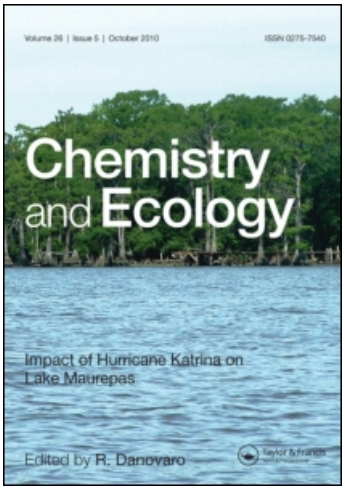

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Coral calcification: Use of radioactive isotopes and metabolic inhibitors to study the interactions with photosynthesis and respiration

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Coral calcification: Use of radioactive isotopes and metabolic inhibitors to study the interactions with photosynthesis and respiration

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In order to characterize the process of calcification in scleractinian corals, a series of laboratory experiments were conducted using radioactive isotopes. Labelled calcium, bicarbonate and glucose were used and the fates of the labelled tracers were followed in the skeleton and the tissue fractions of the coral *Galaxea fascicularis.* In addition, a variety of metabolic inhibitors were used to test the effects of various enzymes and processes on the incorporation rates. The incorporation rate of ^{45}Ca into the coral skeleton decreased to about one-fifth upon inhibition of metabolic respiration by the specific inhibitor NaCN suggesting a major role of metabolic respiration in coral calcification, and decreased to one-half upon inhibition of carbonic anhydrase by the specific inhibitor acetazolamide indicating a role of the enzyme in the process. The results obtained have also shown that corals are able to incorporate carbon from seawater bicarbonate and added glucose in both skeleton and tissue fractions. The process of incorporation was influenced by light conditions, carbonic anhydrase, respiration and photosynthesis. The incorporation rate of ^{14}C-HCO_3^- was reduced to about one-tenth in the skeleton, and one-fifth in the tissue, upon inhibition of carbonic anhydrase suggesting a major influence of the enzyme in the incorporation process. The inhibition of photosynthesis had more influence on the tissue incorporation rate of the tracer than the skeleton suggesting that photosynthesis is the main process responsible for tissue use of seawater bicarbonate in the coral. ^{14}C-glucose incorporation into the skeleton was mainly affected by NaCN addition and to a lesser extent by dichlorophenyldimethylurea (DCMU) addition, while the tissue fraction was mainly affected by NaCN addition. It was concluded that respiration and photosynthesis, in addition to the enzyme carbonic anhydrase, are limiting factors for the process of calcification in the coral, and that various forms and sources of carbon can be used in coral calcification and tissue growth.

Keywords: Coral; Calcification; Photosynthesis; Respiration; Metabolic inhibitors; Radioactive isotopes

1. Introduction

Corals live in an obligate, mutualistic, symbiotic relationship with multi-species communities of the dinoflagellate *Symbiodinium* sp. (commonly known as zooxanthellae) contained within

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the endodermal cells of the animal host [1]. With respect to feeding behaviour, both autotrophy and heterotrophy co-exist in corals [2–4].

The main metabolic processes in the coral which influence the carbon chemistry in the sea are photosynthesis by the symbiont, respiration by the animal host and the symbiont, and calcification by the coral. In photosynthesis, CO_2 is fixed into organic carbon and O_2 is released as a byproduct, while in respiration, organic carbon and O_2 are consumed and CO_2 is released. In calcification, $CaCO_3$ in the form of aragonite [5] is deposited to form supportive skeletons. The association between calcification and photosynthesis was acknowledged after the observations that many calcifying marine organisms have the ability to photosynthesize, and that the rate of calcification is higher in light conditions than in dark conditions [6, 7]. Because of the strong relationship between these metabolic processes and the seawater chemistry—which is affected by increased atmospheric CO_2 and global climate change—it is important to understand this relationship for better prediction of future impacts of global climate change on the world's coral reefs. Several controversial hypotheses have been proposed to explain the interactions between photosynthesis and calcification. Among these are: zooxanthellae enhance calcification by supplying energy and removing the metabolic wastes [6, 8, 9]; calcification helps assimilation of bicarbonate and nutrients by generating protons [10]; calcification does not enhance photosynthesis [11, 12]; and both processes are more efficient in the co-existing system than when reactions are isolated [13].

