THE ROYAL SOCIETY

Meeting the photosynthetic demand for inorganic carbon in an alga–invertebrate association: preferential use of CO_2 by symbionts in the giant clam *Tridacna gigas*

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Unlike most marine invertebrates which excrete respiratory CO_2, giant clams (*Tridacna gigas*) must acquire inorganic carbon (C_i) in order to support their symbiotic population of photosynthetic dinoflagellates. Their capacity to meet this demand will be reflected in the C_i concentration of their haemolymph during periods of high photosynthesis. The C_i concentration in haemolymph was found to be inversely proportional to irradiance with a minimum C_i concentration of 0.75 mM at peak light levels increasing to 1.2 mM in the dark. The photosynthetic rate of isolated zooxanthellae under conditions that prevail in the haemolymph at peak light levels was significantly less than the potential P_{max} (maximum photosynthetic rate) indicating that zooxanthellae are carbon limited *in hospite*. This is consistent with previous studies on the hermatypic coral symbiosis. The P_{max} was not affected by pH but there was a dramatic increase in the half-saturation constant for C_i ($K_{0.5}(C_i)$) with increasing pH (6.5–9.0) and only a small decrease in $K_{0.5}(CO_2)$ over the same range. These results indicate that zooxanthellae in giant clams use CO_2 as the primary source of their C_i in contrast to symbionts in corals, which use bicarbonate. The physiological implications are discussed and comparison is made with the coral symbiosis.

Keywords: symbiosis; zooxanthellae; inorganic carbon; photosynthesis; dinoflagellate

1. INTRODUCTION

Dinoflagellates of the genus *Symbiodinium*, commonly referred to as zooxanthellae, live in symbiotic association with a number of, mainly tropical, marine invertebrates including corals, giant clams and sea anemones. In all cases the dinoflagellates are enclosed within part of the digestive system of the host. In the majority of these symbioses the symbiont is located intracellularly within the perialgal vacuole of cells lining the digestive cavity (Trench 1987). In the giant clam (*Tridacna gigas*) they are intercellular within tubules (Z-tubes) emanating from a diverticulum of the stomach (Norton *et al.* 1992). The metabolism of these dinoflagellates is therefore extremely interesting given that their cellular environment is significantly different from that of free-living marine algae.

In the giant clam symbiosis the Z-tubes proliferate in the mantle. Haemal sinuses pervade this tissue and the circulating haemolymph is both the medium from which zooxanthellae acquire nutrients and through which nutrients are recycled between host and symbiont (Rees *et al.* 1993*a*). Inorganic carbon (C_i) is one such nutrient. While most marine invertebrates excrete respiratory CO_2, giant clams (and other dinoflagellate symbioses) need to supply C_i to their symbiotic zooxanthellae for carbon fixation, since photosynthetically derived carbohydrate is the principal source of energy for all but some small clams (Klumpp *et al.* 1992). The importance of an efficient C_i supply system is evident given that zooxanthellae photosynthetic CO_2 fixation would completely deplete clam haemolymph of C_i in less than 13 min unless replaced by respiratory CO_2 or C_i from seawater (Rees *et al.* 1993*b*).

To date only Denis Allemand and co-workers have addressed the impact of C_i concentration and the relative concentrations of both CO_2 and HCO_3^- on photosynthesis in zooxanthellate symbioses (Al-Moghrabi *et al.* 1996; Goiran *et al.* 1996). They concluded that both corals and their freshly isolated zooxanthellae take up bicarbonate, as well as CO_2, from seawater and that zooxanthellae *in hospite* appear to be carbon limited. The proportion of the C_i species available, which is pH dependent, and C_i concentration are therefore major influences on zooxanthellae photosynthesis in hermatypic corals.

Although the same genus of dinoflagellates populates both giant clams and corals, the symbiosis differs in that the algae are intercellular rather than intracellular and therefore may differ in the mechanism of CO_2 supply. Previous studies have indicated that the photosynthetic rate of the intact clam is proportional to the concentration of haemolymph C_i (Yellowlees *et al.* 1993). However, that study did not take into account the pH of the haemolymph and therefore the relative concentrations of CO_2 and HCO_3^-. We have since demonstrated that the pH of the haemolymph fluctuates and is inversely proportional to the photon flux density (Fitt *et al.* 1995). The pH increases during the day (maximum pH 8.1) and decreases at night (minimum pH 7.2). This is presumably caused by the depletion of C_i in the haemolymph due to zooxanthellae photosynthesis (Fitt *et al.* 1995). This change will affect the equilibrium between CO_2 and HCO_3^- in the haemolymph and Z-tubes, and hence the supply of C_i to the zooxanthellae.

