



# Diurnal cycles of coral calcifying fluid aragonite saturation state

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## Abstract

The sensitivity of corals to ocean acidification depends on the extent to which they can buffer their calcifying fluid aragonite saturation state ( $\Omega_{cf}$ ) from declines in seawater pH. While the seasonal response of the coral calcifying fluid  $\Omega_{cf}$  to seawater pH has been studied previously, relatively little is known about  $\Omega_{cf}$  dynamics on shorter (daily) timescales, particularly whether it is sensitive to seawater pH. Here, we use alizarin dye to mark 4 days of skeletal growth in the corals *Acropora nasuta* and *Pocillopora damicornis* living in situ on Ningaloo Reef in Western Australia. Exploiting newly developed confocal Raman spectroscopy techniques, we imaged the alizarin stains and quantified  $\Omega_{cf}$  between them. We report the first observations of diurnal  $\Omega_{cf}$  cycles, which were found in both species. Our results are consistent with either external control of  $\Omega_{cf}$  by seawater pH or light, or alternatively that  $\Omega_{cf}$  follows an endogenous circadian rhythm.

## Introduction

Invasion of anthropogenic CO<sub>2</sub> into the ocean has already decreased seawater pH (pH<sub>sw</sub>) by ~0.1 units, with a further decline of ~0.3 units expected by the year 2100 under a business-as-usual CO<sub>2</sub> emissions scenario (Hoegh-Guldberg et al. 2014). Commonly known as ocean acidification, these changes in seawater pH are expected to have severe impacts on marine calcifying organisms such as reef-building corals (Kleypas et al. 1999; Ries et al. 2009; Kroeker et al. 2010). Corals build their skeletons with the calcium carbonate mineral aragonite, and although aragonite is supersaturated ( $\Omega > 1$ ) in tropical surface waters,  $\Omega$  decreases as pH declines, making precipitation of aragonite less favorable. While many laboratory experiments have verified that coral calcification is negatively affected by reduced pH<sub>sw</sub> (Gattuso et al. 1998; Langdon et al. 2000; and summarized

in Kroeker et al. 2010; Chan and Connolly 2013; Kornder et al. 2018), other laboratory and field-based experiments have found mixed results or no sensitivity (Fabricius et al. 2011; Crook et al. 2013; Barkley et al. 2015, 2017; Georgiou et al. 2015; Comeau et al. 2017b; Schoepf et al. 2017). Together, the confounding sensitivities of corals to pH<sub>sw</sub> among experiments, and especially between field and laboratory studies, has established little consensus on whether, or to what extent, ocean acidification will negatively impact coral calcification over the next century.

There are several possible reasons for the mixed responses of corals to pH<sub>sw</sub>. One explanation is that corals accrete their skeletons from an extracellular calcifying fluid (cf), which originates as seawater, but undergoes modification to elevate  $\Omega_{cf}$  and thus favor rapid aragonite nucleation and growth (Barnes 1970; Venn et al. 2011; Gagnon et al. 2012). As a consequence, changes in pH<sub>sw</sub> may not necessarily lead directly to changes in  $\Omega_{cf}$  (McCulloch et al. 2012; Georgiou et al. 2015; DeCarlo et al. 2018a). Alternatively, or in combination, the responses of corals to pH<sub>sw</sub> in natural reef environments may be overridden by other factors, such as temperature or light (Price et al. 2012; Kline et al. 2015; Silbiger and DeCarlo 2017). Distinguishing between these two possibilities requires knowledge of whether  $\Omega_{cf}$  tracks pH<sub>sw</sub> or some other environmental factor. However, to date, techniques for determining  $\Omega_{cf}$  of corals within natural reef environments have been restricted to seasonal or annual resolution (D'Olivo and McCulloch 2017; McCulloch et al. 2017; Kubota et al. 2017; Ross et al. 2017, 2018a), leaving

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gaps in our understanding of  $\Omega_{cf}$  dynamics on shorter (diurnal) timescales.