Both photosynthesis and calcification require a continuous supply of inorganic carbon. The concentration of dissolved inorganic carbon in the sea is about 2.4 mM, mostly present in non-permeable forms, HCO_3^- and CO_3^{2-}. The carbonate ion formation is governed by the following reactions:

$$H_2O + CO_2 \Longleftrightarrow H_2CO_3 \Longleftrightarrow H^+ + HCO_3^- \Longleftrightarrow 2H^+ + CO_3^{2-}$$

The direction of the above reactions is subject to a number of environmental parameters such as atmospheric CO_2 concentration and seawater pH; two parameters expected to change in future. The key photosynthetic enzyme, ribulose bisphosphate carboxylase oxygenase (RuBisCO) uses CO_2 as a substrate [14, 15]. Calcification precipitates CO_3^{2-}, which must be available at the calcifying site (*i.e.*, between the tissue layer and the skeleton). Corals, like many other marine photosynthetic organisms, face the problem of supplying CO_2 to the enzyme RuBisCO. Therefore, a carbon-concentrating mechanism, through which active HCO_3^- transport elevates CO_2 concentrations around RuBisCO to increase the photosynthetic efficiency, has evolved in corals and many marine organisms [15–18]. The enzymes carbonic anhydrase and H^+-ATPase help in the uptake process of inorganic carbon from the sea. The CO_2 produced in the metabolic respiration of the photosynthates, and the uptake of particulate matter from the sea, are potentially used in photosynthesis and calcification [19]. However, it is not known where the exact source of carbon in the $CaCO_3$ skeleton comes from. In this study, a series of incubation experiments of the coral *Galaxea fascicularis* were conducted in the presence of labelled bicarbonate and glucose in both light and dark conditions to determine the sources of carbon used in coral calcification and the role of light in the process. The importance of the different enzymes and metabolic processes in coral calcification were also tested through the use of specific inhibitors.

2. Materials and methods

2.1 *Biological samples*

Galaxea fascicularis colonies were collected by SCUBA from a depth of 5 m, south of the Marine Science Station in the Gulf of Aqaba, Jordan. The collected colonies were maintained

under optimal conditions in an aquarium of about 600 L supplied with synthetic seawater (35 salinity, pH around 8.2, light intensity of 140 μmole photons m^{-2} s^{-1}, temperature around 26°C) and were illuminated with a HQI-lamp with a light spectrum and intensity similar to the natural light (12hr:12hr light–dark cycle). Single polyps were fixed on small glass vials with underwater epoxy and left in the aquarium until small colonies developed for use in the incubation experiments.

2.2 *Inhibitors*

The specific inhibitor of photosystem II (PSII), dichlorophenyldimethylurea (DCMU), was dissolved in ethanol and added to a final concentration of 1 μM. Acetazolamide (AZ), a specific inhibitor of carbonic anhydrase, was dissolved in dimethylsulfoxide (DMSO) and added to a final concentration of 600 μM. NaCN, a specific respiratory inhibitor, was dissolved in water and added to a final concentration of 1 mM. The final concentration of DMSO and ethanol was 0.1%.

2.3 *Incubation with radioactive tracers*

Coral colonies were incubated in a beaker containing 400 ml of filtered artificial seawater prepared from tropical marine salt (35 salinity, pH 8.2). The water was stirred and the temperature was maintained at 26°C. The incubation period was 5 hours in all experiments. Prior to addition of the radiotracer, the coral pieces were left for at least 1 hr to recover from the transfer and acclimatize to the incubation conditions. Radioactive tracers, ^{45}Ca as $CaCl_2$, ^{14}C-glucose and ^{14}C-HCO_3^- (Amersham Pharmacia Biotech, UK) were added to a final activity of 3 KBq/ml in all experiments. The specific activities were 74.0 MBq/ml for calcium, 2.0 GBq/mmol for bicarbonate and 12.0 GBq/mmol for glucose.

Two experimental conditions were applied; the first was illumination with 140 μ mole photons m^{-2} s^{-1}, and the second was dark incubation. Controls for isotopic exchange were done with dead specimens killed by 2% formaldehyde.

