The combined effects of ocean acidification and copper on the physiological responses of the tropical coral *Stylophora pistillata*.

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Highlights

- Exposure to increased Cu concentrations suppressed coral calcification.
- Calcification was suppressed further when exposed to Cu under high pCO_2.
- Respiration decreased after two weeks when stressors were applied in combination.

Abstract

A decrease in ocean pH of 0.3 units will likely double the proportion of dissolved copper (Cu) present as the free metal ion, Cu^{2+}, the most bioavailable form of Cu, and one of the most common marine pollutants. We assess the impact of ocean acidification and Cu, separately and in combination, on calcification, photosynthesis and respiration of sub-colonies of a single tropical *Stylophora pistillata* colony. After 15 days of treatment, total calcification rates were significantly decreased in corals exposed to high seawater pCO_2 (~1000-µatm, 2100 scenario) and at both ambient (1.6 - 1.9 nmols) and high (2.5 - 3.6 nmols) dissolved Cu concentrations compared to controls. The effect was increased when both stressors were combined. Coral respiration rates were significantly reduced by the combined stressors after 2 weeks of exposure, indicating the importance of experiment duration. It is therefore likely rising atmospheric CO_2 will exacerbate the negative effects of Cu pollution to *S. pistillata*.

Keywords: Coral, Copper, Ocean Acidification, Calcification, Respiration

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1. Introduction

The living coral cover of tropical coral reefs has shown significant decline in the last three to four decades (Birkeland 2015) reflecting the impacts of fishing, climate change, and decreasing water quality. This coral loss is likely to increase as reefs face multiple

stressors both locally and globally. Rising atmospheric CO_2 is not only causing rising sea surface temperatures, but simultaneously decreasing ocean pH. Since the beginning of the pre-industrial era, the ocean has taken up approximately 25% of the emitted anthropogenic CO_2 (Friedlingstein et al., 2019), causing a decrease in present ocean pH of 0.1 units (Doney et al., 2014). The majority of studies indicate that coral calcification is reduced by a decrease in ocean pH (Erez et al., 2011) but the mechanisms are not fully constrained (Erez et al., 2011; Jokiel et al., 2016).

Altering ocean pH affects the speciation of metals in the ocean (Millero et al., 2009) and thus their bioavailability. Copper (Cu) is an essential element for all organisms (Mitchelmore et al., 2007), but is toxic at high concentrations (Richards et al., 2011). In seawater, dissolved inorganic Cu complexes are dominated by carbonate species, such as $CuCO_3$ and $Cu(CO_3)_2^{2-}$ (Millero et al., 2009). Ocean acidification has reduced the carbonate ion [CO_3^{2-}] content by ~30% since the pre-industrial era (Hoegh-Guildberg et al., 2007) and continued increases in atmospheric CO_2 will further reduce the availability of the carbonate ion to complex Cu^{2+}. Under the IPCC RCP 8.5, 'business as usual' scenario, by 2100 atmospheric CO_2 will reach 1000-µatm and ocean pH will decrease a further 0.3-0.32 units (IPCC, 2014). This will change dissolved Cu speciation with a large increase in the proportion of the free metal ion, Cu^{2+} (Figure 1). Cu^{2+} is the most bioavailable form of Cu in seawater (Richards et al., 2011) and ocean acidification may thereby enhance the bioavailability and subsequent toxicity of Cu to marine organisms.

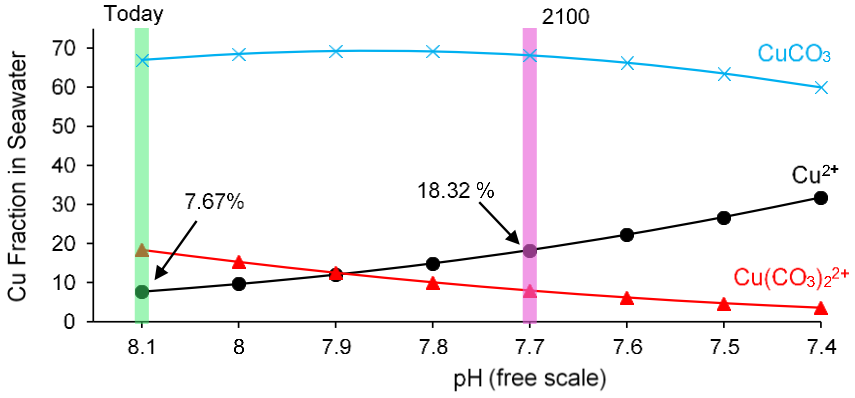

Figure 1: Percentage of three Cu species in seawater. On the free pH scale pH is predicted to be 7.7 in 2100 (Caldeira and Wickett, 2003). Created from information in Millero et al. (2009).

Cu in the surface ocean is typically 0.5-1 nmol/kg, increasing with depth (Biller and Bruland, 2012). In the coastal environment, Cu is a widespread marine pollutant and higher concentrations are observed in waters affected by effluents (1-2.4 µmol/L, Marges et al., 2011; Mishra et al., 2008), sewage discharge (3.1-7.9 µmol/L, Islam and Tanaka, 2004) and antifouling paints (0.01-0.2 µmol/L, Karlsson et al., 2010). The responses of corals to Cu exposure is dependent on Cu speciation and exposure time and include decreases in physiological processes such as calcification (Bielmyer et al., 2010),

photosynthesis (Alutoin et al., 2001; Banc-Prandi et al., 2022; Banc-Prandi and Fine, 2019; Bielmyer et al., 2010), respiration (Nyström et al., 2001), enzyme activity (Bielmyer et al., 2010), DNA damage (Schwarz et al., 2013), increased oxidative stress (Fonseca et al., 2021), coral microbiome structures (Gissi et al., 2019) and larval metamorphis (Negri and Hoogenboom, 2011). Little research has explored the combined effects of Cu^{2+} exposure and ocean acidification on corals. Increasing seawater pCO_2 (to 1000 µatm) enhanced Cu accumulation (at 20 ug/L) in the Caribbean coral *Acropora cervicornis*, but not in the Indo-Pacific coral *Pocillopora damicornis,* with anti-oxidant enzyme activities increasing in both corals (Bielmyer-Fraser et al., 2018). In contrast, interactions between elevated Cu (1, 1.6, 2.3, 3.2 µg/L) and decreased seawater pH (8.1, 7.8, 7.5, 7.2) were mostly antagonistic in the South Atlantic Reef building coral *Mussismilia harttii* (Marangoni et al. 2019).