Here, we show how alizarin staining combined with confocal Raman spectroscopy can be used to evaluate the relationship between  $\Omega_{cf}$  and  $\text{pH}_{sw}$ , temperature, and light on diurnal timescales. The beginning and end of our 4-day in situ coral growth experiment was marked in the skeleton of the corals *A. nasuta* and *P. damicornis* using alizarin stains, which were subsequently visualized with fluorescence confocal microscopy. While alizarin staining of coral skeletons has commonly been used to mark monthly growth rates (DeCarlo and Cohen 2017; Anderson et al. 2017), our novel fluorescence imaging technique shows that it may now be applied at diurnal resolution. Furthermore, measurements of  $\Omega_{cf}$  at small spatial scales (1  $\mu\text{m}$  or less) have only recently become possible with Raman spectroscopy (DeCarlo et al. 2017), and our mapping of sub-daily  $\Omega_{cf}$  variability represents an unprecedented temporal resolution of calcifying fluid dynamics for corals living in their natural environment.

## Methods

This study was conducted on the Ningaloo reef flat (23.1555°S, 113.7520°E) near Coral Bay, Western Australia. Water flow on the reef flat is controlled by a combination of wave setup on the reef crest and semi-diurnal tidal currents (Zhang et al. 2012). Since carbonate chemistry and temperature vary spatially across the reef flat and lagoon due to benthic metabolism and local heating (Falter et al. 2012; Zhang et al. 2012, 2013), the tidal currents can produce semi-diurnal variability of carbonate chemistry and temperature at a fixed location as the upstream current direction changes.

One colony of each of *A. nasuta* and *P. damicornis* living at 2 m depth on the reef flat was covered with a translucent plastic bag and exposed to approximately 6 mg L<sup>-1</sup> alizarin Red dye for 80 min between 15:55 and 17:15 local time on 27 May 2017. At the end of the experiment, this staining procedure was repeated between 15:55 and 17:30 local time on 31 May 2017. After this second dye exposure, a branch was collected from each colony, the tissue was removed with a water jet, and the skeletons were dried in an oven at 50 °C for 24 h. Petrographic thin sections were prepared, oriented along the primary growth axes.

Alizarin stain lines were first visualized with a Nikon A1Si confocal fluorescence microscope using a 561 nm laser with a 20 $\times$  objective, superimposed on a transmitted light image (Fig. 1a, d). In addition, the stain lines were imaged with a WITec Alpha 300RA+ confocal Raman microscope using a 532 nm laser and a 100 $\times$  objective with 0.9 numerical aperture (Fig. 1b, e). The alizarin fluorescence was detected as the background intensity in the 2700–3000 cm<sup>-1</sup>

