Author response to comments by Anonymous Reviewer #2

In summarising, the reviewer questions the likelihood that alga-cnidarian symbioses are ever CO_2-limited, stating quite rightly that dissolved inorganic carbon (DIC = CO_2 + HCO_3^- + CO_3^{-2}) is abundant in seawater (2.0 – 2.2mM); and noting that future predicted changes in atmospheric pCO_2 will only serve to further increase the readily diffusible supply of CO_2 used in photosynthetic carbon-fixation. In response, I will endeavour here to strengthen the arguments outlined in the manuscript, which propose that: (i) CO_2-limitation can occur in alga-cnidarian symbioses at high irradiances, (ii) CO_2-limitation can trigger bleaching (= alga expulsion), and (iii) Bleaching triggered by CO_2-limitation can be exacerbated by future increases in pCO_2.

Meeting the photosynthetic demand for CO_2 in alga-cnidarian symbioses

(a) CO_2-limitation in alga-cnidarian symbioses

The dinoflaggelate algae (= zooxanthellae) within cnidarians symbioses utilise the Calvin–Benson cycle to fix CO_2 (Streamer et al., 1993). However, unlike other oxygenic phototrophs, zooxanthellae possess a Form II Rubisco enzyme that has a poor ability to discriminate between CO_2 and O_2 (Rowan et al., 1996). This enzymatic constraint requires that an elevated concentration of CO_2 be maintained around Rubisco to ensure continuous carbon fixation by the 'dark reactions' of photosynthesis (Leggat et al., 2002). The intracellular location of the zooxanthellae dramatically affects the source and reliability in supply of this CO_2. Muscatine et al. (1989) explained that at low levels of solar irradiance, respiratory CO_2 arising from zooxanthellae and host metabolism is largely sufficient to meet the photosynthetic demand for CO_2. This contrasts with the high solar irradiance condition, when the zooxanthellae become heavily reliant on the host to supplement the internal metabolic supply with CO_2 obtained from the much larger seawater pool (Muscatine et al., 1989). Although seawater CO_2 can freely diffuse across the lipid bilayers of the host, at typical seawater pH (8.1-8.2) it represents only a small fraction (1-2%) of the dissolved inorganic carbon (DIC) available from sea water. The much more abundant HCO_3^- (>90%), however, is largely inhibited from diffusing into the host cells due to its ionic charge. Entry into the host cell via passive diffusion is further restricted by an unstirred boundary layer that surrounds the surface of the host, which dramatically slows the sea water–coral transfer rate for both CO_2 and HCO_3^- (Smith and Walker, 1980). For the intracellular zooxanthellae, the problem of CO_2 assimilation is thus the form of DIC and its delivery, as opposed to its availability in sea water.

It is increasingly understood that both the host and zooxanthellae have carbon concentrating mechanisms (CCMs) to help maximise the availability of CO_2 (over O_2) at the site of photosynthesis (see references in the manuscript; Goiran et al., 1996). However, these CCMs are always subject to the initial delivery constraint imposed by the presence of the unstirred boundary layer, which dramatically slows the supply rate - especially in low flow conditions. Moreover, as outlined in the manuscript, a number of the host CCMs require cellular energy in the form of ATP, which ultimately derives (over short time periods ~minutes) from the supply of fixed-carbon from the zooxanthellae. Thus, anything that restricts the photosynthetic processes of the zooxanthellae (e.g., chronic photoinihibition) can act to disrupt the efficiency of the host CCMs. This linkage of host CCMs to the tight-cycling of photosynthetic carbon is therefore an easily identified Achilles' heel in the intracellular CO_2 supply chain.

The early study of Burris et al. (1983) is commonly cited as evidence that coral zooxanthellae are not CO_2-limited. However, this study manipulated the carbonate chemistry in a very unrealistic way. Numerous other studies provide evidence that CO_2 is often in short supply at the site of photosynthesis:

- Streamer et al. (1986) suggest that a lag period in fixation of ^{14}C-bicarbonate by the branching coral *Acropora formosa* might be due to rate-limiting delivery of CO_2.
- Dennison and Barnes (1987) observed a significant increase in rates of photosynthesis and calcification in the branching coral *Acropora formosa* when surrounding water was stirred. However, when photosynthesis was near compensation, stirring had no effect, suggesting that at high rates, photosynthesis was limited by the diffusion of substrate.
- Muscatine et al. (1989) concluded from $\delta^{13}C$ values in corals that at high rates of photosynthesis, virtually all of the CO_2 that reaches the zooxanthellae is assimilated.

- Weis (1993) showed that net photosynthesis of the sea anemone *Aiptasia pulchella* was DIC-limited at present seawater concentrations (~2mM), and that photosynthesis increased up to a DIC of 5mM.
- Lesser et al. (1994) demonstrated that the coral *Pocillopora damicornis* was DIC-limited for a fixed colony morphology exposed to low flows. The data indicated that the biochemical augmentation of DIC delivery by the host/zooxanthella CCMs was unable to compensate for low flow conditions.
- Goiran et al. (1996) concluded that the zooxanthellae within the coral *Galaxea fascicularis* were DIC-limited at photosynthesis saturation, which occurred at irradiance levels of 200-300 µmol photons m^{-2} s^{-1}.
- Langdon and Atkinson (2005) showed that the net primary production from an assemblage of corals increases by 23% due to a doubling of seawater CO_2.
- Hertford et al. (2008) demonstrate for two common reef-building coral species that photosynthetic rates do not saturate until the exceedence of seawater DIC beyond 4-6mM.
- Crawely et al. (2010) demonstrate that the zooxanthellae of the branching coral *Acropora formosa* are DIC-limited at present seawater concentrations even under subsaturating (100 µmol photons m^{-2} s^{-1}) light conditions.

