ci concentration, pH and Ca^{2+}. It is suggested that unlike green algae and cyanobacteria, *F. shiloi* does not possess a CO_2 concentrating mechanism.

2. Methods

Fragilaria shiloi has been isolated many times as the symbiont of *Amphistegina lobifera* (Lee et al., 1980b; Lee, 1983; Lee and McEnery, 1983; Koestler et al., 1985). Cultures of *F. shiloi* were grown in Erdschreiber medium based on seawater (Lee et al., 1975), and harvested when still in the exponential growth phase. Cultures to be used in experiments with varying inorganic carbon (Ci) concentration were preincubated in Ci free seawater for one hour. Ci free seawater was prepared by acidifying filtered seawater with 1N HCl to pH 3.5 and flushing it for 1.5 hr with N_2 and for about 1 min with O_2, to give 80–90% of the air equilibrated value. The pH was restored to 8.1–8.2 by addition of NaOH solution. Medium of different Ci levels was prepared by the subsequent addition of $NaHCO_3$ to give the desired concentration. The final concentrations were determined with a precision better than 1% (1σ) with the alkalinity titration method (Gran, 1952; Gieskes, 1974). To seawater prepared this way 1 mM $NaNO_3$ and 0.1 mM K_2HPO_4 and 10 mM Tricine buffer pH 8.15 and 5 ml soil extract per liter were added. Attempts to induce a Ci concentrating mechanism were made by growing *F. shiloi* for 24 hr in medium containing 0.15 mM Ci. An earlier attempt using Ci free medium exposed to air led to severe photoinhibition of the algae. The effect of pH on photosynthetic rates was determined in medium prepared by acidifying seawater containing 10 mM Bis-Tris Propane under tightly sealed conditions, keeping the total Ci concentration constant (2.1 mM), with addition of soil extract and nutrients as above. Artificial sea-

water containing 2.1 mM Ci and different calcium concentrations was used to determine the sensitivity of the algae to calcium. The incubations were conducted in sealed Erlenmeyer flasks in a shaker incubator at 28°C at a light intensity of 8.5 mW cm^{-2}, which saturates the photosynthetic rate. Carbon uptake was measured as ^{14}C (added as $NaH^{14}CO_3$) fixation after 2, 4 and, in some experiments, 6 hr. Duplicate samples of 10 ml were filtered onto 0.4 μm Nuclepore filters according to Goldman and Dennet (1985) and counted with 10 ml liquid scintillation fluid. A calibration curve was made to correct for quenching by photosynthetic pigments. Acidified 1 ml samples of the wash-water contained less than 1% of the radioactivity that remained on the filter, indicating that very little photosynthate had leaked out of the algae during the rinsing procedure. Triplicate samples for specific activity were taken at the end of the incubation. The volume taken for sampling was replaced by nitrogen gas mixed with air to give the same pCO_2 in the medium as in the gas above. The chlorophyll content was determined on duplicate 10 ml samples according to Strickland and Parsons (1968). Calculations suggested that even at the incubations with low Ci concentrations less than 15% of the Ci initially present was removed from the medium during the experiments.

The internal accumulation of Ci was determined with the silicone oil centrifugation method (Kaplan et al., 1980; Marcus et al., 1982). A 50 μl sample of cells preincubated in Ci-free seawater was mixed with 200 μl medium and 2 μl of a $NaH^{14}CO_3$ solution of known concentration and specific activity in a microfuge tube containing 50 μl of 3 N NaOH as a killing solution on the bottom and 100 μl silicone oil on top of that. The medium was prepared from Ci free incubation medium and medium with the Ci concentration of seawater to give the final Ci concentrations indicated in Fig. 2. The incubations were in triplicate for 10 and 60 sec in the light (6 mW cm^{-2}, 400–700 nm) at room temperature. The incubations were terminated by centrifugation (Beckman microfuge B), giving an almost instantaneous separation of the cells from the medium. The microfuge tubes thus obtained were frozen in liquid nitrogen and the pellet removed with a razor blade. The pellet was shaken in a vial containing 0.45 ml 0.1 N NaOH solution. Of this, 0.2 ml was counted in a liquid scintillation counter and 0.2 ml was added to an equal volume of 0.5 N HCl. The non-fixed ^{14}C was allowed to evaporate overnight in a hood and the resulting sample was counted the next day. The difference between the untreated and the acidified sample is the internally accumulated

inorganic carbon, combined with some tracer that was present in the medium centrifuged down with the cells.