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The presence of a form II ribulose bisphosphate carboxylase–oxygenase (Rubisco) in *Symbiodinium* sp. (Whitney *et al.* 1995; Rowan *et al.* 1996) places additional demands on the C_i supply system. Rubisco catalyses the fixation of CO_2 in all photosynthetic organisms and the form II enzyme was previously believed to be restricted to anaerobic bacteria as it exhibits relatively low specificity for CO_2 over O_2. Indeed when the form II enzyme is expressed in cyanobacteria it is unable to fix carbon productively without CO_2 supplementation (Pierce *et al.* 1989). Therefore for an oxygenic phototroph such as *Symbiodinium* sp. to fix C_i productively, given that it only expresses a form II Rubisco, the ratio of CO_2 to O_2 at the site of Rubisco is critical (Whitney & Andrews 1998). A number of factors will have an effect on this including the concentration of available C_i, the pH (therefore the ratio of CO_2 to HCO_3^-) and the presence of a CO_2-concentrating mechanism (CCM) in the alga (Badger *et al.* 1998). The latter can take several biological forms but whatever the mechanism the net aim is to ensure that Rubisco is supplied with sufficient CO_2 to support fixation.

The purpose of this study was to determine if the haemolymph C_i concentration, like some other metabolic parameters, was influenced by symbiont photosynthesis and if this could potentially influence the rate of photosynthesis. While zooxanthellae *in hospite* are not bathed directly in haemolymph, previous results indicate that the haemolymph composition reflects the environment surrounding these dinoflagellates (Rees *et al.* 1993*a*; Fitt *et al.* 1995). The haemolymph C_i concentration is therefore a measure of the size of the C_i pool available to the zooxanthellae. With knowledge of the pH and C_i concentrations it is possible to test our hypothesis that, unlike corals, the zooxanthellae in clams preferentially absorb CO_2. This was achieved by determining the effect of C_i on the kinetics of photosynthesis over a pH range in which the ratio of CO_2 to HCO_3^- varies. The K_m and V_{max} values for photosynthesis provide measures of the affinity and uptake rates of CO_2 and HCO_3^- by the zooxanthellae.

2. MATERIAL AND METHODS

(a) *Maintenance of clams and isolation of zooxanthellae*

Clams, *T. gigas*, were obtained from the Australian Centre for International Agricultural Research (ACIAR) Giant Clam Project at the James Cook University Orpheus Island Research Station, Queensland, Australia. After collection, clams were acclimatized in the James Cook University aquarium for three weeks before experiments were commenced. Zooxanthellae were isolated from freshly killed clams by blending the mantle tissue in 0.45 µm-filtered seawater (MFSW), filtering through cheese cloth to remove any large pieces of tissue, centrifuging the filtered homogenate ($600 \times g$) for 2.5 min at room temperature, followed by washing the zooxanthellae pellet twice with MFSW. Zooxanthellae were resuspended in MFSW and cell density determined using a haemocytometer.

(b) *Haemolymph C_i determination*

Clams (12 cm) were maintained in a shallow raceway (15 cm deep) with flowing seawater in natural sunlight. Photon flux densities (photosynthetically active radiation) were measured using a Licor LI-185B meter (fitted with a 2π probe; Licor: New England, USA). Clams were removed at intervals and completely drained of seawater before collection of haemolymph. The pericardial sac was pierced and haemolymph allowed to drain into a beaker. The total C_i of seawater was measured with a CO_2 gas-sensing electrode supplied by Activon (Sydney, Australia). The electrode was sensitive within the 10–1000 ppm concentration range (approximately 0.2–30 mM), and was calibrated against standards before and after each use. Haemolymph samples (20 ml) were stirred and then adjusted to pH 4.5 with 1 M citrate buffer. The millivolt response was measured after the meter had stabilized. All solutions were at $23 \pm 1^\circ$C to eliminate temperature effects on the results.

