Crustose coralline algae and associated microbial biofilms deter seaweed settlement on coral reefs

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Keywords: Crustose coralline algae, Antifouling mechanisms, Bacteria, Allelopathy, Macroalgal spores, Great Barrier Reef

Abstract

Crustose coralline algae (CCA), a group of calcifying red algae found commonly in benthic marine ecosystems worldwide, perform essential ecological functions on coral reefs, including creating benthic substrate, stabilizing the reef structure and inducing coral settlement. An important feature of CCA is the ability to keep their surfaces free of epiphytic algae, thereby reducing algal overgrowth and allowing them access to light. However, the mechanisms by which CCA prevent settlement of opportunistic seaweeds (fleshy macroalgae) are not fully understood, nor is whether these mechanisms vary among CCA species. In our study based on the Great Barrier Reef, we demonstrate that three common CCA species (*Titanoderma pustulatum, Porolithon onkodes* and *Neogoniolithon* sp.) have a remarkable ability to deter settlement of seaweed spores. We provide experimental evidence that the CCA use allelopathy and microbial inhibition against the settlement of spores of the brown seaweed *Padina boergesenii*. Methanol extracts of allelopathic compounds from *T. pustulatum, Po. onkodes and Neogoniolithon* sp. significantly reduced the settlement of *Pa. boergesenii* spores by 4.3 times, 3.0 and 3.8 times, respectively. Further, we found that microbial biofilms, while having a lower inhibitory effect than allelopathic compounds, also reduced seaweed settlement of *Pa. boergesenii.* Our study demonstrates that allelopathy and microbial inhibition, in addition to epithallial tissue sloughing, are mechanisms employed by CCA to prevent the settlement of epiphytic algae. Understanding the mechanisms by which CCA avoid seaweed overgrowth contributes to our understanding of the dynamics of seaweed proliferations on reefs and to the ecological knowledge of this important group of reef-building organisms.

Introduction

Crustose coralline algae (CCA) are important in coral reef ecosystems as they produce calcium carbonate, stabilize the reef by binding coral rubble (Matsuda 1989), and contribute to reef resilience by inducing the settlement of coral larvae (Harrington et al. 2004; Diaz-Pulido et al. 2010). An important characteristic of CCA is the capacity to keep their epithallus free of epiphytes (Keats et al. 1994, 1997), allowing them to grow and successfully compete for space with other benthic organisms (Kim et al. 2004). For example, Vermeij et al. (2011) found that mixed communities of CCA (mainly *Porolithon* spp. and *Hydrolithon* spp.) suppress the growth and recruitment of the abundant Hawaiian seaweed *Ulva fasciata.* However, little is known about the type of antifouling mechanisms involved in the prevention of seaweed settlement on CCA (Vermeij et al. 2011). A better understanding of the nature of these mechanisms is crucial and timely because many coral reefs worldwide are currently undergoing phase shifts from reef-building organisms such as corals and CCA to dominance by fleshy seaweeds (Hughes 1994; Hoegh-Guldberg et al. 2007).

To date, two mechanisms have been suggested as possible strategies used by CCA to prevent the settlement of spores from competing seaweeds. The first, epithallial sloughing, is of a mechanical nature and refers to the shedding of epithallial cells either in sheets or individually; this mechanism is common in many CCA species (Gordon et al. 1976; Steneck 1986; Keats et al. 1993, 1994; Littler and Littler 1997, 1999). Seaweeds already attached to the CCA surface can be removed by sloughing of the outer layer of cells (Keats et al. 1997). The second mechanism is a chemical strategy whereby production of allelopathic compounds can reduce the settlement of seaweed spores (Denboh et al. 1997; Suzuki et al. 1998; Quoc-Hai et al. 2009). For instance, the temperate CCA *Lithophyllum yessoense* produce multiple substances with inhibitory activity against the settlement and germination of spores of several seaweed species in Korea (Kim et al. 2004). A potential third mechanism might be related to the antifouling properties of microbes associated with the surface of the CCA, since marine