Combining increased seawater Cu with low seawater pH exacerbates negative effects on a range of metrics in different marine organisms including the Cu immune response (decreasing total hemocyte count, esterase activity, lysosomal content and increasing hemocyte mortality, phagocytosis activity and reactive oxygen species) in the oyster *Crassostrea rivularis* (Huang et al., 2018), DNA damage in the mussel *Mytilus edulis* and sea urchin *Paracentrotus lividus* (Lewis et al., 2016), fecundity in copepods (Fitzer et al., 2013) and sperm damage and survival in the polychaete *Arenicola marina* (Campbell et al., 2014). Relationships between seawater pCO_2 and Cu toxicity may be non-linear e.g. increasing pCO_2 up to 1000 µatm alleviated Cu toxicity in the green tide algae *Ulva prolifer* but further pCO_2 increases were detrimental (Gao et al., 2017).

Here we present a study to measure the effects of Cu exposure and ocean acidification on respiration, photosynthesis and calcification in the branching coral Stylophora pistillata over a two week period. *Stylophora sp.* has a widespread distribution, is an important reef building coral in the Indo Pacific (Veron, 1993) and grows well under laboratory conditions. We use this extended experimental period to explore the interactions between the physiological processes (light and dark calcification, respiration and net photosynthesis) rates in response to ocean acidification and Cu exposure.

2. Materials and Methods

We studied a colony of the branching coral species *Stylophora pistillata* supplied by MailOrderCorals, Rosyth, UK. All individuals in the study were sourced from the same parent colony, originally collected in Indonesia and maintained in an aquarium for several years. Using a single colony removes the variability in stress response that occurs due to genetic differences between colonies and is common in other studies, e.g. Bielmyer-Fraser et al. (2018). Removing this biological noise increases the potential to resolve changes in relationships between physiological processes in the coral e.g. shifts in the relationship between calcification and photosynthesis, but reduces the ecological relevance of the

study as stressor effects are tested on only one genotype. This parent was divided into 16 small colonies which were glued to ceramic bases or 50 ml centrifuge tube caps and left to recover for 5-8 days. At the start of the study corals were randomly divided into 4 groups (3 treatments and 1 control) and were cultured in ambient conditions (unaltered seawater [Cu] and seawater pCO_2 = ~400-µatm) for 13 – 17 days, with temperature of 26°C and salinity of 35. The calcification, respiration and net photosynthesis rates of each sub colony were measured 7 days before treatment began (the pre-treatment measurements). Corals were then moved into one of four treatment tanks (on Day 0) and exposed to treatments for 15 days with physiological rates measured on Days 1, 8 and 15. There was one tank per treatment with four coral sub-colonies in each tank. To limit the numbers of alkalinity samples produced each day the start day of treatment exposure was staggered between the treatments e.g. Day 0 was 11.6.2018 for the high pCO_2 corals, 12.6.2018 for the high pCO_2 + high Cu corals, 13.6.2018 for high Cu corals and 14.6.2018 for controls corals. The control was ambient pCO_2 + ambient Cu and the three treatments were high pCO_2 + ambient seawater Cu; ambient pCO_2 + high Cu and high pCO_2 + high Cu. Ambient pCO_2 was ~400-µatm and high pCO_2 was ~1000-µatm to reflect predicted 2100 pCO_2 concentrations (IPCC, 2014). Total ambient [Cu] varied from 1.6-1.9 nmol while high [Cu] varied from 2.5-3.6 nmol. These concentrations exceed those observed in open ocean water (Biller and Bruland 2012), but are lower than observed in rivers (>1 µmol) entering reef environments (Cheevaporn and Menasveta, 2003).

2.1 Coral Culturing

Seawater for our study was made by combining ~900 L of natural seawater (collected from Crail, Scotland) with ~150 L of artificial seawater (Red Sea Salt, Red Sea Aquatics, UK) to yield a salinity of 35 and a total alkalinity of ~2200 µeq/kg. Seawater was thoroughly mixed, stored at 25°C, divided between four large tanks (called the reservoir tanks) made of high density polyethylene and bubbled continuously with air mixtures of the target atmospheric CO_2 compositions (as in Cole et al., 2018). Corals were kept in 21 L cast acrylic tanks (called the coral tanks) with seawater recirculating from the reservoir tanks at a rate of 7 L/min i.e. the residence time of water in the coral tanks was ~ 3 minutes. The acrylic tanks drained back into the reservoir tanks under gravity. Water flow in the acrylic tanks was produced using Vortech MP10w pumps (Ecotech Marine). Lighting was (Maxspect R420R 160W-10000k) set to 100% A (white) and 100% B (blue) provided a light intensity of 230-270 µmol photons/m^2/s at coral height on a 12 h light and 12 h dark cycle. Corals were not fed during the experiment. Seawater Cu concentrations were increased in 2 of the reservoir tanks by the addition of a $CuSO_4$ stock solution on a single occasion. Dissolved [Cu] was measured at the start and end of the experiment and remained approximately constant (within 15%) in each treatment. The sulfate concentration [SO_4^{2-}]

of seawater is ~28 mM (Wilson, 1975) and the increase in [SO_4^{2-}] associated with the addition of the Cu standard solution was negligible.

2.2 Estimation of Physiological Rates

Two techniques were used to estimate physiological rates during the experiment. The total coral calcification for all corals in each treatment (n=4) was calculated by summing the additions of Na_2CO_3 required to maintain constant alkalinity in each reservoir and assuming all alkalinity changes reflected calcification (Chisholm and Gattuso, 1991). These data provide an integrated estimate of light and dark calcification combined over each week of the study.