(c) *Rate of photosynthesis*

The photosynthetic and respiration rates of zooxanthellae were measured as oxygen evolution or uptake in a Hansatech (Norfolk, UK) oxygen electrode at 29° C. The electrode was calibrated using published values for oxygen concentrations (Riley & Skirrow 1975). Zooxanthellae were isolated in MFSW as previously described, washed twice in MFSW, pelleted ($600 \times g$) and resuspended in 8 mM buffer containing 428 mM NaCl or 856 mM mannitol as a salt substitute. The buffers used were glycine (pH 9.2, 9.0 and 8.5), N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid (HEPES) (pH 8.0, 7.5 and 7.3), NaH_2PO_4/Na_2HPO_4 (pH 7.0) and 2-(N-morpholino)ethanesulphonic acid (MES) (pH 6.5). The suspension medium was bubbled with N_2 gas in the electrode prior to addition of zooxanthellae (final volume = 1.4 ml). A final zooxanthellae density of 5×10^6 cells ml^{-1} was used to simulate the relative ratio of cell density to haemolymph volume in the clam (Fitt *et al.* 1995). Following addition of zooxanthellae the cells were allowed to photosynthesize at a photon flux density of > 450 µE m^{-2} s^{-1} provided by a Schott KL1500 (Massachusetts, USA) optic fibre light source until the CO_2-compensation point was reached. The photon flux density of > 450 E m^{-2} s^{-1} is known to saturate photosynthesis in zooxanthellae (Fisher *et al.* 1985; D. Yellowlees, unpublished data). The reaction was initiated by the addition of $NaHCO_3$ solution (0.1 ml) bringing the total volume in the cuvette to 1.5 ml. Samples were stirred continuously and data were recorded and processed using the computer program Datacan v. 5.1^{TM} (Sable Systems, UT, USA). Following stabilization of the electrode response (about 1.5 min) a linear regression was fitted to the oxygen trace (> 2 min of data). If the r^2-values were less than 0.99, measurements were repeated. At low C_i concentrations (< 0.05 mM) it was not possible to obtain 2 min of data due to the rapid depletion of available C_i. In these cases only 30 s of linear data were used in the analysis; r^2-values were still greater than 0.99. Respiration rates were determined for zooxanthellae in the dark. The software package Regression M 1.23^{TM} (Blackwell Scientific Publications, Oxford, UK) was used to fit the data and determine the kinetic parameters P_{max} (maximum photosynthetic rate), $K_{0.5}(C_i)$ (half-saturation constant for C_i) and $K_{0.5}(CO_2)$ (half-saturation constant for CO_2).

(d) *Carbonic anhydrase (CA) assay*

The extra- and intracellular CA activities of zooxanthellae were determined using a modification of the method of Yang *et al.* (1985) in the presence and absence of Cl^-. Zooxanthellae (3.2×10^7 cells ml^{-1}) were suspended in either MFSW or 35 mM veronal–H_2SO_4 buffer (pH 8.3) containing 428 mM NaCl or

856 mM mannitol as a salt substitute. Total cellular CA activity (internal + external) was determined on lysed zooxanthellae which had twice been passed through a French Press (200 kPa) at 2 °C (>90% cell lysis). Extracellular CA was determined on whole cells and the difference between this and total CA activity was used to calculate intracellular CA levels. The assay mixture consisted of either MFSW, 428 mM NaCl or 856 mM mannitol buffered with 35 mM veronal–H_2SO_4 pH 8.3 (1.4 ml), zooxanthellae suspension (0.5 ml) and 80% ethanol (0.1 ml) used to solubilize the CA inhibitors acetazolamide or ethoxyzolamide. The reaction was initiated by the addition of distilled water (2 ml) saturated with CO_2 at 2 °C. All assays were performed in triplicate and appropriate blanks run in each case. A unit of CA activity was calculated from the formula $T_0/T - 1$ where T and T_0 are the times for the pH to change from 8.3 to 7.3 with and without sample.

(e) *Zooxanthellae photosynthesis after isolation*

The photosynthetic characteristics of freshly isolated zooxanthellae were measured over a period of 12 days after isolation. Zooxanthellae were isolated as previously described (§2(a)) and washed five times in MFSW before being resuspended in 1-l MFSW to a cell density of approximately 10^6 cells ml^{-1}. Penicillin G (125 mg l^{-1}), streptomycin sulphate (62.5 mg l^{-1}) and chloramphenicol (12.5 mg l^{-1}) were added to minimize bacterial growth. The culture was kept at a photon flux density of 100 μE m^{-2} s^{-1} on a 14 L:10 D cycle. After seven days the culture media was exchanged for MFSW containing penicillin G, streptomycin sulphate and chloramphenicol at the above concentrations.