bacteria inhabiting biofilms can produce compounds with algicidal properties (Holmstrom et al. 1996; Lovejoy et al. 1998; Armstrong et al. 2001; Egan et al. 2002; Patel et al. 2003; Bowman 2007; Silva-Aciares and Riquelme 2008). However, evidence for the role of microbes as a mechanism for avoiding epiphytic settlement on CCA is still lacking, although it is well recognized that microbes play an important part in inducing coral settlement (Webster et al. 2004; Siboni et al. 2012). Furthermore, the role of allelopathy against fleshy macroalgae in tropical CCA has not been demonstrated.

In this study, we first determine whether common and ecologically important CCA species from the Great Barrier Reef (GBR), Australia, are able to deter settlement of seaweed spores. Second, we investigate the antifouling mechanisms used by these CCA species to prevent the settlement of spores of an abundant fleshy brown macroalga. We hypothesize that the antifouling mechanisms exhibited by these CCA against macroalgal settlement are primarily driven by the production of allelopathic compounds acting in conjunction with the microbial community living in the biofilms on the CCA surfaces.

Materials and methods

General approach

We collected living CCA from shallow coral reefs of Heron Island, southern GBR, and exposed the CCA to macroalgal spores in a series of laboratory assays at Heron Island Research Station (HIRS). The first experiment (Experiment 1) tested whether two common CCA species of the GBR (*Porolithon onkodes* and *Titanoderma pustulatum*) were able to prevent the settlement of spores of the brown seaweed *Padina boergesenii*, and thus explored the antifouling competence. We chose these species because of their ecological importance: *Po. onkodes* is an important reef builder in the Pacific and GBR (Littler 1971; Dean et al.

2015) and *Titanoderma* spp. are crucial for reef resilience as they have a remarkable capacity to induce the recruitment of corals (Harrington et al. 2004; Price 2010; Doropoulos et al. 2012). We used *Pa. boergesenii* because it is a common species in the studied reefs, the reproductive biology and ecology is well known (Ganesan et al. 2000; Diaz-Pulido et al. 2007), and the release of its spores can be induced under controlled conditions in the laboratory (Ganesan and Rao 1999). Once the ability of the CCA to avoid spore settlement was established, we conducted a second experiment (Experiment 2) to investigate (1) the role of allelopathy by extracting chemical compounds from CCA, and (2) the role of microbial biofilms associated with the surface of the CCA in preventing settlement of *Pa. boergesenii* spores in laboratory experiments.

Experiment 1: Antifouling competence

Crusts of *Po. onkodes* and *T. pustulatum* were collected from Tenements II on the reef slope of Heron Island (23°26.24S, 151°55.23E) and the reef flat in front of the HIRS (23°26.53S, 151°54.98E), respectively. Both species were collected using hammer and chisel. After collection, the specimens were transported to HIRS where they were acclimatized in flow-through aquaria for 1 week before the experiment, which was conducted during March 2014. Reproductive thalli of *Pa. boergesenii* were collected from the Heron Island reef flat and maintained in separate flow-through aquaria at the outdoor facilities of HIRS.

To induce the release of spores by *Pa. boergesenii*, adult, foliose thalli (120 g wet weight) were rinsed several times with sterilized seawater in the laboratory to eliminate sediments and unwanted material, epiphytes were removed carefully by hand, and thalli were subsequently pat dried using paper towel. Immediately after, thalli were pre-treated in the dark for 24 h at ambient temperature (26–28 °C) to maximize spore release (Kim et al. 2004). Spore release was induced by placing the dried thalli in sterilized seawater in 15-L plastic

tanks under a fluorescent light (Aqua Medic - Ocean Light Plus: 150 W aqualine 10000, colour temperature 13,000K + 2 x 24 W T5 Ocean Blue Actinic) of 80 μmol m^{-2} s^{-1} photon flux density for 72 h. After spore release, 10 aliquots of 1 mL were taken to count spores using a Sedgwick–Rafter chamber (50 mm long x 20 mm wide x 1 mm deep) and a compound microscope.