Light and dark calcification, photosynthesis and dark respiration were also estimated for each individual colony by moving the colonies into individual (225 ml) acrylic chambers (called the chambers, (Allison et al., 2011) held within a water bath maintained at 26.2 ± 0.3°C (hourly measurements, TinyTag Aquatic, Gemini Data Loggers, UK). The chambers were fed with seawater from the corresponding reservoir tank by automated solenoid diaphragm pumps. Seawater was pumped into the bottom of each chamber and exited the chamber at the top whilst water movement was maintained by magnetic stirrers at the chamber bases. Light and dark calcification rates were estimated from the change in total alkalinity of the water entering and exiting the chambers (with the corals present) while net photosynthesis and dark respiration were calculated from the change in dissolved oxygen (DO_2), in the light and dark respectively (as in Allison et al. 2011). DO_2 was measured using a Thermo Orion 5-star meter with RDO sensor. Duplicate DO_2 measurements were within <1%.

Chamber measurements were made 7 days before corals were moved into the treatments/control (the pre-treatment measurements) and on Days 1, 8 and 15 of treatment. Coral sub-colonies were moved into the chambers 14 hours before measurements were made to allow a recovery period from handling. Estimates of light calcification and photosynthesis were made after exposure of the corals to light for 8 hours. Dark calcification and respiration measurements were taken 4 hours after lights were turned off. These timings ensured that samples could be collected at 8 am (light had been on for 8 hours) and 4pm (lights had been off for 4 hours) each day. Chamber measurements provide a snap-shot of metabolic rates for individual colonies at key intervals. After measurements, sub-colonies were returned to the 21 L acrylic tanks. Seawater was pumped into the chambers through platinum-cured silicon tubing (which is biocompatible but has high CO_2 permeability), itself sealed inside poly vinyl chloride tubing (with low CO_2 permeability). To test the potential for CO_2 to diffuse into and out of the seawater passing along the tubing we compared the dissolved inorganic carbon concentration ([DIC]) of the high pCO_2 seawater in the reservoir tank with that of the high

pCO_2 seawater exiting the chamber (with no corals present), after passage through the tubing. We observed little change in [DIC] (reservoir = 2015 ± 1µmol/kg; chamber = 2003 ± 3 µmol/kg) and conclude that little CO_2 is lost/gained from the seawater during transport to the chambers.

2.3 Seawater Monitoring

The physical and chemical characteristics of the seawater used in the study are summarized in Table 1. The salinity and temperature of the reservoir containers were measured daily. Temperature was measured using a Thermo Orion 5-star probe and salinity was measured using a refractometer (ATC Range, Bellingham + Stanley). Seawater salinity and temperature in each reservoir was maintained at 35 psu and 25.8°C ± 0.4°C (Table 1). The water temperatures in the coral tanks were monitored hourly (TinyTag Aquatic, Gemini Data Loggers, UK).

The pCO_2 of the gas streams used to bubble the reservoirs were measured twice each day using a non-dispersive infra-red CO_2 analyser (WMA04, PP systems, USA). Total alkalinity of each reservoir was measured 3-4 times a week by automated Gran titration (Metrohm, 888, Titrando) and calibrated against a natural seawater certified reference material (CRM; A. Dickson, Scripps Institution of Oceanography). Each sample was run in duplicate and precision (standard deviation) was typically better than 10 µeq/kg. The total alkalinity, [Ca] and [Sr] of the culture seawater was maintained by additions of 0.6 M Na_2CO_3 and a mixture of 0.58 M $CaCl_2$ + 0.02 M $SrCl_2$ (Cole et al., 2016) every 1-2 days. DIC was measured in each reservoir twice (Week 1 and Week 2) during the experiment using a CO_2 differential, non-dispersive, infrared gas analyser (Apollo SciTech; AS-C3) also calibrated with the natural seawater CRM. The DIC was additionally measured in the coral tanks (all treatments) and individual coral chamber (in the high pCO_2 treatment) to check DIC was constant at all locations. The precision of repeat DIC injections was typically better than 0.2%.

Seawater samples for Cu analysis were collected twice during the treatment period (on days 3 and 15 of treatment), filtered through acid washed 0.22 µm polyether sulfone membranes, stored in acid washed HDPE bottles and acidified to pH<2. Acidified seawater samples were diluted 20-fold in 1 M HNO_3 (150µL sample in 3 mL 1M HNO_3) and analyzed by ICP-MS (ThermoXR) using an external regression. Cu standards were prepared in 1M HNO_3 and ranged from 0.1 to 250 nM. The error of the Cu analysis was determined by the standard deviation replicate samples and was <6%. Deionized water blanks and procedural blanks (deionized water aliquots filtered, acidified and stored as samples) were used to correct for contamination during handling.

2.4 Data Processing and Analysis

Coral physiological rates are frequently normalised to colony surface area (Edmunds and Gates, 2002). However the surface area normalised physiological responses of small experimental corals are not representative of larger colonies (Edmunds and Burgess, 2016) suggesting that colony size and physiological rates are not linearly related. Furthermore, calcification in branching corals is focused at the branch tips (Goreau et al., 1979) and estimates of entire colony surface area may not provide a good indication of the abundance of growing tips. In this study we normalise the calcification, photosynthesis and respiration rates measured in each coral sub-colony during treatment to the rates measured for the same sub-colony before treatment. This removes the effect of variations in coral surface area between the sub-colonies used in different treatments (Allison et al., 2011).

2.4.1 Statistical Analysis

We pooled the observations on the multiple sub-colonies in each control and treatment (n=4 in each case) and tested for variations between physiological rates (light calcification, dark calcification, net photosynthesis and dark respiration) in each of the seawater pCO_2 and seawater Cu treatments using a one way ANOVA and Tukey's pairwise comparison. We conducted this test using the datasets produced for Days 1, 8 and 15 separately. We used ANCOVA to test if relationships between physiological processes e.g. light and dark calcification varied significantly between the controls and each of the treatments. In all tests a significant difference was identified when $p < 0.05$.

3. Results

Treatment conditions are summarised in Table 1. Differences in the [DIC] of the seawater in the reservoirs and the associated coral tanks were small (<10 µmol/kg, Table 1), indicating coral metabolism had little effect on the seawater DIC during the short residence time of the water in the coral tank.

Table 1: Physical and chemical characteristics of experimental seawater. [DIC] was measured in both the reservoirs and the coral tanks to test how coral metabolism affected DIC. Cu was measured twice during the experiment, once during week 1 of treatment and once during week 2.