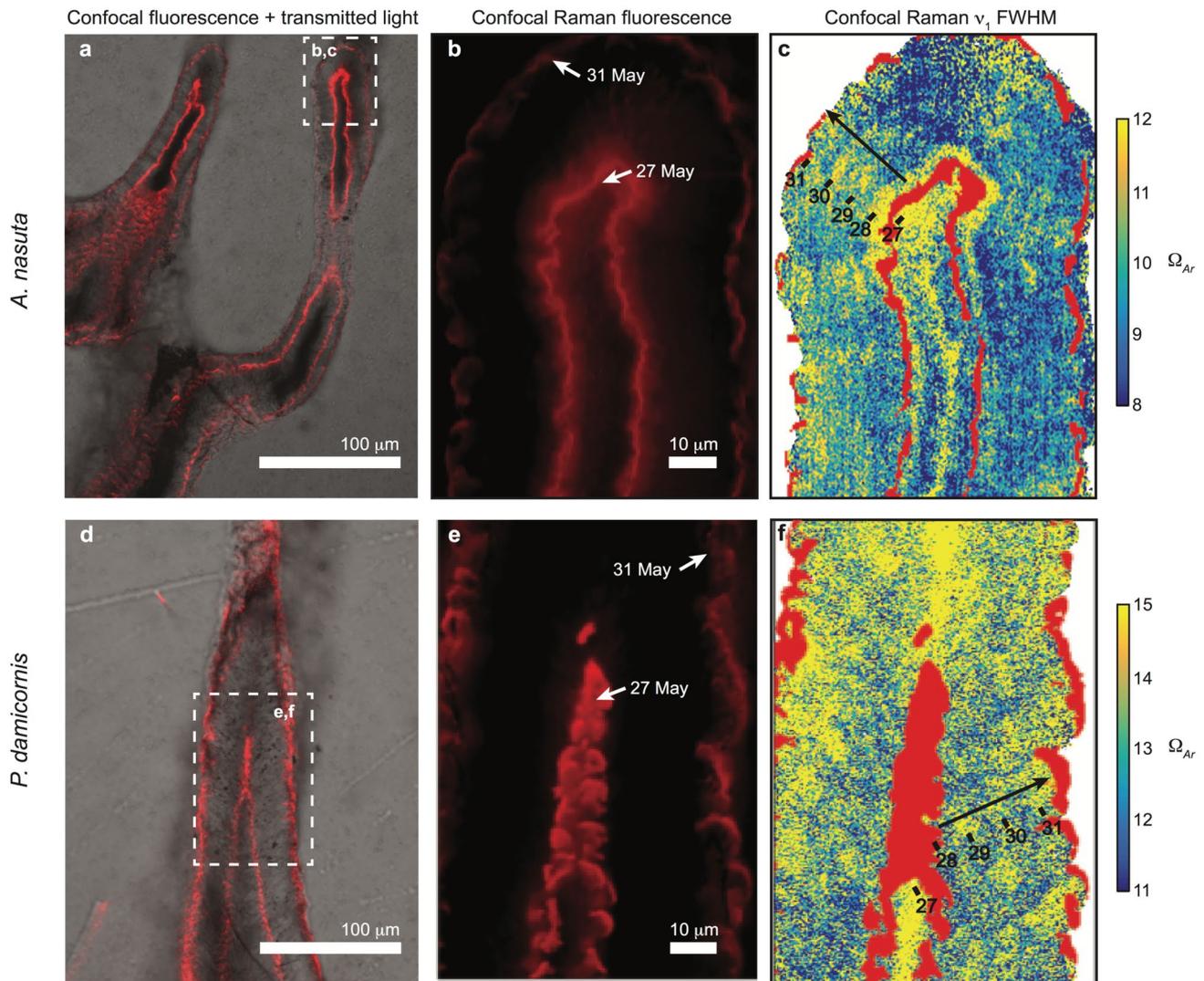
Raman Shift region (see DeCarlo et al. 2018b for analysis of Raman fluorescence). Finally, the same area was mapped with 250 nm (for *A. nasuta*) or 333 nm (for *P. damicornis*) pixels with 0.5 s integrations using the Raman microscope with the same objective but a 785 nm laser (theoretical spatial resolution of 530 nm). At each pixel,  $\Omega_{cf}$  was calculated from the full-width at half-maximum (FWHM) intensity of the  $\nu_1$  peak based on a previous calibration with abiotic aragonites, whereby more disordered crystals (higher FWHM) are precipitated from higher- $\Omega$  fluids (DeCarlo et al. 2017).

Seawater temperature and pH (reported on the total scale) during the 4-day experiment were measured within 2 meters of the corals using a SeaFET (Satlantic) sensor, calibrated at the start and end of the deployment with Tris buffer using the calculations of the seacarb R package (Lavigne et al. 2011). Photosynthetically active radiation (PAR) data (above water) were acquired from the Australian Institute of Marine Sciences (AIMS 2018), and converted to PAR reaching the benthos based on tidal changes in sea level and using a time-averaged light attenuation coefficient of 0.14 m<sup>-1</sup> determined between 1 and 9 m depth on the same reef for several days during the month of July (Zhang et al. 2012).

We tested for coherence between the  $\Omega_{cf}$  time series of the two corals and the potential environmental drivers of temperature,  $\text{pH}_{sw}$ , and PAR. This analysis was conducted using AnalySeries 2.0.8 (Paillard et al. 1996), and included re-sampling to consistent timescales followed by filtering with a Bartlett window prior to coherency computations.