The ability of CCA to avoid settlement of seaweed spores was tested by adding a spore solution of 25 mL containing an average of 134 ± 8 (SE) spores mL^{-1} to 50-mL flasks containing living *Po. onkodes* and *T. pustulatum*, and comparing the number of spores settled on the living surface of the CCA with that of control flasks containing dead CCA. There were seven replicates of each treatment (living *Po. onkodes*, living *T. pustulatum*, or dead CCA). Each replicate consisted of a CCA fragment measuring 1 x 1 cm, placed in the bottom of a 50-mL flask. Species of CCA and controls were kept separate (not intermixed in each flask) and treatments were randomized among the flasks. The spore solution was added to each level by gentle pipetting. The resulting spore suspensions were placed in the dark for 24 h at 26 °C to allow for even settlement of spores on the experimental substrates. At the end of this period, the non-settled spores were removed from the experimental substrates by seawater jets using a waterpik (water flosser model WP-100); consequently only the spores firmly attached (settled spores) to the experimental substrates were counted under a stereomicroscope (Fig. 1).

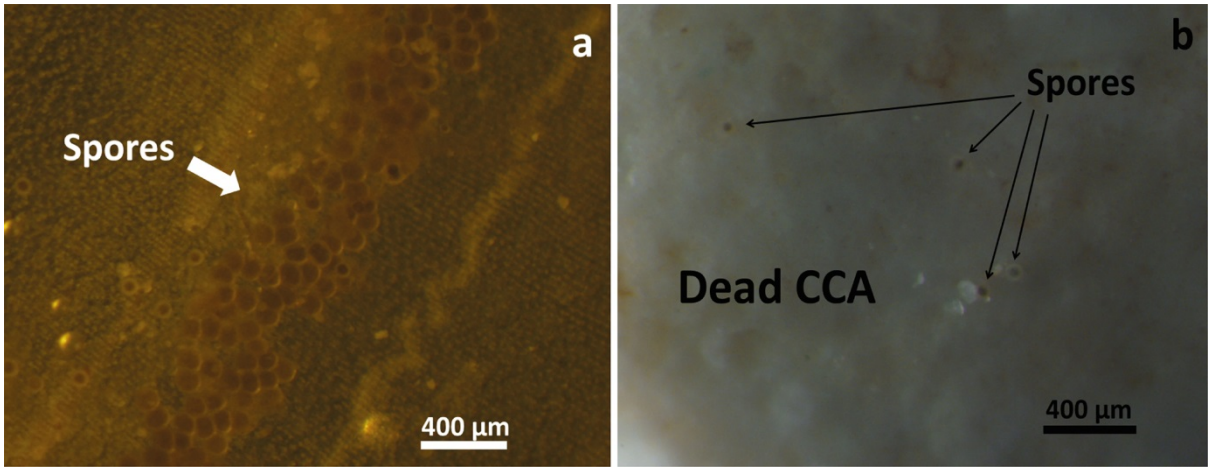

Fig. 1 a Upper side of a mature frond of the brown alga *Padina boergesenii* showing a band of reproductive structures (sporangia) containing spores. **b** *Padina boergesenii* spores (indicated by *arrows*) successfully settled on a dead fragment of the crustose coralline alga *Porolithon onkodes*

Experiment 2: Antifouling mechanisms

In this experiment we examined the role of allelopathy and microbial biofilms associated with CCA as antifouling mechanisms against seaweed spores. CCA collection and fragment preparations were conducted during June 2014 in the same way as described in Experiment 1. Allelopathic interactions were tested by extracting allelochemicals of three CCA species, applying the extracts to fragments of dead CCA in the laboratory, and comparing the number of settled spores from this treatment level to that on fragments of (untreated) living algae and untreated dead CCA. We used the same two CCA species from Experiment 1, *Po. onkodes* and *T. pustulatum*, but included a third species, *Neogoniolithon* sp. *Neogoniolithon* sp. was used because it is also an important reef-building taxon and the epithallial sloughing is quite conspicuous, allowing us to compare the allelopathic and bacterial mechanisms in taxa with different levels of mechanical shedding. Allelochemicals were extracted using the following