2.4 *Processing of the samples after incubation*

After the incubation, the coral colonies were extensively washed in 2 L filtered seawater (two successive times, 5 minutes each). After washing, single polyps were separated from the mother colony with a wire cutter. The skeletons that were not covered with tissue were discarded. Following this, the colonies and the polyps were heated in 2 N NaOH at 90°C for 20 minutes to hydrolyze the tissue. The skeletons were washed several times with NaOH solution to remove the tissue within the corallite. Decay per minute (dpm) in the hydrolysate was determined using a Packard TR 2500 scintillation counter operating in efficiency tracing mode to correct for quench. The skeletons were then washed with distilled water to remove nonspecifically bound tracer and dried overnight at 50°C. The next day, the skeletons incubated in ^{45}Ca were weighed and hydrolyzed in 12 N HCl solution and the activity was counted. CO_2-trapping (for the colonies labelled with ^{14}C) was done according to the method of Boetius *et al.* [20].

3. Results

The incorporation of ^{45}Ca in the coral skeleton was higher in light than in dark incubated colonies. Both treatments had much higher incorporation rate compared with the dead

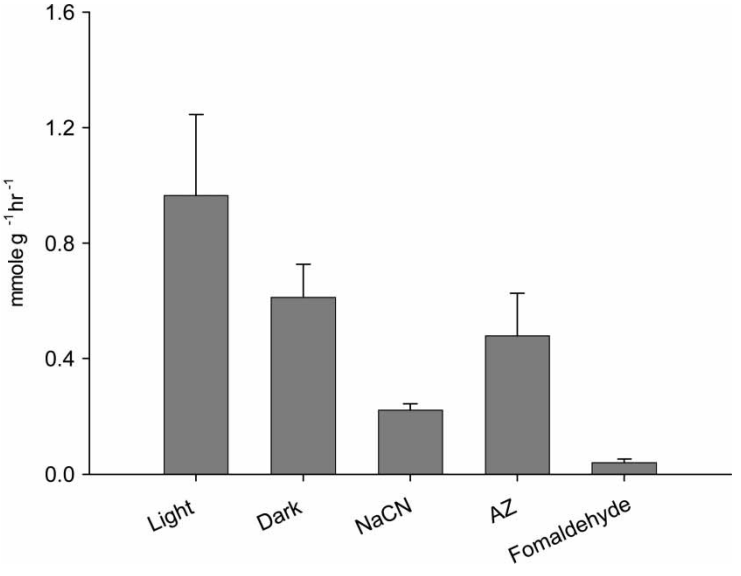

Figure 1. Rates of ^{45}Ca incorporation into the coral skeleton under light and dark conditions and addition of NaCN, acetazolamide (AZ) and formaldehyde in light.

(*i.e.* formaldehyde treated) colonies. Upon addition of NaCN, a specific respiratory inhibitor, ^{45}Ca incorporation rate into the coral skeleton was reduced to about 23% relative to the original control rate in light (figure 1). Addition of acetazolamide, the specific carbonic anhydrase inhibitor, decreased the rate of ^{45}Ca incorporation into the coral skeleton to about 50% relative to its rate in light (figure 1). On the other hand, light and dark treatments had no effect on the incorporation rate of the radioactive tracer in the coral tissue as both light and dark treated colonies had similar incorporation rates as the dead colonies (figure 2).

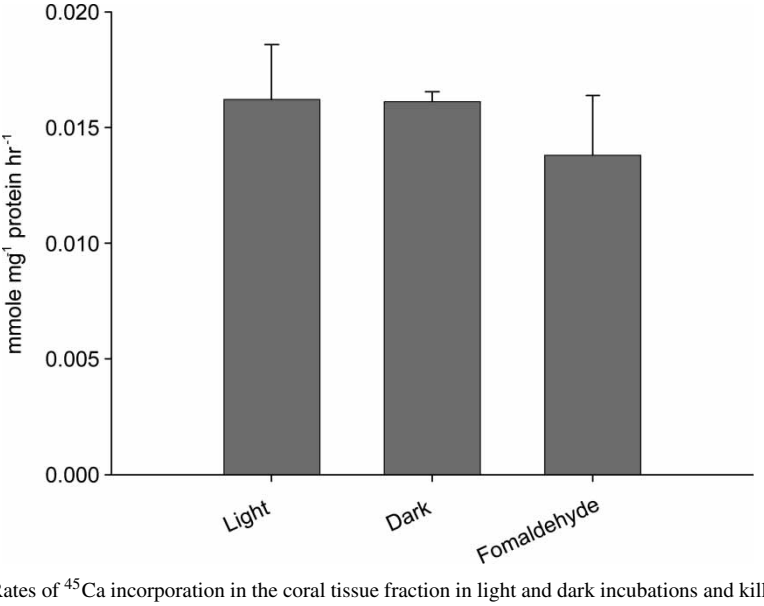

Figure 2. Rates of ^{45}Ca incorporation in the coral tissue fraction in light and dark incubations and killed colonies.