It is also important to keep in mind, that the majority of these studies were performed at irradiance levels that are known to maximise rates of photosynthesis (~200-300 µmol photons m^{-2} s^{-1}). However, corals in natural reef setting are frequently exposed to irradiance levels that are considerably higher than this. For example, summer irradiance levels can often exceed ~1000-1500 µmol photons m^{-2} s^{-1} for corals located in water depths less than 10m (Yentsch et al., 2002; Frade et al., 2008).

Beyond the CO_2-supply side dynamics, an increased demand for CO_2 from a nutrient-driven enlargement of the zooxanthellae population is an equally important factor in the potential onset of CO_2-limitation at the site of photosynthesis:

- Cumming and McCarty (1982) demonstrated from $\delta^{13}C$ values in corals that larger zooxanthellae populations lead to a significantly higher depletion of CO_2.

- Dubinsky et al. (1990) proposed that CO_2-limitation within the coral *Stylophora pistillata* was the most plausible explanation for the inverse correlation between zooxanthella density and photosynthesis per cell.
- Snidvongs and Kinzie (1994) used the cellular composition of in hospite zooxanthellae to propose that intracellular CO_2 was a limiting nutrient when zooxanthellae densities increase due to external nutrient additions.
- Davy and Cook (2001) proposed that the balance between zooxanthella density and CO_2 availability in the sea anemone *Aiptasia pallida* was the most likely explanation for the observed increase in photosynthetic rate per cell as the density of zooxanthellae decreased by 50% with starvation.
- Zhu et al. (2010) observed that a starvation-induced reduction in the number of zooxanthellae within the sea anemone *Stichodactyla mertensii* caused a significant increase in the photosynethic yield of the remaining zooxanthellae, suggesting a link between CO_2 availability and the quantum yield of photochemistry in photosystem II; which is the proposed site of damage in the zooxanthellae chloroplast that triggers coral bleaching (see next).

(b) CO_2-limitation and coral bleaching

A cellular model for the warm-water breakdown of the coral–algae endosymbiosis has been developed in recent years and includes algal photoinhibition, oxidative damage and host-cell disruption as underlying processes (Gates et al., 1992; Lesser, 1996; Jones et al., 1998; Warner et al., 1999). As the terminal electron acceptor of photosynthesis, theoretical considerations do permit CO_2-limitation within the 'dark reactions' of photosynthesis to be proposed as a potential trigger for the classic bleaching sequence of photoinhibition, oxidative damage and zooxanthellae expulsion. In this case: (i) lack of CO_2 substrate required for the 'dark reactions' can reduce the rate of consumption of the products of photosynthetic electron transport (ATP and NADPH), subsequently causing the photosynthetic electron transport components of the 'light reactions' to become blocked (Takahashi and Murata, 2006); (ii) continued funnelling of excitation energy into the over-reduced electron transport chain can then trigger the onset of photoinhibition (Jones and Hoegh-Guldberg, 2001), damage essential photosynthetic components, (principally photosystem II, PSII), and generate damaging reactive oxygen species (ROS) (Lesser, 1996; Warner et al., 1999; Takahashi and Murata, 2006); and (iii) the excess production of ROS beyond the antioxidant defence strategies of the coral host (and zooxanthellae) has been linked to the host-cell necrosis and detachment that underpins zooxanthellae expulsion (Gates et al., 1992; Dunn et al., 2002). Importantly, this sequence of events is consistent with observations that the bleaching process begins with impairment of the CO_2-fixation mechanism within the zooxanthellae and that the severity of the bleaching impact is a direct function of light intensity (Jones et al., 1998; Buxton et al., in press).

Cyanide (NaCN) inhibits the 'dark reactions' of the Calvin cycle, specifically the Rubisco enzyme (Wishnik and Lane, 1969). As such, exposure of symbiotic corals to cyanide can be expected to replicate the likely sequence of events initiated by intracellular CO_2 limitation. Consistent with this expectation, the effects of cyanide on zooxanthellae are very similar to the impacts of elevated water temperature. As outlined by Jones and Hoegh-Guldberg (1999), in both instances there are high levels of non-photochemical quenching and a lowering of dark-adapted photosynthetic yields symptomatic of damage to PSII. In the case of cyanide-mediated bleaching, light is a secondary variable that is essential to elicit loss of zooxanthellae; a similar interaction between light and temperature has also been reported for corals during laboratory experiments. Whilst damage to PSII appears to be the trigger for cyanide-induced bleaching (as is the case for warm-water coral bleaching), it is likely not to be the primary site of action but a secondary effect, which is light-dependent and subsequent to 'sink' limitation in electron transport.

In this way, any mismatch in the intracellular supply and demand of CO_2 that leads to sink limitation within the photosynthetic 'light reactions' of the zooxanthellae must be considered a valid triggering mechanism leading to bleaching.

(c) Bleaching triggered by CO_2-limitation may be exacerbated by future increases in pCO_2

A central tenet of the hypothesis outlined in the manuscript is that the modern envelope of seawater conditions found within many coral reef ecosystems (characterised by elevated temperatures, rising pCO_2, and enriched nutrient levels) are antagonistic toward the dominant host processes that restrict excessive symbiont proliferation. Far from being beneficial, an enlarged zooxanthellae population is predicted to become a metabolic burden to the coral host during periods of high irradiance and temperature (and low flows), leading to the deleterious onset of intracellular CO_2-limitation and zooxanthellae expulsion (= symbiosis breakdown). References and supporting evidence are provided in the manuscript.

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