procedure: CCA fragments of each of the three species were treated with an antibiotic mixture (see below) to minimize the effects of bacteria on the allelochemical extraction. The pink, living CCA tissue was then carefully scraped off using scalpels, weighed (by means of an analytical balance) and immersed in a 1:10 (v/v) methanol solution. Following Kim et al. (2004), the slurry containing CCA tissue and methanol was kept in darkness for 24 h at room temperature (26–28 °C). The resulting solution was filtered using a pore size of 0.45 μm (47 mm diameter, Sigma Aldrich). The extracts were completely evaporated using a rotary evaporator at a constant temperature of 40 °C. These extracts were diluted with 5 mL methanol and added at natural concentration to 1-cm^2 dead CCA fragments. Dead CCA were obtained by drying 1 x 1 cm CCA fragments in an oven at 60 °C for 72 h. There were seven replicates per treatment and seven control fragments (same solvent used but no extracts, i.e. methanol controls) to test for possible effects of the solvent and extraction protocol on spore settlement.

The role of the microbial biofilm was tested by reducing the number of bacteria on the surface of each of the three species of (living) CCA using antibiotics, and comparing the amount of spore settlement among dead CCA inoculated with microbial biofilms, dead CCA with no microbial inoculation (untreated dead CCA), and living CCA. To reduce the number of CCA-associated bacteria, living CCA were treated with antibiotics using the following method: CCA were submerged in sterile seawater with a mixture of chloramphenicol (50 mg L^{-1}), tetracycline (30 mg L^{-1}) and streptomycin sulphate (30 mg L^{-1}) for 24 h. It has been previously shown that this combination of antibiotics reduces the number of bacteria living on CCA by 20-fold (Johnson and Sutton 1994), and parallel experiments in this study showed this method to be effective in reducing bacterial numbers (data not shown). After 24 h, CCA fragments were rinsed vigorously for several minutes with sterile seawater (four times) and soaked a fifth time for 60 min to ensure that antibiotics were removed from the CCA. Dead

CCA were inoculated with microbial biofilms using the following protocol: surfaces of living CCA (1 x 1 cm fragments) of each species were washed with 50 mL of sterile seawater by pressure flow using a waterpik (water flosser model WP-100) to remove the associated epiphytic microbial community. Dead CCA were incubated in the obtained solution containing seawater and microbial organisms for 4 d. As for Experiment 1, replicates were randomly assigned to treatments. Replicates consisted of a 50-mL plastic container filled with seawater, each containing 25 mL of spore solution (36 ± 2.3 spores mL^{-1}), and a corresponding CCA fragment for each treatment and CCA species. Containers were placed in the dark for 24 h at an ambient temperature (24–25 °C) to allow for even settlement. The number of settled spores was counted under a stereo microscope at the end of the 24-h period. Due to logistic limitations, we were unable to test for interactions between the two mechanisms in our experiment.

Data analysis

For Experiment 1, one-way ANOVA, followed by Dunnett's post hoc test for multiple comparisons was used to test for differences among treatments. Dunnett's test was chosen because it allows the comparison of a number of treatments (living crusts of *Po. onkodes* and *T. pustulatum*) with a single control (dead CCA) (Dunnett 1964). For Experiment 2, data were analysed using two-way ANOVAs, with CCA species as one factor (three species), allelochemicals (four levels) or bacteria (four levels) as the other factor, and fragments as replicates. When significant interactions occurred among treatments, one-way ANOVAs were subsequently performed followed by Tukey tests. Statistical analyses were performed using SPSS v.22.