3. Results and Discussion

The photosynthetic rates of *F. shiloi* as a function of the external Ci concentration (Fig. 1) can be described by the Michaelis-Menten parameters (Lineweaver-Burk plot, $V_{max} = 150$ μmol C mg chl^{-1}h^{-1}, $k_{1/2} = 500$ μM for total Ci, corresponding to about 10 μM for CO_2). The value of $K_{1/2}$ is similar to that of the high CO_2 grown cyanobacterium *Anabaena variabilis* (Kaplan et al., 1984), but much higher than for low CO_2 grown *Anabaena* or green algae, which possess a CO_2 concentrating mechanism. It is in the low range of values quoted by Raven (1984) for the Rubisco (ribulose 1,5-biphosphate carboxylase) enzyme. Cells grown at low Ci concentrations (0.15 mM instead of 2.1 mM) had a similar affinity, but a slightly higher V_{max} (Fig. 1). In similar experiments cyanobacteria were shown to adapt to low Ci concentrations by strongly reducing the $K_{1/2}$ for external Ci (increasing affinity) (Kaplan et al., 1980, 1984; Marcus et al., 1982). This effect was attributed to the induction of a Ci concentrating mechanism. In *F. shiloi* we could not observe significant differences in the photosynthetic performance (rate versus concentration) in cells grown under 0.15 or 2.1 mM Ci. Thus we conclude that *F. shiloi* does not undergo adaptation to various CO_2 levels, at least in the range examined here. An attempt to grow *F. shiloi* at even lower Ci levels lead to severe photoinhibition. The possible presence of a constitutive Ci concentrating mechanism was further investigated in an experiment measuring the internal accumulation of Ci. For this purpose, the presence of Ci in samples of cells preincubated in Ci free seawater was determined after incubations of 10 and 60 sec with varying Ci concentrations (see Methods). Experiments on green algae (Badger et al., 1980) and cyanobacteria (Kaplan et al., 1980; Volokita et al., 1984; Miller and Canvin, 1985; Ogawa and Ogren, 1985) suggest that if a Ci concentrating mechanism is present, a considerably higher internal Ci concentration would be expected after 60 than after 10 sec. This was not observed (Fig. 2), though the Ci content after 60 sec was slightly higher at all external Ci concentrations measured. Possibly the equilibrium distribution of the label was not completely reached after 10 sec. The data of Figs. 1 and 2 suggest that *F. shiloi* does not actively concentrate inorganic carbon. However, under normal seawater conditions (Ci=2.1 mM) and also within the host (Ci=1.3 mM; ter Kuile et al., in press), Ci seems

not to be a growth limiting factor (Fig. 1). The same was observed for the symbionts of the coral *Seratopora hystrix* (Burris et al., 1983).

The purpose of the experiments measuring photosynthetic rates as a function of the external pH was to examine whether the effect of the pH is exerted directly or through changes in the distribution of the Ci species. The rates of photosynthesis at pH 7.5 or less were lower than those at pH 8.0–8.5 This effect was even more pronounced after 4 than after 2 hr, suggesting a direct inhibitory effect of pH at values below 8.0. It cannot be explained by changes in the CO_2 concentration, since these increases with decreasing pH, or the HCO_3^- concentration, because the same HCO_3^- levels below and above pH 7.5 give different rates. The relatively high rates at pH 9.0 and 9.5 suggest that either *F. shiloi* can use HCO_3^- for photosynthesis, or the $K_{1/2}$ for CO_2 transport is very low. In the latter case our conclusions based on Fig. 2 must be revised, and some CO_2 concentrating mechanism may be present. Under the experimental conditions the rate conversion of HCO_3^- to CO_2 (Gavis and Ferguson, 1975; Stumm and Morgan, 1981; Volokita et al., 1983) was sufficient to account for the photosynthetic rates of *F. shiloi* we measured. In contrast, similar calculations for the data of Borowitzka and Larkum (1976) on *Halimeda tuna* and those of Borowitzka (1981) on *Amphiroa foliacea* do suggest that the pH dependence of photosynthesis is caused by changes in the CO_2 concentration (assuming Michaelis-Menten uptake kinetics). The changes in photosynthetic O_2 evolution of *Anabaena variabilis* as a function of extracellular pH (Zenvirth et al., 1984) seem to depend on the bicarbonate concentration.