## Results and Discussion

Within the 4-day duration of the experiment, we found four oscillations of  $\Omega_{cf}$  between the alizarin stain lines in both species (Figs. 1c, f, 2), consistent with diurnal cycles of  $\Omega_{cf}$ . Temperature,  $\text{pH}_{sw}$ , and PAR all exhibited diurnal cycles, although temperature and  $\text{pH}_{sw}$  also showed some sub-diurnal variations, likely due to changes in current direction. Qualitatively, these sub-diurnal changes in temperature and  $\text{pH}_{sw}$  were not recorded in  $\Omega_{cf}$ . However, this does not necessarily preclude a link between  $\Omega_{cf}$  and either temperature or  $\text{pH}_{sw}$  for two reasons. First, the sub-diurnal variations in temperature and  $\text{pH}_{sw}$  were relatively small compared to the dominant diurnal cycles, and thus, they may not impose large enough changes in  $\Omega_{cf}$  to be detected. Second, the spatial resolution of our Raman mapping may not be sufficient for capturing  $\sim$ hourly  $\Omega_{cf}$  variability. Thus, we must limit our interpretations to the daily oscillations of  $\Omega_{cf}$  that were successfully detected. On these diurnal frequencies,  $\Omega_{cf}$  of both species was significantly coherent (at 95% confidence) with  $\text{pH}_{sw}$  and PAR (Fig. 2g, h). Conversely, temperature was significantly coherent with  $\Omega_{cf}$  of *P. damicornis*, but



**Fig. 1** Alizarin staining and  $\Omega_{cf}$  variability in **a–c** *A. nasuta* and **d–f** *P. damicornis*. Confocal fluorescence images (red; **a**, **b**, **d**, **e**) superimposed on transmitted light images (grayscale; **a**, **d**) show two alizarin stain lines marking 27 and 31 May. **c**, **f**  $\Omega_{cf}$  mapping (blue, green, and yellow) with the alizarin stains overlaid (red). An approximate timescale is indicated as the calendar days in May with tick marks. The black arrows show growth direction for the transects plotted in Fig. 2