Upon incubation of the coral in seawater containing ^{14}C-labelled HCO_3^-, the coral skeleton showed a higher incorporation rate of the radioactive tracer in light compared with dark conditions (figure 3). The tissue rate of incorporation of the radioactive tracer was influenced more greatly by the light and dark incubations than the skeleton. The rate of incorporation was about 10 folds higher in light compared with dark incubated colonies (figure 4). Addition of acetazolamide greatly reduced the incorporation rate of ^{14}C from bicarbonate in the coral skeleton in both light and dark conditions. The rate was reduced to about 12% and 19% relative

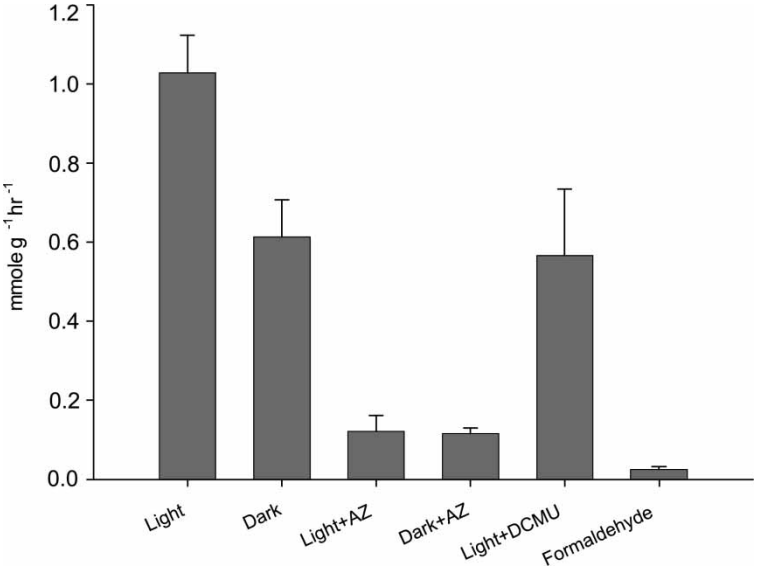

Figure 3. Rates of ^{14}C-bicarbonate incorporation into the coral skeleton under different incubation conditions. (AZ – acetazolamide.)

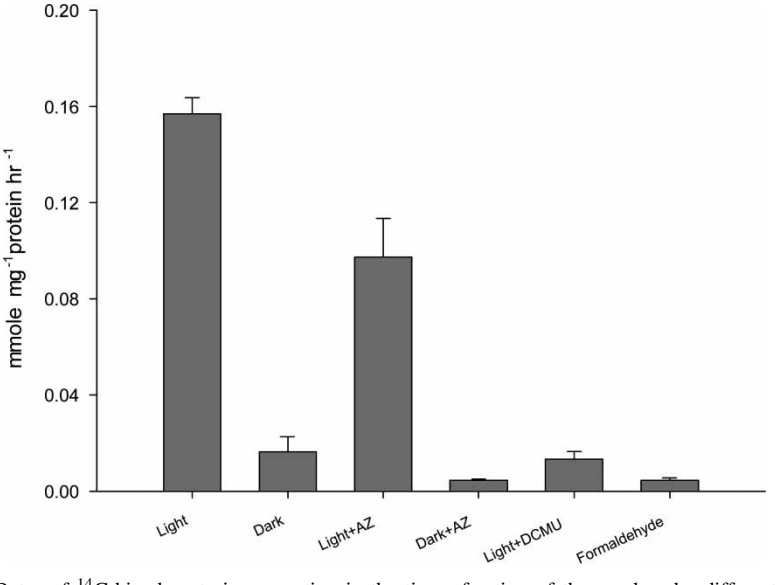

Figure 4. Rates of ^{14}C-bicarbonate incorporation in the tissue fraction of the coral under different incubation conditions. (AZ – acetazolamide.)