Over a period of 12 days zooxanthellae were removed from the culture, pelleted at 600 × *g* and washed twice with MFSW before being resuspended in the assay media (428 mM NaCl, 25 mM 1,3-bis(tris(hyroxymethyl)methylamino)-propane(BTP) pH 8.0). Oxygen evolution was measured and analysed as described above (§2(c)).

Extracellular CA activity was also measured as previously described over the first four days of the experiment.

3. RESULTS

(a) *Depletion of haemolymph C_i*

Figure 1 shows the data from one experiment to determine the change in C_i concentration in clam haemolymph during the natural light period of a summer day. The experiment was repeated on similar-sized clams on different days and the same trends were obtained. In all experiments the haemolymph C_i concentration declined as photon flux densities increased with the minimum C_i occurring before maximum values of photon flux densities were reached. C_i concentrations slowly increased as soon as the photon flux density began to decrease. On a bright day the C_i concentration dropped to between 0.6 and 0.8 mM. Normal nocturnal values for C_i concentrations in the clams studied were around 1.2 mM.

(b) *CA activity*

CA activity was measured in suspensions of intact and lysed zooxanthellae. When zooxanthellae were suspended in mannitol the external CA activity was approximately two to seven times higher than with either NaCl or MFSW (table 1). Chloride inhibition of algal external CA has also been demonstrated in a number of other marine

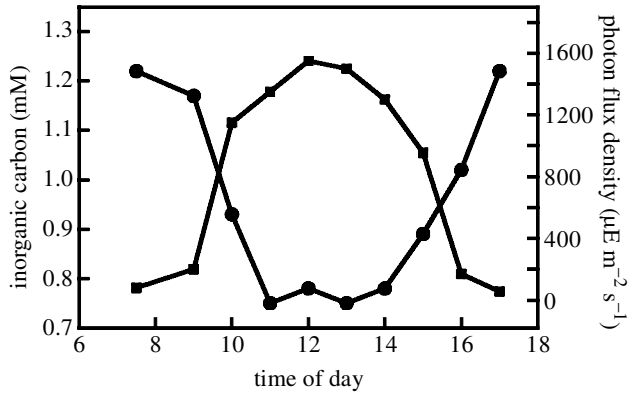

Figure 1. Mean light levels (filled squares) and haemolymph [C_i] (filled circles) of *T. gigas* over a diel cycle. Clams were killed and the haemolymph drained throughout the day and the [C_i] determined with a gas-sensitive electrode.

microalgae (Dionisio-Sese & Miyachi 1992). Internal CA levels in mannitol suspensions (0.004 ± 0.002 U per 10^6 cells) were approximately one-third of external levels (0.014 ± 0.001 U per 10^6 cells) and were not significantly different from internal activities in NaCl and MFSW suspensions (table 1). The external CA activities in MFSW reported here are similar to those found by Yellowlees *et al.* (1993); however, internal CA levels are slightly less (0.004 compared with 0.016 U per 10^6 cells). Acetazolamide (50 μM) completely inhibited external and internal CA activity in both NaCl and MFSW suspensions and produced between 50 and 96% inhibition in mannitol suspensions (data not shown).

The concentrations of acetazolamide and ethoxyzolamide that produced a 50% inhibition of extracellular zooxanthellae CA activity in MFSW suspensions were found to be 1×10^{-10} and 1×10^{-9} M, respectively (data not shown).

(c) *Variation of the rate of photosynthesis with pH*

We investigated the effect of pH on the photosynthetic rate of zooxanthellae because of the diel fluctuation of haemolymph pH, between pH 7.2 and 8.1 (Fitt *et al.* 1995), and the pH-dependent variation in C_i species.

The photosynthetic rate increased with decreasing pH when zooxanthellae were suspended in either NaCl or mannitol (figure 2). Addition of CA (100 U per 10^6 cells) had little effect on the photosynthetic rate at pH 6.5 and 7.2, but marginally increased the rate at pH 8.0 and 9.2 (figure 2).