Inside the host, the symbionts might be exposed to higher calcium concentrations than in seawater. Possibly algal species that are tolerant to high calcium levels are more suitable as symbionts in a calcifying system than calcium sensitive species. As can be seen in Fig. 4, calcium concentrations up to 20 mM hardly affected photosynthesis of *F. shiloi*. This indicates that possible changes in the internal calcium concentration of the host do not influence the carbon fixation by the symbionts. Internal free calcium concentrations exceeding 20 mM are unlikely to occur because calcium seems to diffuse inwards initially, before being concentrated for calcification (ter Kuile et al., in press). Furthermore, such high internal calcium concentrations might result in uncontrolled precipitation of calcium salts.

The photosynthetic rates of *F. shiloi* are higher inside the host than isolated in culture. The maximum photosynthetic rate in culture is between 140–170 μmol C mg chl^{-1}h^{-1} (Figs. 1,3 and 4). Inside the host 21 nmol C

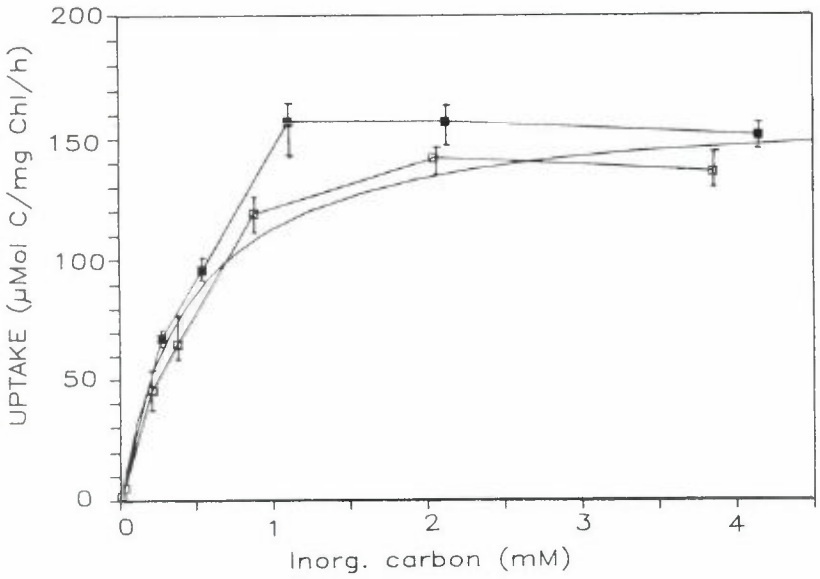

Figure 1. Photoassimilation of ^{14}C by *F. shiloi* as a function of inorganic carbon (Ci) concentration in the medium (□). Without preincubation; (■) preincubated at 0.15 mM Ci. Error bars indicate the extremes of 6 values (duplicates after 2,4 and 6 hr). The smooth line represents a Michaelis Menten plot (V_{max} = 150 μmol C mg chl^{-1}, $K_{1/2}$ = 500 μM for total Ci). Their radiance was 8.5 mw cm^{-2}, the temperature was 28°C, the pH was 8.1.

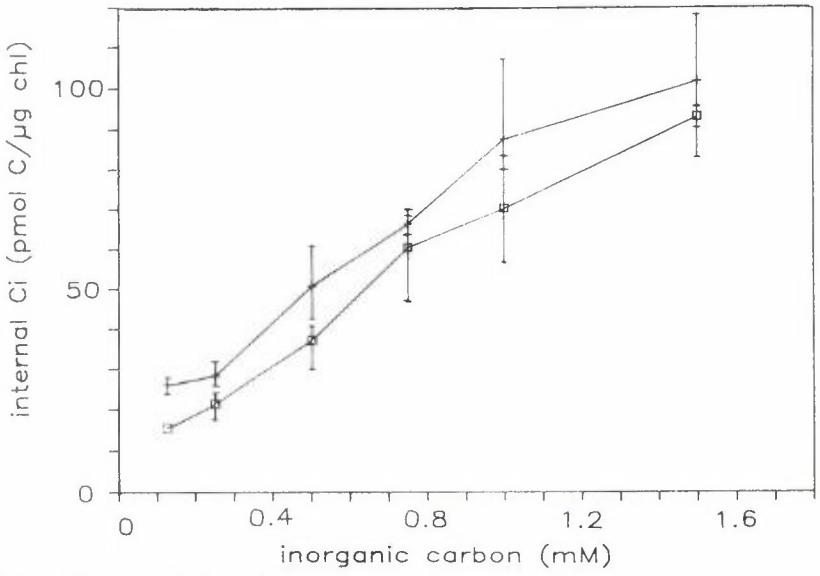

Figure 2. Internal accumulation of Ci by *F. shiloi* after 10 (□) and 60 (+) seconds as a function of external Ci concentration. Error bars indicate extreme values of triplicates. The irradiance was 6 mW cm^{-2}, the temperature was 26°C, the pH was 8.1