Results

Experiment 1: Antifouling competence

Results of this assay clearly demonstrated that two common CCA species on the GBR can deter the settlement of seaweed spores. The number of *Pa. boergesenii* spores that settled on the living CCA *Po. onkodes* and *T. pustulatum* following 24 h of incubation was significantly lower (1.75 ± 0.6 and 0.4 ± 0.1 spores cm^{-2}, respectively) than that recorded on dead CCA (16.7 ± 3.7 spores cm^{-2}). However, the mean number of spores settled on the two CCA species was not significantly different (Table 1; Figs. 1b, 2). The results indicate that these CCA species use effective antifouling mechanisms against seaweeds.

Table 1 One-way ANOVA of the effects of experimental substrates on seaweed (*Padina boergesenii*) spore settlement to assess the antifouling ability of CCA. Treatments: dead CCA crusts, living crusts of *Porolithon onkodes* and *Titanoderma pustulatum.* MS: mean square

Source of variation	df	MS	*F*	p	Conclusion – Dunnett test
Treatments	2	1325.25	315.52	<0.001	Dead CCA > *P. onkodes* = *T. pustulatum*
Error	47	4.20			

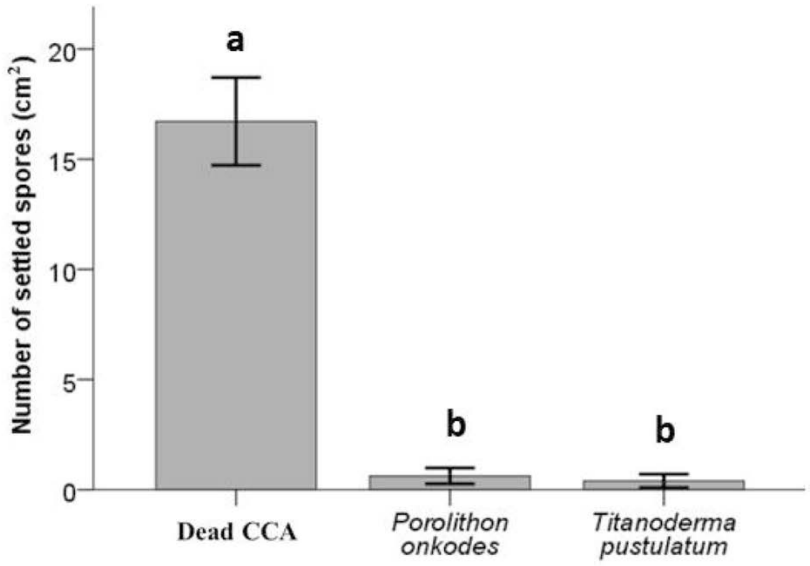

Fig. 2 Number of spores of the brown alga *Padina boergesenii* successfully settled on living thalli of the crustose coralline algae (CCA) *Porolithon onkodes* and *Titanoderma pustulatum* and dead fragments of CCA. Values are means ± SE, n = 7. Letters indicate significant differences among treatments (Dunnett's test)

Experiment 2: Antifouling mechanisms

Effects of CCA allelopathy on seaweed settlement

CCA deterred the settlement of *Pa. boergesenii* spores by allelochemical inhibition. There was a significant effect of the allelochemical treatment on *Pa. boergesenii* spore settlement, however, there were significant interactions between CCA species and the allelochemical treatment ($p = 0.018$; Table 1). The number of spores settled on dead CCA treated with allelochemicals was lower than the number of spores settled on dead CCA, indicating that bioactive compounds produced by CCA deterred seaweed spores from settlement; this result was consistent across the three CCA species (Fig. 3;Table 2). Nonetheless, the fact that spore settlement was even lower on living *T. pustulatum* and *Neogoniolithon* (but not on *Po.*

onkodes) suggests that there are antifouling mechanisms other than allelopathy in these species (see below). There was no significant difference between the number of spores settled on the dead CCA treatment and the methanol control for any of the CCA species examined, demonstrating that the solvent used for the allelochemical extraction (methanol) did not affect spore settlement (Fig. 3; Table 2).