	Control		high pCO_2			high Cu		high pCO_2 + high Cu	
	Reservoir	Coral tank	Reservoir	Coral tank	Chamber	Reservoir	Coral tank	Reservoir	Coral tank
[DIC] Week 1									
Mean (µmol/kg)	1891	1892	2056	2063		1924	1931	2035	2032
Standard Deviation	2	2	1	2		3	2	2	2
n	9	8	9	9		8	9	9	9
[DIC] Week 2									
Mean (µmol/kg)	1894		2015	2015	2003	1905		2005	
Standard Deviation	2		1	3	3	4		14	
n	5		9	4	9	8		6	
Total Alkalinity									
Mean (µeq/kg)	2179		2190			2216		2181	
Standard Deviation	12.6		18.2			58.3		29.9	
n (Days 1-15)	7		9			9		7	
Temperature									
Mean (°C)	25.7	26.4	25.8	26.4		25.8	26.1	25.8	26.0
Standard Deviation	0.6	0.4	0.5	0.2		0.3	0.5	0.3	0.5
n	12	541	15	262		13	672	14	323
Gas Stream [CO_2]									
Mean (µatm)	401		1008			401		1008	
Standard Deviation	6		15			6		15	
n	20		17			20		17	
Dissolved [Cu]									
Mean (Day 3 & Day 15) (nmol/L)	1.63		1.90			2.54		3.64	
Standard Deviation	0.09		0.34			0.04		0.56	
n	2		2			2		2	

3.1 Chamber Experiments

Relative calcification rates in the control corals increased (~1.5 times) between the pre-treatment compared to the treatment weeks (Figure 2). This may reflect changes in the conditions in the coral culture system e.g. light availability, compared to the tank in which the corals were kept prior to obtaining for this experiment. The colony growth tips, the most actively calcifying regions of the colonies, became bigger during the experiment and this may contribute to the rate increase (Figure 3).

The behaviour of the individual colonies within each treatment during the chamber experiments was highly variable (Figure 2) and no significant differences in light

calcification, dark calcification or net photosynthesis were resolved between the different treatments compared to the controls on any of the 3 days (Table 2, one-way ANOVA with Tukey's pairwise comparison, p≤0.05). Dark respiration rate in the high pCO_2 + high Cu treatment was significantly reduced compared to the control corals on Day 15 (Figure 4, Table 2) and was also significantly lower compared to the corals in the high Cu treatment (p =0.02). Significant variations in respiration were not observed between treatments on Day 8 (Table 2). There was not a significant difference in relative respiration rates between Day 15 and Day 1 or Day 8 (paired t-test, p >0.05).

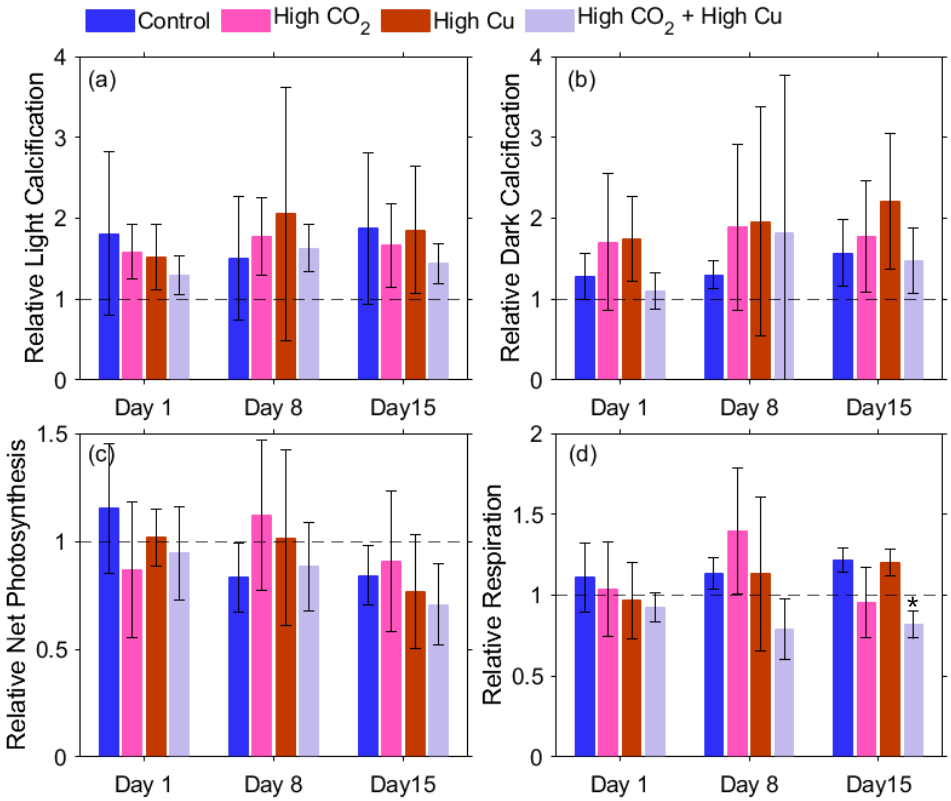

Figure 2: The mean physiological responses (from chamber experiments) of coral sub-colonies in each condition after 1, 8 and 15 days of exposure ± 1 standard deviation. Each sub-colony was normalised to a baseline rate from pre-treatment measurements in ambient conditions, this was then averaged for the four coral sub-colonies within a treatment and plotted. Four physiological responses were calculated - (a) relative calcification rate in the light period, (b) relative calcification rate in the dark period, (c) relative net photosynthesis rate and (d) relative dark respiration rate. Significant differences between treatment and control marked by asterisks (one-way ANOVA with Tukey's pairwise comparison).

Table 2: Results of Tukey's pairwise comparison ran after a one-way ANOVA. Significant differences (p≤0.05) are highlighted in bold.