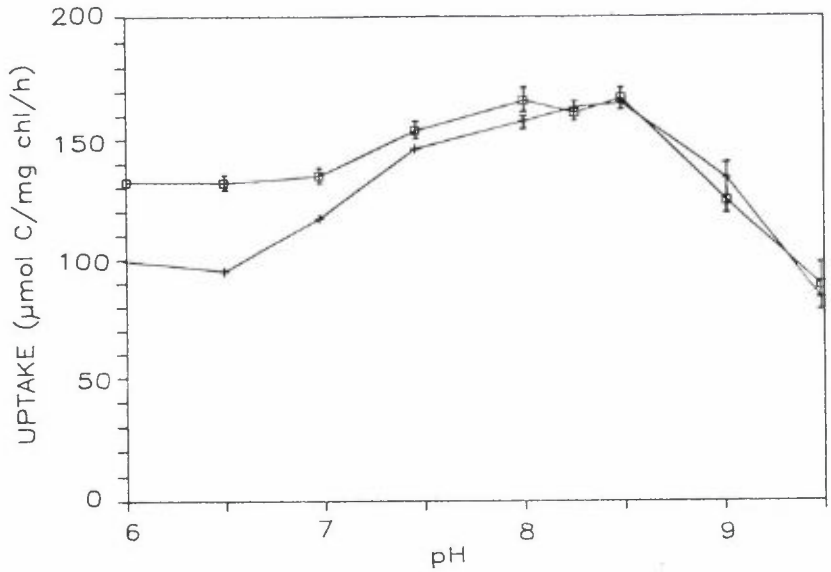

Figure 3. Dependence of the photosynthetic rates of *F. shiloi* on the pH after 2 (□) and 4 (+) hours. Error bars indicate the differences between duplicates. The irradiance was 8.5 cm^{-2}, the temperature was 28°C, the total Ci concentration was 2.1 mM.

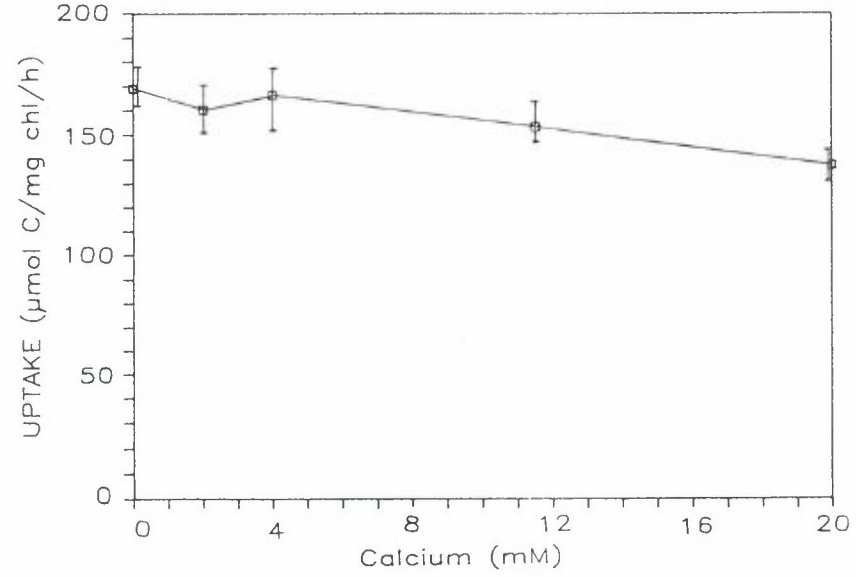

Figure 4. Photosynthetic rates of *F. shiloi* as a function of the external calcium concentration. Error bars indicate extremes of 4 values (duplicates after 2 and 4 hr). The irradiance was 8.5 mW cm^{-2}, the temperature was 28°C, the total Ci was 2.1 mM and the pH was 8.1.