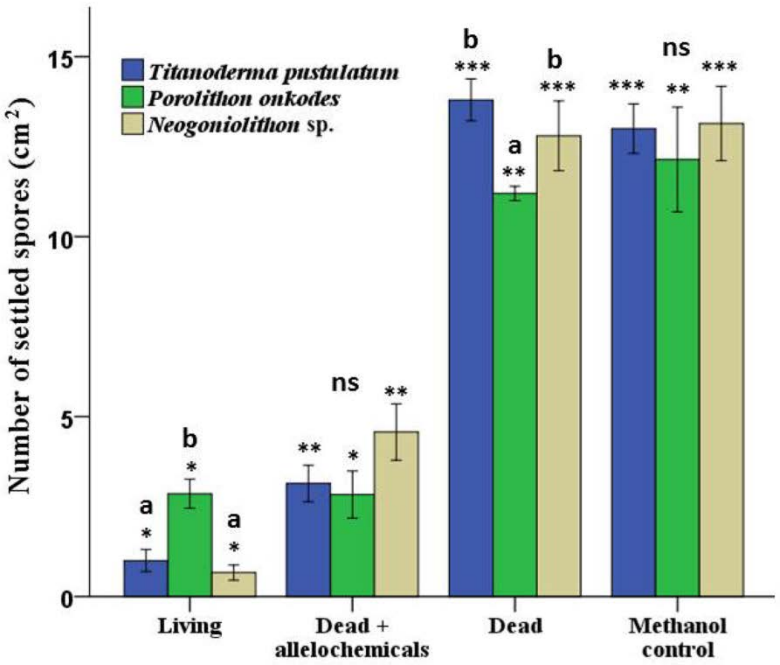

Fig. 3 Number of spores of the brown alga *Padina boergesenii* settled on experimental substrata used to test the antifouling activity of allelochemicals extracted from crustose coralline algae (CCA). The experimental treatments were living CCA, dead CCA with allelochemicals added, dead CCA fragments (without allelochemicals added), and methanol control (procedural control). The CCA species tested were *Neogoniolithon* sp. (*beige*), *Porolithon onkodes* (*green*) and *Titanoderma pustulatum* (*blue*). Asterisks indicate significant differences among treatments and letters denote significant differences among CCA species. Values are means ± SE, n = 7

Table 2 Two-way ANOVA to test for the effect of allelochemicals produced by CCA on the number of *Padina boergesenii* spores successfully settled. Treatments: *alleloc*: dead CCA treated with allelochemicals; *living*: living CCA (untreated); *dead*: dead CCA; *metha*: methanol control. *Neog: Neogoniolithon* sp.; *P. onk: Porolithon onkodes*; *T. pus: Titanoderma pustulatum.* MS: mean square. ns: not significant. Since significant interactions occurred between treatments in the two-way ANOVA, further one-way ANOVAs were conducted within treatment combinations.

Source of variation	**df**	**MS**	***F***	**p**	**Conclusion—Tukey test**
Treatments	3	737.599	219.451	*Neog* <0.001 *P. onk* <0.001 *T. pus*<0.001	dead=metha>alleloc>living dead=metha> alleloc=living dead=metha> alleloc>living
CCA species	2	2.714	0.808	alleloc 0.145 living <0.001 dead 0.002 metha 0.789	ns *P. onk* > *Neog* = *T. pus* *Neog* = *T. pus* > *P. onk* ns
Treatments * CCA species	6	9.302	2.767	0.018	
Error	72	3.361			

Effects of CCA-associated microbes on seaweed settlement

The CCA-associated microbial treatment had a significant effect on the number of seaweed spores settled on the CCA (Table 3), although this effect varied among the CCA species examined (significant interactions treatment x algae, p= 0.001; Table 3). The number of spores that settled on dead CCA crusts that had been inoculated with a microbial biofilm (from the corresponding living CCA species) was significantly lower than on dead CCA crusts without added bacterial biofilm; this result was consistent across all CCA species (Fig. 4; Table 3). Spore settlement was 2.4 and 2.8 times greater on dead CCA crusts inoculated with microbial biofilm than that on living CCA treated with antibiotics, for *T. pustulatum* and

Neogoniolithon sp. respectively (Fig. 4; Table 3). There was no difference between the number of spores settled on CCA treated with antibiotics and the number on living CCA in all CCA species tested (Fig. 4; Table 3).