Treatment	Day 1	Day 8	Day 15
Relative Light Calcification			
High pCO_2	0.94	0.98	0.97
High Cu	0.9	0.84	1
High CO_2 + High Cu	0.61	1	0.8
Relative Dark Calcification			
High pCO_2	0.67	0.92	0.97
High Cu	0.61	0.89	0.48
High CO_2 + High Cu	0.96	0.94	1
Relative Net Photosynthesis			
High pCO_2	0.41	0.54	0.98
High Cu	0.87	0.82	0.97
High CO_2 + High Cu	0.66	1	0.86
Relative Respiration			
High pCO_2	0.97	0.66	0.06
High Cu	0.8	1	0.99
High CO_2 + High Cu	0.63	0.47	**0.004**

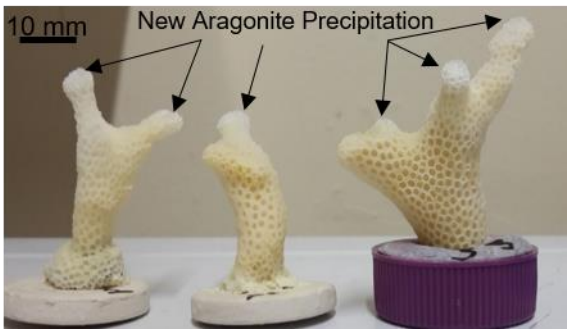

Figure 3: Coral skeletons, from high pCO_2 treatment, showing areas of new coral growth and aragonite precipitation.

We explored the effects of treatment on the interactions between different metabolic processes by plotting the relationships between light and dark calcification, between net photosynthesis and total chamber calcification (from light and dark measurements) and between dark respiration and total chamber calcification after 15 days of treatment (

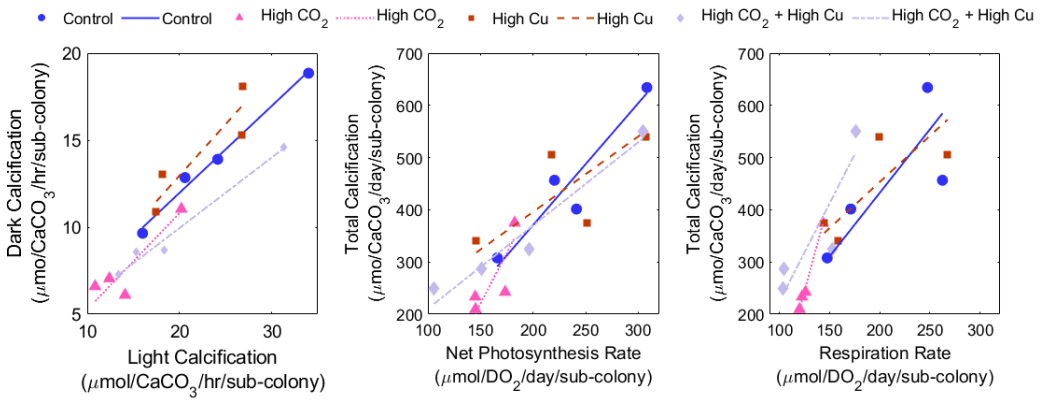

Figure 4, Table 3). We observed significant positive correlations between total chamber calcification rate and both net photosynthesis and dark respiration and between light and dark calcification in all corals pooled into a single group during the pre-treatment measurements (Table 3). These processes continued to exhibit positive correlations after 15 days of treatment (

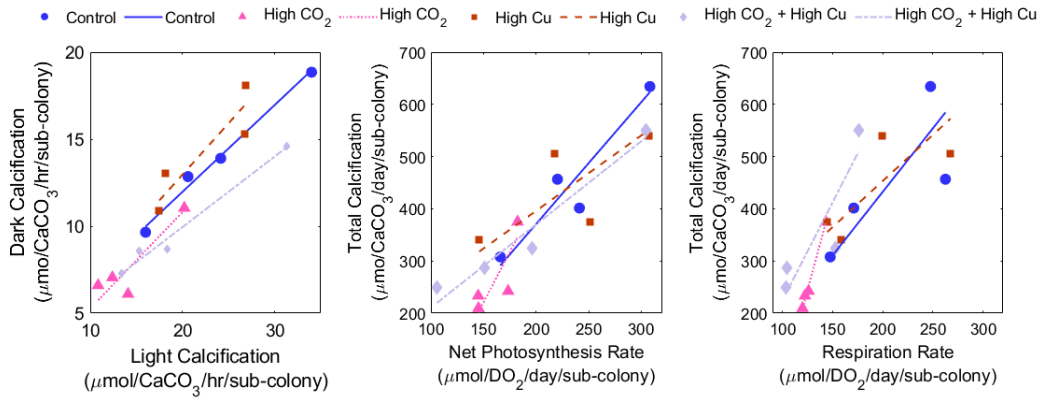

Figure 4), although due to the small numbers of corals in each treatment (n=4), in many cases these correlations are not significant (Table 3). We observed a significant difference in the relationship of light to dark calcification in the corals cultured in high seawater pCO_2+ high Cu compared to the controls (ANCOVA, Table 4). No other significant differences were observed.

Table 3: R^2 value and p value calculated from regression analysis for all sub-colonies pooled prior to treatment and for each coral treatment group on Day 15. Significant relationships (p≤0.05) are highlighted in bold.

	Light & Dark Calcification		Net Photosynthesis & Total Calcification		Dark Respiration & Total Calcification	
Treatment	R^2	p	R^2	p	R^2	p
All sub-colonies prior to treatment.	0.57	**0.00078**	0.49	**0.0024**	0.72	0.**000030**
Control	0.99	**0.0054**	0.89	0.055	0.60	0.22
High pCO_2	0.81	0.098	0.64	0.20	0.99	**0.0042**
High Cu	0.81	0.099	0.50	0.30	0.56	0.25
High pCO_2 + High Cu	0.98	**0.0097**	0.95	**0.02**	0.77	0.12