mg foram^{-1}h^{-1} is fixed (ter Kuile and Erez, 1987). The chlorophyll content of *A. lobifera* is 0.10 μg chl mg foraminifer^{-1} (ter Kuile and Erez, 1984), giving a photosynthetic rate of 210 μmol C mg chl^{-1}h^{-1}. If this small difference is indeed significant, it cannot be explained by increased availability of Ci, since Ci is not growth rate limiting, nor does the host concentrate Ci actively (ter Kuile et al., in press). The photosynthetic V_{max} *in vivo* might be affected by the nutrient status of the cells. Possibly the host stimulates photosynthesis of the symbionts by concentrating nutrients. The nutrient requirements of *F. shiloi* are very high (Lee, 1983), while the nutrient levels in the Gulf of Eilat are very low (Levanon-Spanier et al., 1979). Feeding by the host may serve as a nutrient source for the symbionts of both benthonic (Lee et al., 1980a; ter Kuile et al., 1987) and planktonic foraminifera (Jorgensen et al., 1985), thereby providing the symbionts with the correct growth conditions. Possibly the suppression of frustule formation inside the host (Lee, 1980; 1983) also leads to higher photosynthetic rates as no energy is spent on their formation.

Acknowledgements

The authors wish to thank Drs. J. Erez and E. Padan for helpful discussions and Dr. J. Erez for his comments on an earlier version of this manuscript. This study was supported by the U.S.-Israel Binational Science Foundation through grant 3418-83 to Drs. J. Erez and J.J. Lee.

REFERENCES

Badger, M.R., Kaplan, A., and Berry, J.A. 1980. Internal inorganic carbon pool of *Clamydomonas reinhardtii*: Evidence for a carbon dioxide concentrating mechanism. *Plant Physiol.* **66:** 407–413.

Borowitzka, M.A. 1981. Photosynthesis and calcification in the articulated coralline red algae *Amphiroa anceps* and *A. foliacea*. *Mar. Biol.* **62:** 17–23.

Borowitzka, M.A. and Larkum, A.W.D. 1976. Calcification in the green alga *Halimeda*. III. The sources of inorganic carbon for photosynthesis and calcification and a model of the mechanism of calcification. *J. Exp. Bot.* **279:** 879–873.

Broecker, W.S. and Peng, T.H. 1982. *Tracers in the Sea.* Eldigio Press, New York.

Burris, J.E., Porter, J.W., and Laing, W.A. 1983. Effects of carbon dioxide concentration on coral photosynthesis. *Mar. Biol.* **75:** 113–116.

Gavis, J. and Ferguson, J.F. 1975. Kinetics of carbon dioxide uptake by phytoplankton at high pH. *Limnol. Oceanogr.* **20:** 211–221.

Gieskes, J.M. 1974. The alkalinity-total carbon dioxide system in seawater. In: *The Sea.* E.D. Goldberg, ed. Vol. 5. Wiley, New York, pp. 123–151.

Goldman, J.C. and Dennet, M.R. 1985. Susceptability of some marine phytoplankton species to cell breakage during filtration and post-filtration rinsing. *J. Exp. Mar. Biol. Ecol.* **86:** 47–58.

Gran, G. 1952. Determination of the equivalence point in potentiometric titrations, Part 2. *Analyst* **77:** 661–671.

Jorgensen, R.B., Erez, J., Revsbech, N.P., and Cohen, Y. 1985. Symbiotic photosynthesis in a planktonic foraminiferan *Globigerinoides sacculifer* (Brady), studied with microelectrodes. *Limnol. Oceanogr.* **30(6):** 1253–1267.

Kaplan, A., Badger, M.R., and Berry, J.A. 1980. Photosynthesis and the intracellular inorganic carbon pool in the blue-green alga *Anabaena variabilis*: Response to external CO_2 concentration. *Planta* **149:** 219–266.

Kaplan, A., Volokita, M., Zenvirth, D., and Reinhold, M. 1984. An essential role for sodium in the bicarbonate transporting system of the cyanobacterium *Anabaena variabilis. FEBS Lett.* **176:** 166–168.

Koestler, R.J., Lee, J.J., Reidy, J., Sheryll, R.P., and Xenophontes, X. 1985. Cytological investigation of digestion and re-establishment of symbiosis in the larger benthic foraminifera *Amphistegina lessonii. Endocyt. C. Res.* **2:** 21–54.