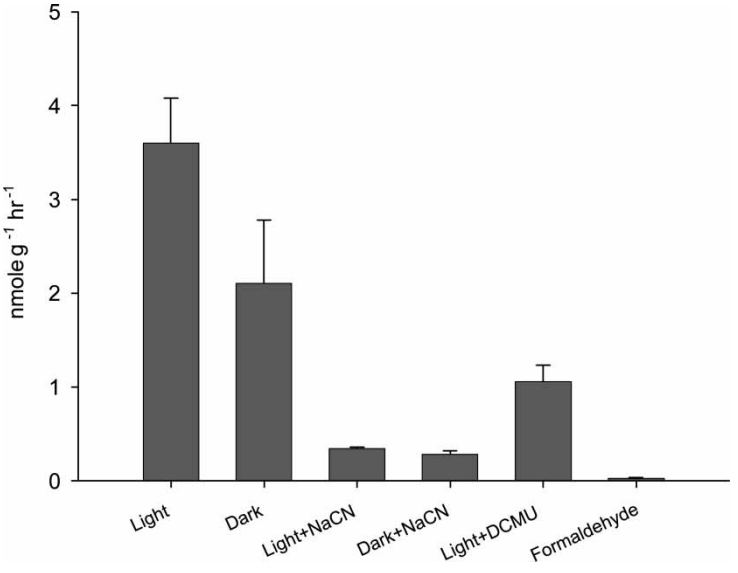

Figure 5. Rates of ^{14}C-glucose incorporation into the coral skeleton under different incubation conditions.

to the control rates in light and dark conditions, respectively (figure 3). The tissue fraction was influenced by acetazolamide addition to a lesser extent. The rate was reduced to about 62% and 27% relative to the control rates in light and dark conditions, respectively (figure 4). DCMU, the specific PSII inhibitor, reduced the rate of ^{14}C-HCO_3^- incorporation to about 55% in the skeleton and 8% in the tissue fraction (figures 3 and 4).

When coral colonies were incubated in seawater containing ^{14}C-labelled glucose, the skeleton fraction was more active in light than in dark in the uptake process of the tracer, although the rates of incorporation in both conditions were very high compared with the killed colonies

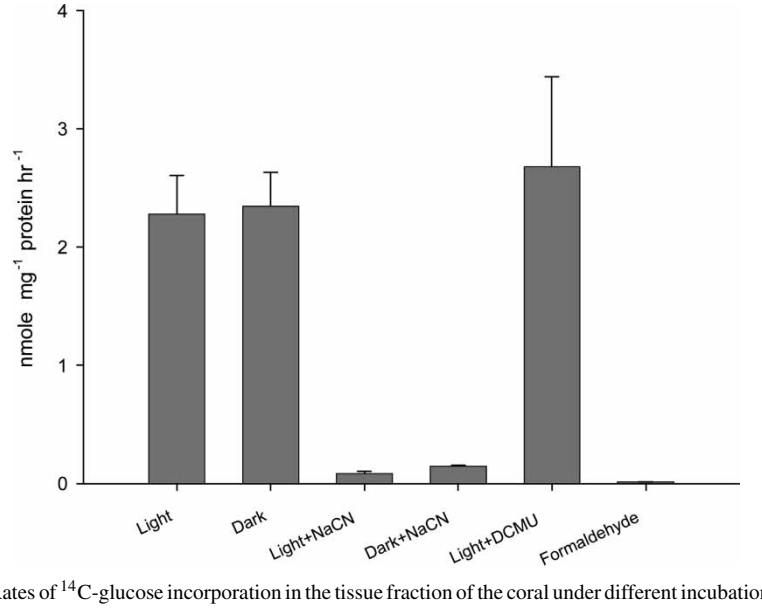

Figure 6. Rates of ^{14}C-glucose incorporation in the tissue fraction of the coral under different incubation conditions.

(figure 5). Addition of NaCN to the incubating seawater greatly reduced the rate of ^{14}C incorporation in the coral skeleton to about 10% and 13% relative to the control rates in both light and dark conditions, respectively. Addition of DCMU reduced the rate of incorporation to about 29% relative to the control rate in light (figure 5). Contrary to the skeleton fraction, light and dark treatments had no influence on the rate of ^{14}C incorporation in the tissue fraction. Both treatments had similar incorporation rates, though it was much higher than the rate of incorporation in the killed colonies (figure 6). NaCN greatly reduced the rate of incorporation to about 4% and 6% relative to the control rates in both light and dark conditions, respectively. Upon addition of DCMU, the rate was slightly increased relative to the control rate in light (figure 6).