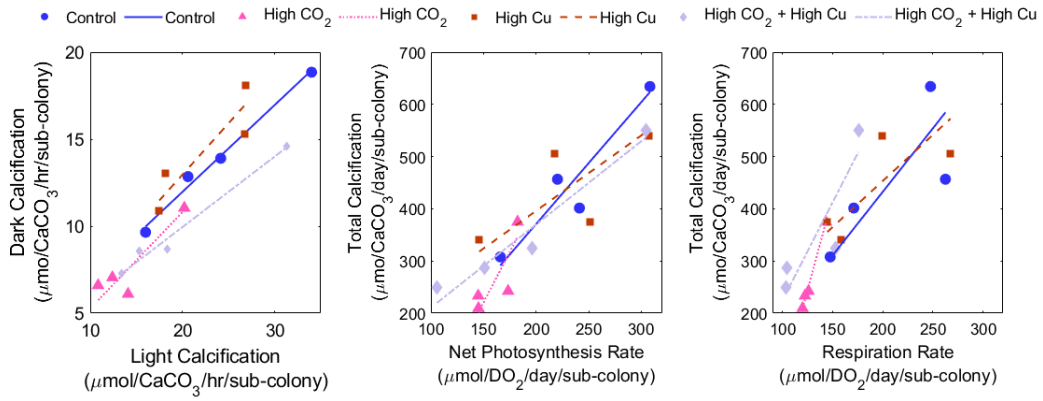

Figure 4: Relationship between different physiological parameters for each coral sub-colony, calculated from

chamber measurements after 15 days of exposure. The R^2 value and p value calculated from regression analysis are found in Table 3.

Table 4: p values of ANCOVA to test for significant variations in the regressions of coral physiological processes on day 15 between treatments and controls. Significant differences between the relationships observed in the control and treatment corals are in bold (p≤0.05).

	Light to Dark Calcification Rate *p* value	Total Calcification Rate to Net Photosynthesis Rate *p* value	Total Calcification Rate to Dark Respiration Rate *p* value
1000-µatm pCO_2	0.11	0.72	0.81
1000-µatm pCO_2 + high Cu	**0.0049**	0.64	0.45
high Cu	0.18	0.93	0.83

3.2 Total Calcification

The total coral calcification (light plus dark) in each treatment was estimated by summing the additions of Na_2CO_3 required to maintain constant alkalinity in the treatment reservoirs and assuming that one mole of Na_2CO_3 was required to replace the CO_3^{2-} used to deposit one mole of $CaCO_3$. An average daily calcification rate was calculated for each week and normalized to pre-treatment rates measured in the chamber experiments 7 days before treatment began giving the relative calcification rates of each treatment (Figure 5).

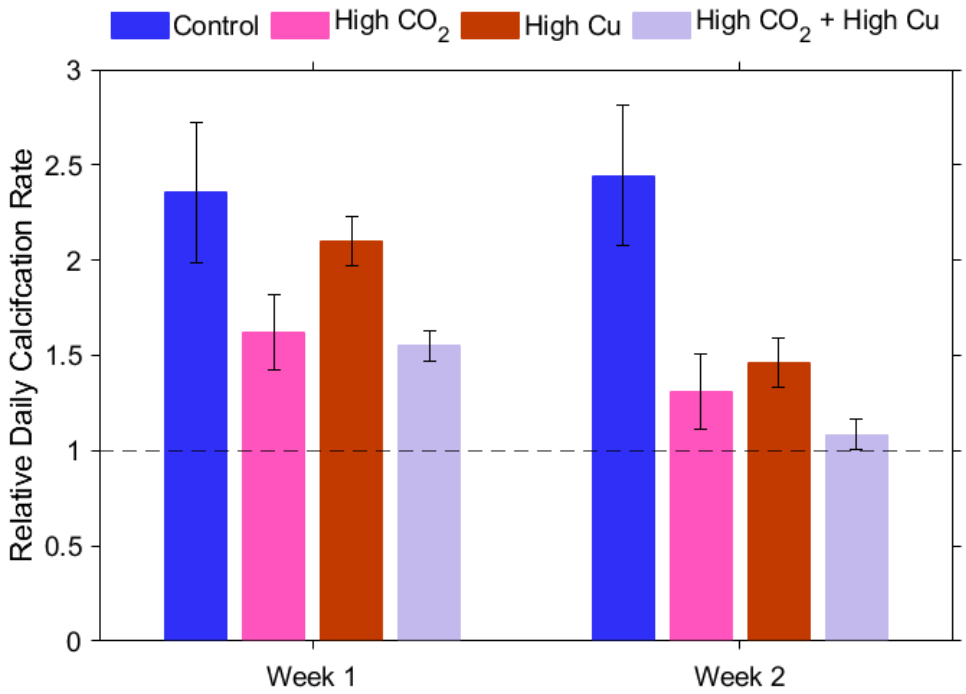

Figure 5: Relative calcification rates in each treatment in weeks 1 and 2 of the experiment. Calcification rates for each colony are normalised to the rate observed in the same colonies before treatment (shown by the dotted line at 1). Error bars are relative percentages calculated from standard deviation of alkalinity measurements.

Relative calcification rates were lower in all treatments compared to the controls and declined between weeks 1 and 2 in all treatments, while rates in the control corals remain almost identical (Figure 5). To assess the significance of variations between treatments

we pool the data from weeks 1 and then from week 2 to create two estimates of calcification for each tank. We test for variations in calcification between the control and treatments using one way ANOVA followed by Tukey's pairwise comparison. This test has low statistical power (n=2) but we note that a significant reduction in calcification is observed in both treatments at high seawater pCO_2 (with low and high Cu, (Table 5)). Calcification rates were suppressed the most when the two stressors were applied in combination by 47% and 61% in weeks 1 and 2 respectively.

Table 5: Summary of p values comparing calcification rates between treatments (one way ANOVA followed by Tukey's pairwise comparisons). Significant differences (p≤0.05) are highlighted in bold.

	High pCO2	High Cu	High pCO2 + Cu
Control	**0.038**	0.099	**0.021**
High pCO2		0.675	0.840
High Cu			0.324

4. Discussion

4.1 Copper and pCO_2 effects

We observed no significant effect of Cu and/or seawater pCO_2 on light or dark calcification in the coral chamber experiments (Figure 2, Table 2) but total calcification was significantly reduced by high seawater pCO_2, both with low and high Cu, in the estimates based on reservoir alkalinity (Figure 5, Table 5). Calcification reductions were most pronounced when the 2 stressors were combined and resulted in decreases of calcification of 47% and 61% after weeks 1 and 2 respectively (Figure 5). High seawater pCO_2 and Cu combined significantly reduced coral respiration rates by the end of the study (Figure 2, Day 15). It is likely that this reflects a deleterious effect of Cu at low pH reflecting changes in Cu speciation (Figure 1), particularly the increase in the free metal ion, Cu^{2+} (Millero et al., 2009) which is probably the most bioavailable Cu species (Richards et al., 2011).