We further investigated the characteristics of zooxanthellae photosynthesis between pH 6.5 and 9.0 and determined P_{max} and $K_{0.5}(C_i)$ values over this pH range. Changes in medium pH had a significant effect on the $K_{0.5}(C_i)$ of zooxanthellae photosynthesis without significantly changing P_{max} or respiration rates (figure 3). Control experiments were conducted to show that changes were due to a pH effect and not the use of different buffers (data not shown). $K_{0.5}(C_i)$ for photosynthesis increased with increasing pH, rising from 0.043 mM at pH 6.5 to 1.4 mM at pH 9.0 (figure 3). However, $K_{0.5}(CO_2)$ showed a slight decline, from 18 to 3 μM CO_2, over the entire pH range (figure 3). This result suggests that CO_2 is the preferred source of C_i for

Table 1. *Extracellular and intracellular CA activity of* Symbiodinium *sp. freshly isolated from the giant clam* T. gigas

(Assays were conducted in MFSW, or an artificial seawater solution containing either 428 mM NaCl or 856 mM mannitol as a salt substitute, using a modified method of Yang *et al.* (1985).)

	CA activity (U per 10^6 cells)		
	856 mM mannitol	428 mM NaCl	MFSW
extracellular activity	0.014 ± 0.001	0.0065 ± 0.0009	0.0023 ± 0.0007
intracellular activity	0.004 ± 0.002	0.002 ± 0.002	0.002 ± 0.002

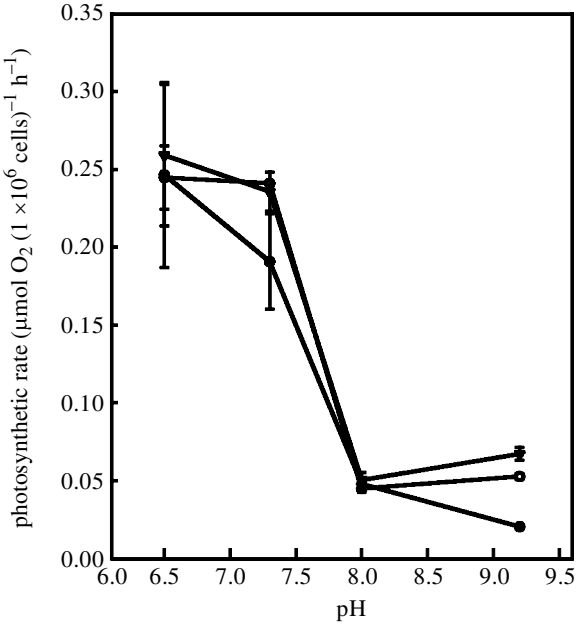

Figure 2. Effect of pH on *Symbiodinium* sp. photosynthesis in mannitol (open circles), NaCl (filled circles) and mannitol with additional CA (100 U per 10^6 cells) (filled inverted triangles) between pH 6.5 and 9.2 with 0.5 mM $NaHCO_3$ as substrate. Oxygen evolution was measured using an oxygen electrode. Error bars represent standard errors ($n = 3$).

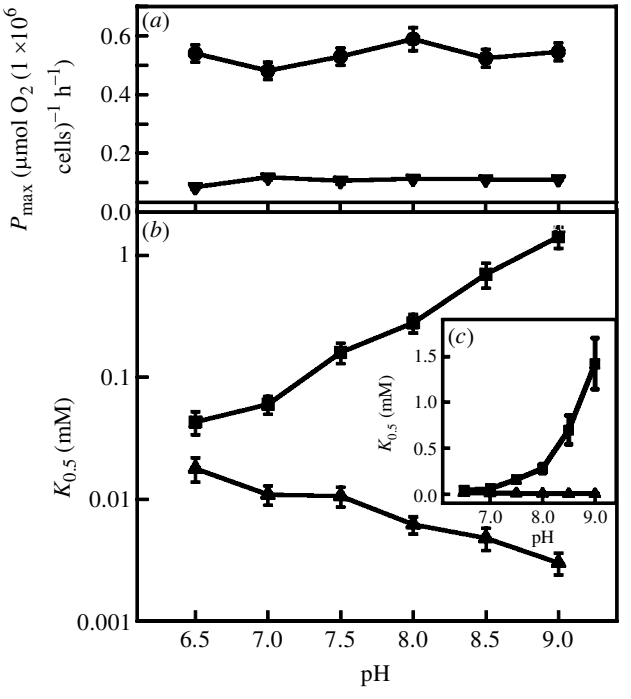

Figure 3. The effect of pH on (*a*) P_{max} (filled circles) and respiration (filled inverted triangles); (*b*, *c*) $K_{0.5}(C_i)$ (filled squares) and $K_{0.5}(CO_2)$ (filled triangles) for *Symbiodinium* sp. freshly isolated from the giant clam *T. gigas*. (*b*) and (*c*) are the same data using log and non-log scales for the $K_{0.5}$ values. Oxygen evolution and uptake were measured using an oxygen electrode and kinetic parameters determined using the program Regression M 1.23. Assays were conducted in 428 mM NaCl with 8 mM buffer, the buffers used were glycine (pH 9.0 and 8.5), HEPES (pH 8.0 and 7.5), NaH_2PO_4/Na_2HPO_4 (pH 7.0) and MES (pH 6.5). Error bars represent standard errors calculated using Regression M 1.23.

zooxanthellae when freshly isolated, while HCO_3^- provides only a small amount of C_i for photosynthesis at higher pH values, indicated by the slight decline in $K_{0.5}(CO_2)$.