4. Discussion

The inhibition of respiration by NaCN reduced the rate of ^{45}Ca incorporation into the skeleton to about one-fifth of its control rate in light (figure 1), which demonstrates the energy requirement for calcification and the role of metabolic respiration in this regard. The results obtained are similar to those obtained by Tambutte *et al.* [21] in corals and ter Kuile *et al.* [22] in foraminifera. The 80% decrease in the rate of calcification upon addition of NaCN suggests that oxidative phosphorylation is supplying most of the ATP used in coral calcification as it was also suggested elsewhere [9, 23, 24]. In coral calcification, energy is required for the transport of ions and synthesis of the organic matrix, which is necessary for crystal nucleation and growth [9, 25, 26–29]. Ca-ATPases and H^+-ATPase, which are involved in ion transport, also require ATP [29, 30–33]. In addition to oxidative phosphorylation, cyclic photophosphorylation and glycolysis also supply some of the ATP used in calcification and other metabolic activities [34–36].

The enzyme carbonic anhydrase catalyzes the interconversion between CO_2 and HCO_3^-. It has been suggested that this enzyme facilitates in the supply of CO_2 for calcification and photosynthesis from the surrounding seawater [17, 18]. Both, external and internal enzymes exist in corals [19]. The use of the impermeable carbonic anhydrase inhibitor, acetazolamide, inhibits only the external enzymes [37]. This enzyme inhibition reduced ^{45}Ca incorporation in the coral skeleton by one-half of its control rate. The rate reduction could have resulted from either a direct inhibition of calcification by reducing carbon supply and/or indirectly by reducing the rate of photosynthesis, which is known to enhance calcification in corals. In either case, the enzyme proved to be necessary for coral calcification. The tissue fraction did not incorporate ^{45}Ca (figure 2) as the coral actively pump Ca^{2+} out of the cells and maintain very low intracellular Ca^{2+} concentrations [30, 38].

Carbonic anhydrase inhibition also inhibited the incorporation of ^{14}C labelled bicarbonate into the coral skeleton (figure 3). Carbon from bicarbonate is incorporated into the skeleton by direct use of bicarbonate ions in calcification [17] or indirectly through the C-cycle, where it is used by photosynthesis and the photosynthates from photosynthesis, and/or the CO_2 from respiration can potentially be incorporated into the coral skeleton [19]. The tissue fraction was less influenced by this enzyme inhibition compared with the skeleton fraction (figure 4). The results obtained suggest that calcification is mainly dependent on seawater for its carbon supply. Although it was estimated by Goreau [39] that seawater bicarbonate supplies 40% of the carbon used in photosynthesis and calcification, and 60% is obtained from recycled respiratory CO_2, it is not possible to give good estimations for the fraction of carbon used in each process. This is due in part to the fact that carbon from various sources is pooled and cycled many times inside the coral before ending up in a given destination [19]. The tissue

incorporation rate of the radiotracer was more affected by carbonic anhydrase inhibition in dark condisions (figure 4). This could be due to the fact that photosynthesis—the main process responsible for bicarbonate incorporation in the coral tissue—ceases in the dark and seawater bicarbonate therefore becomes the main source of carbon used in the coral tissues. As such, upon inhibition of carbonic anhydrase, this source of carbon is greatly reduced (although bicarbonate transporters were also suggested in corals) [17]. Almost all tissue incorporation of ^{14}C-bicarbonate ceased upon inhibition of photosynthesis by DCMU (figure 4). This result confirms the previous conclusion about the role of photosynthesis in incorporating ^{14}C from seawater bicarbonate. On the other hand, photosynthesis inhibition reduced the skeleton incorporation of ^{14}C-bicarbonate by about one-half. This could be due to the fact that either the calcification rate was reduced upon photosynthesis inhibition and/or that carbon supply through photosynthesis was stopped. There are several means by which the decreased photosynthesis rate leads to a decrease in calcification rate, of which the supply of energy needed by calcification is reduced by reducing oxygen and/or the reduced organic carbon (*i.e.* the photosynthates) supply, which are used in respiration to generate ATP to power calcification. This process was suggested to be responsible for the enhanced rate of calcification in light [9, 40]. The corals possess a host factor that induce the release of photosynthates from the symbiotic dinoflagellates [41–43]. It was estimated that the symbiotic algae in corals supply about 70% of the daily respiratory demand of the animal host and the remainder is presumed to be satisfied by the intake of particulate organic carbon [3]. Another way by which photosynthesis influences calcification is through increasing the pH by uptake of CO_2 [44]; a condition required for coral calcification [28]. It was shown that reef corals calcify at a faster rate in the presence of non-calcareous algae [45]. In addition to being a source of energy, the photosynthates produced by the symbiont are used in the synthesis of the organic matrix, which is required for calcification to proceed [39, 46].