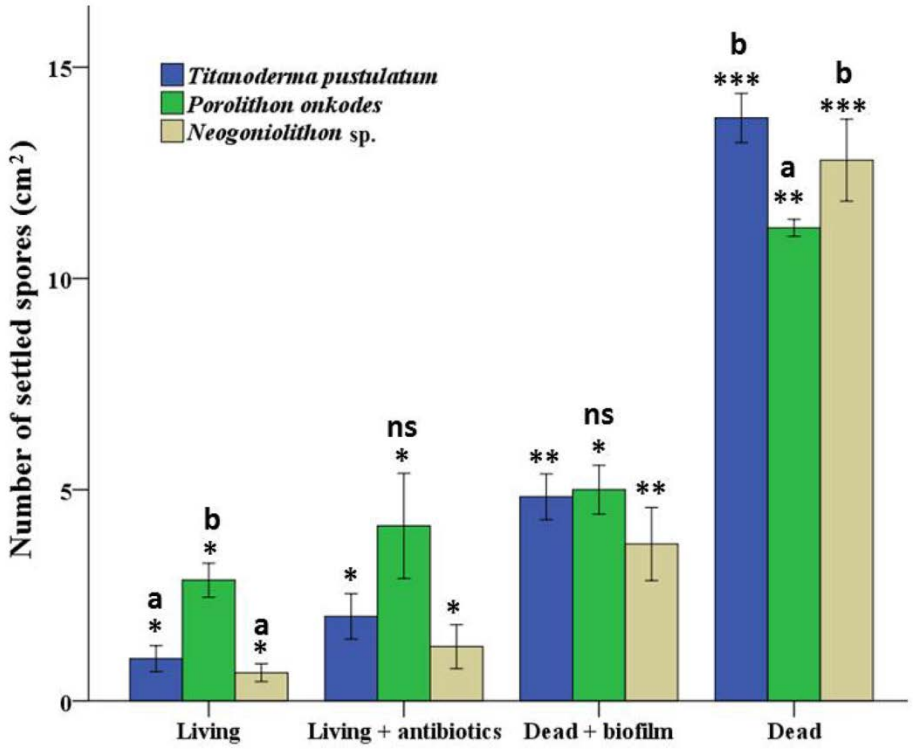

Fig. 4 Number of spores of the brown alga *Padina boergesenii* settled on experimental substrata used to test the antifouling activity of crustose coralline alga (CCA)-associated microbial biofilms. The experimental treatments were living CCA, living CCA treated with antibiotics, dead CCA incubated with microbial biofilms isolated from living CCA, and dead CCA fragments (without biofilms or allelochemicals added). The CCA species tested were *Neogoniolithon* sp. (*beige*), *Porolithon onkodes* (*green*) and *Titanoderma pustulatum* (*blue*). Asterisks indicate significant differences among treatments and letters indicate significant differences between CCA species. Values are means ± SE, n = 7

Table 3 Two-way ANOVA to test for the effect of CCA microbial biofilms on the number of successfully settled *Padina boergesenii* spores. Treatments: *living*: living CCA (untreated); *dead*: dead CCA; *antibio*: living CCA treated with antibiotics; *micro*: dead CCA incubated with microbial biofilms. *Neog: Neogoniolithon* sp.; *P. onk: Porolithon onkodes*; *T. pus: Titanoderma pustulatum.* MS: mean square. ns: not significant. Since significant interactions occurred between treatments in the two-way ANOVA, further one-way ANOVAs were conducted within treatment combinations.