(d) *Zooxanthellae photosynthesis after isolation*

Cultured zooxanthellae have been shown to have a physiology significantly different from that when they are found in symbiosis. These differences include the predominance of the coccoid non-flagellate form in symbiosis (Trench 1993), higher growth rates in culture (Cook & D'Elia 1987; Falkowski *et al.* 1993), thickening of cell walls after isolation (Schoenberg & Trench 1980) and significant changes in the protein constituents, including Rubisco and peridinin-chlorophyll *a* protein (PCP) expression (Stochaj & Grossman 1997). These differences are probably due to symbiotic zooxanthellae being found in a significantly different environment from their free-living counterparts. One major difference is that when in symbiosis zooxanthellae are associated with high levels of CA, which is found around the Z-tubes (Baillie & Yellowlees 1998), which may assist in supplying C_i to the zooxanthellae for photosynthesis. Because of these differences between cultured and symbiotic zooxanthellae and the possible role that clam CA plays in supplying C_i to the zooxanthellae we investigated zooxanthellae photosynthesis for 12 days after isolation to examine how P_{max} and $K_{0.5}(C_i)$ change.

Zooxanthellae photosynthesis was significantly altered after isolation. P_{max} approximately halved after one day, from 0.875 to 0.346 μmol O_2 per 10^6 cells h^{-1}, and remained relatively unchanged for the next 11 days (figure 4). Over the 12 days $K_{0.5}(C_i)$ also decreased from 0.33 to 0.14 mM C_i (figure 4). Extracellular CA activities were determined over the first four days of the experiment and remained constant over this time-period (data not shown).

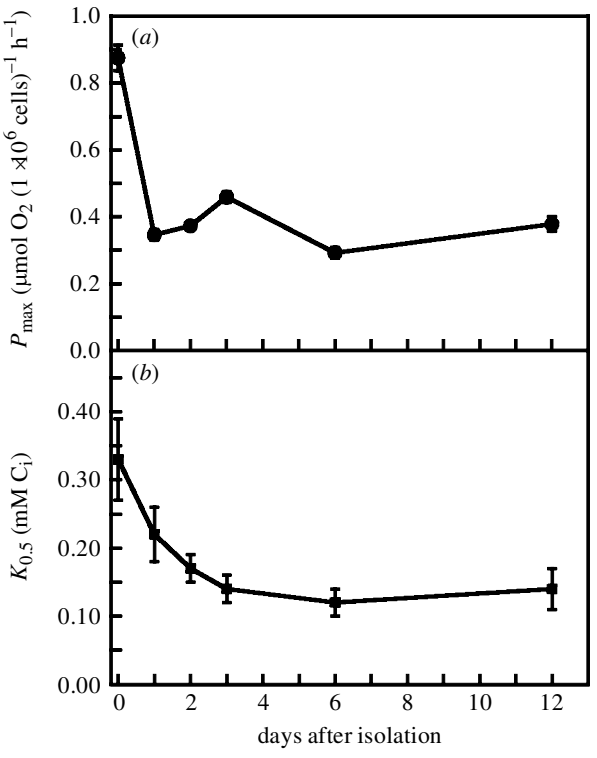

Figure 4. (*a*) P_{max} (filled circles) and (*b*) $K_{0.5}(C_i)$ (filled squares) for *Symbiodinium* sp. after isolation from *T. gigas*. *Symbiodinium* were isolated from the clam and kept in MFSW with antibiotics over the period of the experiment. Assays were conducted in 428 mM NaCl, 25 mM BTP (pH 8.0) using HCO_3^- as a substrate. Error bars represent standard errors calculated using Regression M 1.23.