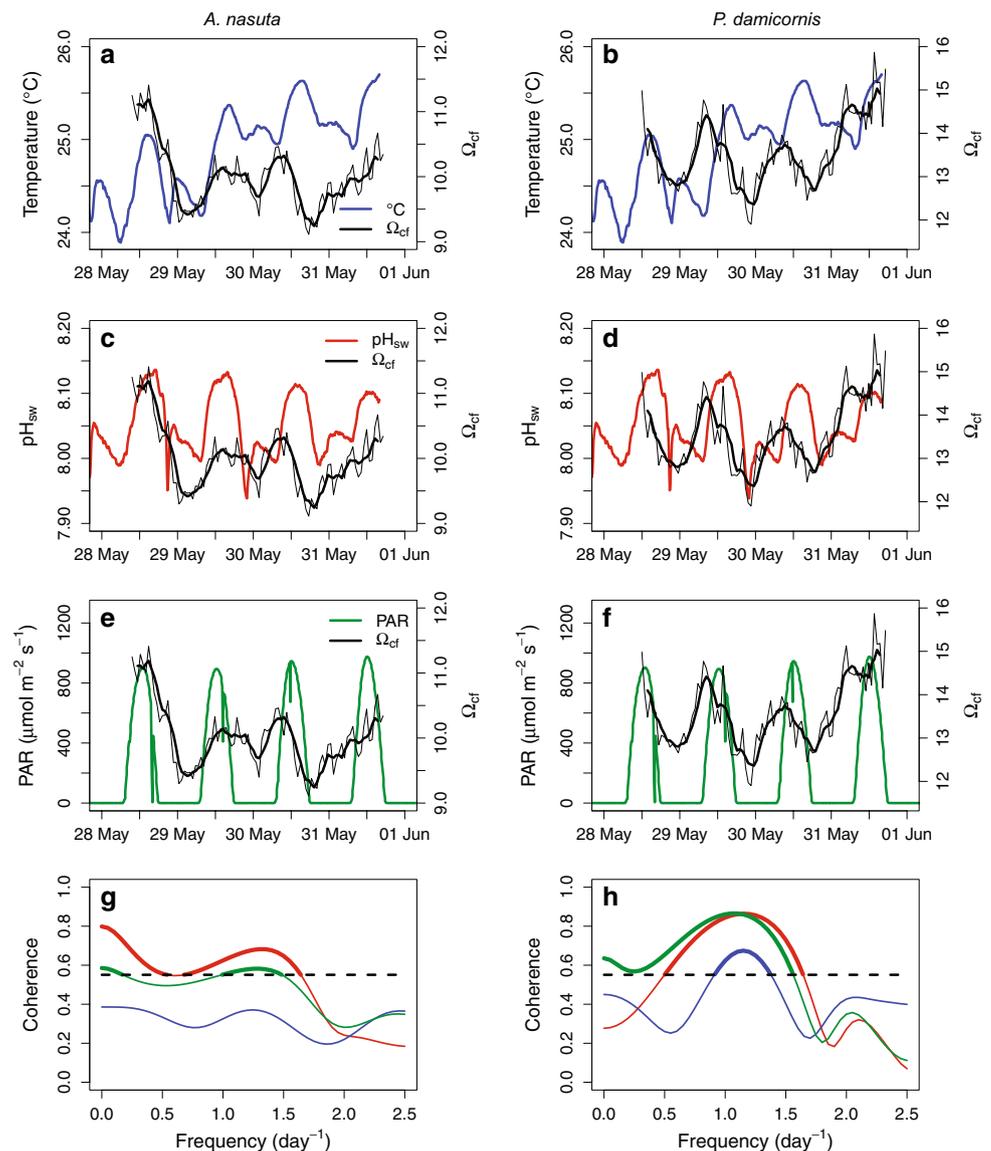
and yellow) with the alizarin stains overlaid (red). An approximate timescale is indicated as the calendar days in May with tick marks. The black arrows show growth direction for the transects plotted in Fig. 2

not *A. nasuta* (Fig. 2g, h). Thus, at the diurnal frequencies that dominate the  $\Omega_{cf}$  time series, we can exclude temperature as a common driver between the two samples, but both  $\text{pH}_{sw}$  and PAR remain potential candidates as environmental controls on  $\Omega_{cf}$ .

There are a variety of explanations for the coherent diurnal rhythms of  $\Omega_{cf}$ ,  $\text{pH}_{sw}$ , and PAR. In seawater, pH and  $\Omega$  are typically positively correlated, and since seawater is the source of the coral calcifying fluid (Gagnon et al. 2012; Tambutté et al. 2012),  $\text{pH}_{sw}$  may be expected to influence  $\Omega_{cf}$ . Yet, recent studies combining Raman spectroscopy with boron systematics have neither found this relationship seasonally nor in laboratory experiments (DeCarlo et al. 2018a; Ross et al. 2018a, b; Comeau et al. 2018; Cornwall

et al. 2018). Conversely, the previous studies using confocal imaging of pH-sensitive dyes or microelectrodes inserted into the calcifying fluid have found short-term (sub-daily) correlations between  $\text{pH}_{sw}$  and the pH of the calcifying fluid ( $\text{pH}_{cf}$ ) (Ries 2011; Venn et al. 2013; Holcomb et al. 2014; Comeau et al. 2017a). There are two possibilities to reconcile our finding of coherent  $\text{pH}_{sw}$  and  $\Omega_{cf}$  daily cycles with the previous Raman/boron and electrode/dye studies. The first is that both  $\text{pH}_{cf}$  and  $\Omega_{cf}$  could be tightly linked to  $\text{pH}_{sw}$  on hourly to daily timescales when the diurnal range of  $\text{pH}_{sw}$  is  $\sim 0.2$  units, but not on monthly timescales when skeletal samples are integrated over many diurnal cycles and the seasonal range of  $\text{pH}_{sw}$  ( $\sim 0.06$  units on the reef flat in Coral Bay; Ross et al. 2018a) is approximately one-third