Kuile, B. ter and Erez, J. 1984. *In situ* growth rate experiments on the symbiont-bearing foraminifera *Amphistegina lobifera* and *Amphisorus hemprichii*, J. Foram. Res. **14:** 262–276.

Kuile, B. ter and Erez, J. 1987. Uptake of inorganic carbon and internal carbon cycling in symbiont-bearing benthonic foraminifera. *Mar. Biol.* **94:** 499–509.

Kuile, B.H. ter, Erez, J., and Lee, J.J. 1987. The role of feeding in the metabolisms of larger symbiont-bearing foraminifera. *Symbiosis* **4:** 335–350.

Lee, J.J. 1980. Nutrition and physiology of the foraminifera. In: *Biochemistry and Physiology of Protozoa 3.* M. Levandrowska and S. Hatner, eds., Academic Press, New York, pp. 43–66.

Lee, J.J. 1983. Perspective on algal endosymbionts in larger foraminifera. *Int. Rev. Cytol.* **14:** 49–77.

Lee, J.J. and McEnery, M.E. 1983. Symbiosis in foraminifera. In: *Algal Symbiosis.* E.L. Goff, ed. Cambridge, Cambridge University Press, pp. 37–68.

Lee, J.J., McEnery, M.E., Kennedy, E., and Rubin, H. 1975. A nutritional analysis of a sublittoral diatom assemblage epiphytic on *Entermorpha* from a Long Island salt march. *J. Phycol.* **2:** 14–49.

Lee, J.J., McEnery, M.E., and Garrison, J.R. 1980a. Experimental studies of larger foraminifera and their symbionts from the Gulf of Elat on the Red Sea. *J. Foram. Res.* **10:** 31–47.

Lee, J.J., Reimer, C., and McEnery, M.E. 1980b. The identification of diatoms isolated as endosymbionts from larger foraminifera from the Gulf of Elat (Red Sea) and the description of two new species, *Fragilaria shiloi* sp. nov. and *Navicula reissi* sp. nov. *Bot. Mar.* **23:** 41–48.

Levanon-Spanier, J., Padan, E., and Reiss, Z. 1979. Primary production in a desert-enclosed sea. *The Gulf of Elat, Red Sea Deep-Sea Research.* **26:** 673–685.

Lucas, W.J. 1983. Photosynthetic assimilation of exogenous HCO_3^- by aquatic plants. *Ann. Rev. Plant Physiol.* **34:** 71–104.

Marcus, Y., Zenvirth, D., Harel, E., and Kaplan, A. 1982. Induction of HCO_3^- transporting capability and high photosynthetic affinity to carbon dioxide by low concentration of CO_2 in *Anabaena variabilis. Plant Physiol.* **69:** 1008–1012.

Miller, A.G. and Canvin, D.T. 1985. Distinction between HCO_3^- and CO_2 dependent photosynthesis in the cyanobacterium *Synechococcus leopoliensis* based on the selective response of HCO_3^- transport to Na^+. *FEBS* **2706 187** 29–32.

Ogawa, T. and Ogren, W.L. 1985. Action spectra for accumulation of inorganic carbon in the cyanobacterium, *Anabaena variabilis. Photochem. Photobiol.* **41:** 583–587.

Raven, J.A. 1984. *Energetics and Transport in Aquatic Plants.* Liss, New York.

Strickland, J.D. and Parson, T.R. 1968. *A Practical Handbook of Seawater Analysis.* Fisheries Research Board of Canada. 310 pp.

Stumm, W. and Morgan, J. 1981. *Aquatic Chemistry.* 2nd ed. Wiley, New York, 583 pp.

Volokita, M., Kaplan, A., and Reinhold, L. 1983. Nature of the rate limiting step in the supply of inorganic carbon for photosynthesis in isolated *Asparagus* mesophyll cells. *Plant Physiol.* **72:** 886–890.

Volokita, M., Zenvirth, D., Kaplan, A., and Reinhold, L. 1984. Nature of the inorganic carbon species actively taken up by the cyanobacterium *Anabaena variabilis*. *Plant Physiol.* **76:** 599–602.

Zenvirth, D., Volokita, M., and Kaplan, A. 1984. Evidence against H^+–HCO_3^- symport as the mechanism for HCO_3^- transport in the cyanobacterium *Anabaena variabilis*. *J. Membrane Biol.* **79:** 271–274.