4. DISCUSSION

Throughout the day significant changes occur in the physiological environment in which symbiotic zooxanthellae live within clams. These include changes in pH of the haemolymph, and most probably the Z-tubes, between 7.2 and 8.1 (Fitt *et al*. 1995), and a depletion of haemolymph C_i when compared with the surrounding seawater (Yellowlees *et al*. 1993; figure 1). These changes have significant effects on zooxanthellae photosynthesis (figure 3). We examined in detail the photosynthetic response of zooxanthellae between pH 6.5 and 9.0. Over this pH range the P_{max} was unchanged, but the $K_{0.5}(C_i)$ increased while $K_{0.5}(CO_2)$ decreased slightly. If either HCO_3^- or CO_2 were used exclusively for the C_i supply to photosynthesis it would be expected that the respective $K_{0.5}$ for the C_i species would remain constant over this pH range. The decreased affinity for CO_2 between pH 6.5 and 9.0 suggests that CO_2 is the preferred source of C_i but that at higher pH values HCO_3^- is used to some extent. This supports our previous contention that CO_2 is the preferred form of C_i for zooxanthellae in symbiosis with clams (Yellowlees *et al*. 1993). However, these results also indicate that there are significant differences between clams and corals since Goiran *et al*. (1996) demonstrated that zooxanthellae from the coral *Galaxea fascicularis* predominantly use HCO_3^-.

As well as providing information on the C_i species used, the data enable predictions to be made about the photosynthetic rates achievable by zooxanthellae when in symbiosis. It is evident from figure 1 that the haemolymph C_i concentration was depleted in clams exposed to high photon flux densities, indicating that the supply of C_i cannot meet the demand of algal photosynthesis. This depletion of C_i is consistent with the alkalinization of the haemolymph reported previously (Fitt *et al*. 1995). It is likely that the C_i depletion is even more pronounced in the Z-tubes than in the haemal sinuses due to the proximity of the algae, and, provided there is no acidification in the diverticulum, the pH will also be higher. Indeed Israel & Beer (1992) have demonstrated that the pH at the surface of the thalli of the red alga *Gracillaria conferta* increases to pH 9.4 during photosynthesis. Using the data from figures 1 and 3 and the pH change in the haemolymph (Fitt *et al*. 1995) it is possible to make some predictions about the zooxanthellae photosynthetic rate throughout the day. Assuming that conditions in the Z-tubes mirror those in the haemolymph, zooxanthellae will be C_i limited throughout the day and the P_{max} will never be attained (table 2). The highest possible rate of photosynthesis could occur at 08.00 (87% of P_{max}), but light levels (80 μE m^{-2} s^{-1}) are not saturating (Klumpp *et al*. 1992) and cannot support this level of photosynthesis. This can be contrasted to 13.00, when light was no longer limiting, but the pH was at a maximum (pH 8.14) while C_i concentration was at a minimum (0.75 mM). These conditions are the most unfavourable for photosynthesis and translate to a photosynthetic rate of only 65% of P_{max}. A more detailed knowledge of the physiological environment of the Z-tubes, to determine if they reflect conditions in the haemolymph, and the nature of any CCM is needed before we can fully understand the C_i supply system to the zooxanthellae *in hospite*. Experiments with other marine symbioses, including sea anemones and corals, have also found that *in hospite* zooxanthellae appear to be C_i limited (Weis 1993; Yellowlees *et al*. 1993; Goiran *et al*. 1996).

The relatively high $K_{0.5}(CO_2)$ of freshly isolated zooxanthellae is indicative of microalgae grown under high C_i. It appears that when isolated, zooxanthellae undergo two changes to their photosynthetic characteristics; P_{max} was halved after one day of isolation while there was a slower decrease in $K_{0.5}$ over four days (figure 4). From this data it is difficult to determine what these changes in photosynthesis are due to. However, the decrease in $K_{0.5}$ is consistent with the induction of a CCM, which would allow use of a greater proportion of the available C_i. More sophisticated methods must be used to determine if zooxanthellae do possess an inducible CCM.