It's unclear how Cu affects coral calcification but some observations are insightful. Cu exposure can inhibit the enzyme carbonic anhydrase in corals (Bielmyer et al., 2010) and is a potential biomarker for acute Cu exposure (Fonseca et al., 2019). This enzyme promotes the conversion of CO_2 to HCO^{3-} and CO_3^{2-} and may be involved in the supply of inorganic carbon for both photosynthesis and calcification (Bertucci et al., 2013). However any relationship between the enzyme activity and calcification is complex. Carbonic anhydrase activity is inhibited at 10 µg Cu/L (equivalent to 158 nmol) in *A.cervicornis* but calcification is not suppressed while the enzyme activity is unaffected at 20 µg Cu/L in *P.damicornis* and calcification is reduced by 4 µg/L Cu in this species (Bielmyer et al., 2010). Elevated Cu inhibits the activity of Ca-ATPase in the symbiont bearing foraminifera, *Amphistegina lessonii* at ambient pCO_2 (Prazeres et al., 2012) and *Amphistegina gibbosa* at low seawater pH (Marques et al., 2017). It is possible therefore that inhibition of this enzyme in corals could cause a decrease calcification due to reduced ability of corals to increase calcification fluid pH.

As photosynthesis and calcification typically exhibit a positive correlation in corals (Bove et al., 2020; Kleypas et al., 1999), suppression of photosynthesis is likely to also affect calcification. We identified no significant effects of high Cu on photosynthesis in this study. Previous studies report no effect of combined high seawater pCO_2 and Cu on photosynthesis over a 35 period in *M. harttii* (Marangoni et al., 2019) or a significant decreases in photosynthesis in the coral *Montastraea faveolata* in response to high Cu only (Bielmyer et al., 2010). Variations of the magnitude previously reported (~10-20% in Bielmyer et al., 2010) would not be apparent in our study given the high variability of rates observed between replicate colonies in the chamber experiments (Figure 2).

We observe a significant change in the relationship between light and dark calcification in corals treated with high seawater pCO_2 and high Cu in combination compared to the controls (

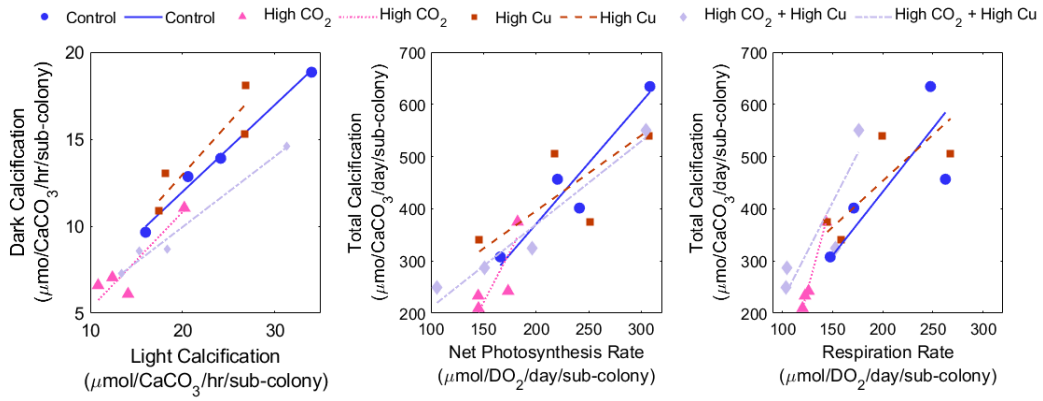

Figure 4, Table 4, p= 0.049). In the control group dark calcification proceeding at about half the rate of light calcification, in common with most other reports (e.g. Gattuso et al., 1999). However, in the treated corals, dark calcification rates are lower than observed in the control corals with comparable light calcification rates. Why dark calcification rates are lower than light rates is unknown. The pH of the extracellular coral calcification media used to form the skeleton may be lower in the dark compared to the light (Al-Horani et al., 2007; Venn et al., 2019) although this is not observed in all coral studies (Sevilgen et al., 2019). Increasing the pH of this media may promote calcification by increasing the concentration of the DIC substrate required for $CaCO_3$ formation (Allison et al., 2018) and could explain why dark calcification is usually slower than in the light. Increasing seawater pCO_2 can cause a more marked reduction in calcification media pH in the dark compared to the light (Venn et al., 2019) and could offer an explanation for the offset in light to dark calcification rate relationships observed here.

Respiration rate decreased significantly in corals exposed to high seawater pCO_2 and high Cu compared to the controls after 15 days. Respiration provides energy for biological processes including the synthesis of skeletal organic matrix and the activity of Ca-ATPase. It's unclear if respiration is suppressed by Cu toxicity (e.g. Cu may induce oxidative

damage in bivalve mitochondria, Collins et al., 2010) or if respiratory demands are less in the treated corals e.g. reflecting a reduction in calcification. Respiration rates are not measured in most studies of Cu toxicity to corals. Alutoin et al. (2001) found no significant effect of Cu on respiration in the massive coral *Porites lutea* during 96 h of exposure. In the current study the respiration decrease was observed after 15 days of exposure indicating the importance of study length in identifying the coral response to the stressors. This has been shown in similar experiments involving Cu exposure, Banc-Prandi et al. (2021) found chlorophyll content was decreased after 3 days of exposure to high Cu, but photosynthesis was not identified to be decreasing till after 14 days and Fonseca et al. (2021) only identified changes in lipid peroxidation and total antioxidant capacity after 12 days of Cu exposure.