**Fig. 2** Temporal variability of  $\Omega_{cf}$  (black) plotted with seawater temperature (a, b; blue),  $\text{pH}_{sw}$  (c, d; red), and PAR (e, f; green) for *A. nasuta* (a, c, e) and *P. damicornis* (b, d, f). The  $\Omega_{cf}$  data are taken from 10  $\mu\text{m}$ -wide transects indicated by the arrows in Fig. 1c, f, interpolated onto a timescale based on the known staining times, and are plotted both as the raw transect data (thin line) and 5-point running mean (thick line). **g, h** Coherence between  $\Omega_{cf}$  and environmental factors (same colors as other panels) across frequencies. The dashed black line indicates the threshold of non-zero coherence (at 95% confidence), and thick (thin) lines indicate coherence above (below) this threshold



of the diurnal range. Alternatively,  $\text{pH}_{cf}$  (reported in dye/electrode studies) and  $\Omega_{cf}$  (reported in boron/Raman studies) can become decoupled due to changes in the calcifying fluid concentrations of dissolved inorganic carbon (DIC) or  $\text{Ca}^{2+}$  (McCulloch et al. 2017; DeCarlo et al. 2018a).

In addition to the potential role of  $\text{pH}_{sw}$ , our results are also consistent with an influence of PAR on  $\Omega_{cf}$ , which may occur in at least two ways. First, the supply of energy from the corals' photosynthetic symbionts can be used to power ion exchanges such as with the  $\text{Ca}^{2+}$ -ATPase enzyme that removes protons and increases  $[\text{Ca}^{2+}]$ , thereby elevating  $\Omega_{cf}$  and driving higher rates of calcification during the day (Al-Horani et al. 2003; Cohen and McConnaughey 2003). Second, the daytime drawdown of  $\text{CO}_2$  by the symbionts can increase  $\Omega_{cf}$  if a concentration gradient is established that enables  $\text{CO}_2$  diffusion out of the calcifying fluid (Allemand

et al. 2004; Frankowiak et al. 2016). These potential links between PAR and  $\Omega_{cf}$  are consistent with the previous measurements of  $\text{pH}_{cf}$  sensitivity to diurnal changes in light (Al-Horani et al. 2003) and with hypotheses that coral symbionts stimulate calcification (Goreau and Goreau 1959; Cohen et al. 2016). Alternatively, diurnal  $\Omega_{cf}$  variability may be an entrained rhythm cued by an environmental stimulus such as light or tides (Gutner-Hoch et al. 2016), or it may be an endogenous circadian rhythm independent of a specific environmental driver (Sorek et al. 2014).

Despite uncertainties remaining in the driver(s) of  $\Omega_{cf}$ , the key result of our study is that  $\Omega_{cf}$  exhibits diurnal oscillations in *A. nasuta* and *P. damicornis* with higher  $\Omega_{cf}$  during the day and lower  $\Omega_{cf}$  during the night. While the existence of daily  $\Omega_{cf}$  oscillations has been predicted (Al-Horani et al. 2003; van de Locht et al. 2013), our technique is the first to

enable imaging of  $\Omega_{cf}$  at sufficiently high resolution to test this hypothesis. Future studies employing similar methods to track  $\Omega_{cf}$  in reef environments where PAR and  $pH_{sw}$  are decoupled, and in controlled laboratory experiments, will be essential to disentangling the drivers of  $\Omega_{cf}$  and thereby improving our understanding of coral sensitivities to ocean acidification.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethics approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The collection of corals for this study was conducted under permission from the Government of Western Australia Department of Parks and Wildlife (now Department of Conservation, Biodiversity and Attractions) with research permits and licenses to take fauna for scientific purposes (#SF010963) and the Government of Western Australia Department of Fishers exemption from the Fish Resources Management Act 1994 (#2944).

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