The above results are at variance with those found by Goiran *et al*. (1996) who compared freshly isolated and cultured zooxanthellae from *Galaxea fascicularis*. They found that P_{max} was approximately 12-fold greater and $K_{0.5}(C_i)$ between two- and fourfold greater in cultured than in freshly isolated zooxanthellae. Lesser & Shick (1989) also found that cultured zooxanthellae from *Aiptasia pallida* had a P_{max} approximately 1.4 times that of freshly isolated zooxanthellae. These differences exhibited by zooxanthellae from the various hosts (clams versus corals–anemones) and their preference for different forms of C_i (CO_2 versus HCO_3^-) probably reflect the acclimation of zooxanthellae to the different symbiotic environments in clams and corals. Indeed Allemand *et al*. (1998)

Table 2. *Calculated photosynthetic rate of* Symbiodinium *sp. when in symbiosis with* T. gigas

(Estimates were made assuming conditions in the Z-tubules are similar to those in the haemolymph. Light and haemolymph C_i levels are from figure 1, haemolymph pH is from Fitt *et al.* (1995), while $K_{0.5}(C_i)$ is from figure 3. The estimated P_{max} of freshly isolated zooxanthellae is 0.54 μmol O_2 per 10^6 cells h^{-1}) (figure 3).)

time	photon flux density ($\mu E\,m^{-2}\,s^{-1}$)	haemolymph pH	haemolymph $[C_i]$ (mM)	$K_{0.5}$ (mM)	estimated photosynthetic rate (μmol O_2 per 10^6 cells h^{-1}) *in hospite*	per cent of estimated P_{max}
08.00	80	7.50	1.22	0.161	0.47	87
10.00	1150	7.60	0.93	0.186	0.44	82
13.00	1500	8.14	0.75	0.410	0.35	65
15.00	170	7.80	0.89	0.249	0.42	77
17.00	56	7.55	1.22	0.173	0.47	86

in reviewing carbon acquisition in corals propose that the host cells have a CCM or 'at least a carbon-transporting mechanism' which incorporates a bicarbonate transporter. This intracellular existence of zooxanthellae clearly represents a different physiological environment from the intercellular zooxanthellae in clams.

The pH sensitivity and relatively high $K_{0.5}(C_i)$ of freshly isolated zooxanthellae are indicative of unicellular microalgae grown at high C_i concentrations. Also, as indicated above, C_i would appear to be transported into the dinoflagellate primarily as CO_2. This begs the question as to how the CO_2 is made available to the zooxanthellae from the total C_i pool and whether it can be supplied in sufficient concentration to obviate the need for a CCM. Given our current lack of knowledge regarding the physical environment of the Z-tubes it is impossible to draw any firm conclusions. However, three possibilities are that the Z-tubes are acidic, ensuring a plentiful supply of CO_2; the C_i is efficiently converted to CO_2 by host CA prior to uptake; or there is a CCM in these dinoflagellates.

Since the Z-tubes are an offshoot of the digestive system it is not unreasonable to suggest that their contents could be acidic. A pH of less than 6.0 would ensure that the majority of C_i would be present as CO_2. The second possibility is supported by the presence of external CA in the zooxanthellae, although activity is low when compared with that in other unicellular algae that use this enzyme for C_i uptake (Burns & Beardall 1987). There is also a high concentration of CA in the host mantle, which could be involved in CO_2 supply (Yellowlees *et al.* 1993; Baillie & Yellowlees 1998). Both sea anemones and corals also possess CA associated with the perialgal vacuole and in the case of the former this is induced upon the establishment of the symbiosis (Weis 1991). Although this CA would not increase $[C_i]$ it may maintain the equilibrium between CO_2 and HCO_3^- thereby maximizing the available CO_2 for uptake. Despite this, CA would not appear to be a critical factor in C_i acquisition in clams since the addition of a high concentration of CA did not significantly increase the rate of photosynthesis at high pH (figure 2). In addition there was no observed difference between P_{max} and $K_{0.5}$ of zooxanthellae suspended in NaCl or mannitol (data not shown). The presence of a CCM in the zooxanthellae is probably required whether the pH is acidic or CA is needed for C_i acquisition; the high $K_{0.5}(C_i)$ combined with the presence of a form II Rubisco would appear to make this obligatory in order to raise the CO_2 levels available to Rubisco. The changes observed in the $K_{0.5}(C_i)$ of zooxanthellae when isolated indicate that the affinity for C_i increases, perhaps due to the induction of a CCM.

The differences in C_i acquisition between zooxanthellae from corals and clams could be due to different taxa or adaptation to either the intra- or intercellular environment. There is no evidence at present to suggest that zooxanthellae taxa differ according to host specificity. Indeed our results support the opposite (M. Ten-Lohuis, personal communication). More likely this is a further illustration of how the host environment influences the metabolic strategy of the zooxanthellae. Given our poor understanding of the immediate environment surrounding the zooxanthellae we would endorse the view of Allemand *et al.* (1998) that this should be a high priority for future research.

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