Our observations that Cu and high seawater pCO2 in combination alters the light/dark calcification relationship (

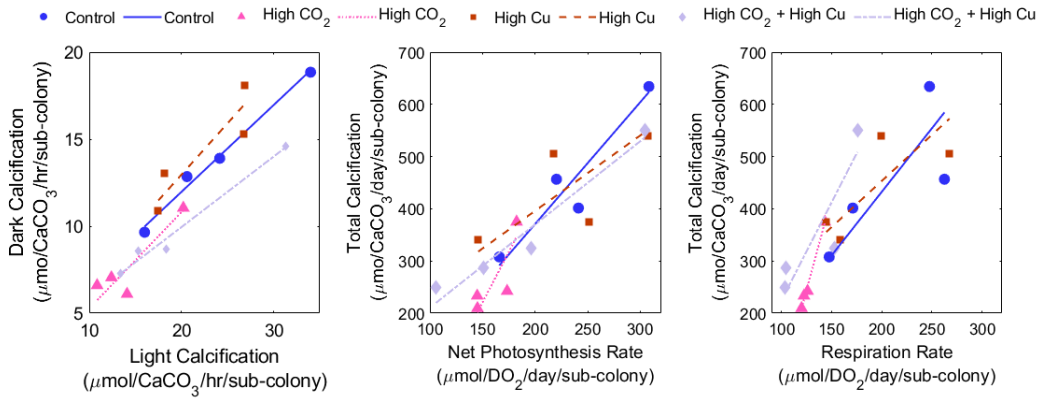

Figure 4), decreasing respiration (Figure 2) and decrease in total calcification (Figure 5) are in agreement with the prediction of increased bioavailable Cu in seawater in future ocean acidification scenarios. There is potential for increasing seawater pCO_2 to potentially reduce Cu toxicity i.e. if the increased seawater H+ competes with Cu^{2+} for cell binding sites (Franklin et al., 2000) as hypothesized to occur in the green algae *Ulva prolifera* (Gao et al., 2017) but this phenomenon was not observed in this study. Corals exposed to high Cu at high seawater pCO_2 had significant decreases in total calcification and respiration. We observe no positive impacts of Cu exposure e.g. due to enhancement of chlorophyll performance (Banc-Prandi et al., 2021) via the essential role of Cu in electron transport (Gaji´c et al., 2018) or due to a positive increase in Ca-ATPase activity in combination with low pH exposure (Marangoni et al., 2019).

We did not observe significant differences between treatments in the calcification rates estimated in the chamber experiments. The relative physiological rates determined in the chambers were highly variable between colonies (see error bars on Figure 2). This may reflect the handling of corals prior to the chamber experiments. We consider that the total

405 calcification data is likely to yield a more representative indication of the response of the
406 corals to the treatments.

407 *4.2 Implications for Reefs*

408 We note that we tested the effect of increased total dissolved Cu on the metabolic
409 processes of a single *S. pistillata* genotype. Earliest reported fossils of *Stylophora* sp. date
410 to 65-70 My (Baron-Szabo, 2006) and *S. pistillata* has now developed both a broad
411 biogeography (covering the Red Sea and Indian and Pacific Oceans) and a high genetic
412 diversity (Keshavmurthy et al., 2013). Stress responses vary between coral genotypes and
413 therefore it is not possible to predict accurately the global responses of this species based
414 on single studies on one (or even several) genotypes. Within same colonies on the Great
415 Barrier Reef (GBR) heavy metal concentrations including Cu have been found to vary
416 substantially (Esslemont, 2000). However, our study does provide some indication of the
417 potential effects of Cu on this important coral species.

418 Cu is mined in multiple countries which host extensive coastal coral reef ecosystems
419 including Indonesia, Papua New Guinea, Philippines and Australia. In Australia the
420 majority of Cu producers are located along the eastern coast (Mudd et al., 2014) and Cu
421 accumulation has been identified in corals in the Great Barrier Reef (Esslemont, 2000).
422 They measured concentrations up to 252 nM/g in the tissue of *Pocillopora Damicornis*
423 tissue from Nelly Bay, GBR where surface water was measured at 9 nM (Esslemont,
424 2000). The Great Barrier Reef is an important source of income and generates $5.7 billion
425 annually to the Australian economy through tourism, commercial fishing, recreation and
426 scientific research (Deloitte Access Economics, 2013), and a decrease in the coral
427 calcification rates which build the reef, could threaten this. The Australian and New
428 Zealand Environment Conservation Council has set a trigger concentration of 1.3 µg/L (20
429 nmols) Cu for 95% species protection (ANZECC & ARMCANZ, 2018). We observe
430 significant reductions in calcification of *S. pistillata* at dissolved Cu concentrations below
431 this (at 0.16-0.23 µg/L) in combination with increased seawater pCO_2 which is likely to
432 occur globally within a few decades.

433 We note that high coral reef seawater pCO_2 is not caused solely by rising atmospheric
434 CO_2. Large natural variations in pCO_2 can occur, particularly in shallow lagoons and on
435 reef flats, in response to diel variations in photosynthesis, respiration, calcification (Shaw
436 et al., 2015) and mixing with ambient seawater. Price et al. (2012) measured pH diurnal
437 variations in excess of 0.2 units on reefs in the central Pacific while Shaw et al. (2015)
438 observed a seawater pCO_2 range of 289-724 µatm on the Great Barrier Reef. These
439 studies demonstrate that large variations in seawater pH already occur across coral reefs
440 and likely drive changes in Cu speciation, with increased bioavailable Cu at night (when
441 pH is lower). As ocean acidification continues to progress the oceans ability to buffer local

pCO_2 increases will decrease (Cai et al., 2011) and we may see greater diurnal variation in pCO_2 and pH, which could exacerbate the effects of Cu.

Our conclusion, that rising seawater pCO_2 enhances the toxicity of Cu to tropical coral and reduces calcification and respiration rates, has implications for many reef sites. A 10-20% decrease in calcification of reefs could create a considerable deficit in calcification (Kleypas et al., 1999). Reported Cu concentration at some Australian reef sites range from ~6 to >100 nmols (Esslemont, 2000) and are far in excess of the concentrations tested in the current study. We conclude that future increases in atmospheric CO_2 and the associated decrease in sea surface pH will serve to increase the toxicity of Cu to coral causing reduced calcification and respiration rates. Reducing coral calcification has consequences for reef health, potentially altering community structure with reef-wide effects (Kleypas et al., 1999) including reducing reef fish density due to lack of hiding places (Hoegh-Guildberg et al., 2007).

Author Contributions

S. Cryer: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing
N. Allison: Conceptualization; Methodology: Formal Analysis; Funding acquisition; Writing - original draft; Writing - review & editing
C.Schlosser: Formal Analysis; Writing - review & editing

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