The tight coupling between photosynthesis and calcification in corals and other marine calcifying photosynthesizing organisms may have important implications on the role this relationship plays in regard to the global increase of atmospheric CO_2. The expected increase in atmospheric CO_2 will lead to a decrease in seawater pH and calcium carbonate saturation state, which in turn will lead to a decrease in calcification and thereby threaten the coral reefs of the world [47, 48]. Because calcification releases 1 mole of CO_2 for each mole of calcium carbonate precipitated, the decreased calcification rate will have a negative impact on the atmospheric CO_2 [49]. On the other hand, photosynthesis, which consumes seawater carbon, may increase in response to increased atmospheric CO_2 [50]. Therefore, the increase in photosynthesis might alleviate the effect of CO_2 increase on calcification since photosynthesis is known to enhance calcification. Further studies are needed to study the effect of increased atmospheric CO_2 on the combined photosynthesis and calcification systems and there effects on the carbon budget of seawater.

Although glucose exists in low concentrations in seawater, it was used here to test the ability of the coral to utilize it as an energy and carbon source. It was demonstrated previously that corals are able to take glucose, glycerol and protein hydrolysate from solution [51–53]. Corals can use glucose either through breaking it in the metabolic pathways to extract energy and CO_2, or through using it in biosynthesis as a building block. It was suggested that glycolysis supplies some of the energy needed by the coral, while the resulting outputs of glycolysis can also be further broken down to CO_2 through the tricarboxylic acid cycle (TCA) where CO_2 and energy are produced [35]. The CO_2 in this case can be recycled through the C-cycle [19], or can be used in calcification through the reactions catalyzed by carbonic anhydrase [31]. When this source of CO_2 is fixed by photosynthesis, it allows the coral to transform glucose into other organic carbon forms [54], which enhance calcification when translocated to growing coral tips [55]. With this in mind, the labelled carbon from glucose might end up in the tissue

fraction and/or in the skeleton fraction of the coral. The use of NaCN, greatly reduced the skeleton rate of tracer incorporation to about 10% in both light and dark conditions (figure 5). This suggests that carbon coming from reduced organic carbon sources is indeed involved in coral calcification and that metabolic respiration is the main process that supplies energy for calcification. The inhibition of photosynthesis reduced the incorporation rate by about 70% relative to the control rate, which suggests that much of the glucose must be recycled through photosynthesis after its breakdown to CO_2. On the other hand, almost all the tissue incorporation rate of the tracer was inhibited by NaCN in both light and dark conditions, indicating that tissue use of glucose is solely controlled by its normal catabolic pathways (*i.e.* glycolysis and the TCA cycle). Upon inhibition of photosynthesis, the tissue rate of tracer incorporation was slightly increased indicating that photosynthesis is not needed for the tissue to utilize glucose, unlike the skeleton. Similar results were obtained by Borowitzka and Larkum [56] where the CO_2 evolution was stimulated in the green alga *Halimeda* upon inhibition of photosynthesis.

In conclusion, calcification in corals is governed by a set of physiological and environmental conditions. The process is dependent on energy supply mainly through metabolic respiration which obtains the reduced carbon compounds either from the symbiont photosynthesis or through uptake from the surrounding environment. Both, organic and inorganic carbon sources are used in calcification with the enzyme carbonic anhydrase being imperative for the uptake of bicarbonate from seawater. Photosynthesis is crucial for the survival of corals in an environment described as oligotrophic and is necessary for supplying energy, organic carbon sources and removal of CO_2. The relationship between photosynthesis and calcification suggests an important role in determining the influence of the expected global increase of atmospheric CO_2 on coral reefs, which should be further investigated.

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