Source of variation	**df**	**MS**	***F***	**p**	**Conclusion- Tukey test**
Treatments	3	530.346	212.338	*Neog* <0.001 *P. onk* <0.001 *T. pus* <0.001	dead>micro>antibio=living dead> micro= antibio=living dead> micro>antibio=living
CCA species	2	9.547	3.822	living <0.001 dead 0.002 antibio 0.067 micro 0.312	*P. onk> Neog= T. pus* *Neog=T. pus> P. onk* ns ns
Treatments * CCA species	6	10.696	4.282	0.001	
Error	71	2.498			

Discussion

We demonstrate that three common reef-building CCA species (*Po. onkodes*, *T. pustulatum* and *Neogoniolithon* sp.) from the GBR have antifouling mechanisms that reduce settlement of spores of a common seaweed species. Spore settlement of the brown seaweed *Pa. boergesenii* was lower on living CCA compared to dead CCA (Figs. 2, 3, 4). Previous studies have shown that reef CCA are usually free of epiphytes (Suzuki et al. 1998; Kim et al. 2004) and a number of mechanisms have been proposed to explain these observations (Tables 4, 5). Allelopathy and microbially mediated interactions have also been suggested to inhibit the settlement of seaweeds (Itoh et al. 1987; Kim et al. 2004). However, the evidence in support of these processes in tropical CCA was scarce. In this study we confirmed that allelopathic

interactions and associated microbial biofilms are two important mechanisms contributing to the control of epiphyte settlement on CCA.

Table 4 Summary of evidence of antifouling mechanisms in CCA against fleshy seaweed settlement. NA: No information available.

CCA species	Epiphyte taxa	Region	Mechanism				Reference
			Mechanical	Allelopathy	Microbial	NA	
Lithophyllum yessoense, L. okamurai, Lithothamnium japonicum Neogoniolithon sp.	*Laminaria*	Temperate (Japan)				•	Masaki et al. 1984
Phymatolithon sp.	*Antithamnion*	Temperate (Nova Scotia)	•				Johnson and Mann 1986
Spongites yendoi	NA	Temperate (S^{th} Africa)				•	Keats et al. 1993
Spongites yendoi, Leptophytum ferox	*Gelidium, Ulva*	Temperate (S^{th} Africa)				•	Keats et al. 1994
Sporolithon ptychoides, Neogoniolithon fosliei, Hydrolithon onkodes	NA	Tropical (Fiji)	•				Keats et al. 1997
Lithophyllum spp.	*Laminaria*	Temperate (Japan)		•			Suzuki et al. 1998
Spongites sp., *Hydrolithon* sp.	*Colpomenia, Sargassum, Ulva, Enteromorpha*	Tropical (Brazil)				•	Villas-Bôas and Figueiredo 2004
Lithophyllum yessoense	17 species of seaweeds	Temperate (Japan)		•			Kim et al. 2004
Lithophyllum yessoense	3 green algae, 9 reds, 4 browns	Temperate (Korea)		•			Quoc-Hai et al. 2009
Porolithon spp., *Hydrolithon* spp.	*Ulva*	Tropical (Hawaii)				•	Vermeij et al. 2011
Unidentified spp.	*Saccharina*	Polar (Alaska)				•	Okamoto et al. 2013
Titanoderma pustulatum,	*Padina*	Tropical		•	•		This study

Porolithon onkodes, *Neogoniolithon* sp.	(Australia)

Our findings indicate that chemical compounds produced by CCA act as antifouling defences significantly reducing the settlement of the brown alga *Pa. boergesenii*. This result was consistent across the three CCA species studied (Fig. 3). There are a number of specific processes by which allelochemicals may inhibit spore settlement. For example, CCA may produce biocidal substances that target structural components of the spores prior to settlement, when the plasma membrane is exposed (Braten 1971). A large majority of fleshy seaweeds release spores that have unprotected cell membranes (Kakisawa et al. 1988) and only develop the protective cell wall upon settlement (Braten 1971). On the other hand, since algal spores can be repelled by a number of chemical stimuli (Bucolo et al. 2012), the CCA's inhibitory component can act as a negative cue, preventing spore settlement until death ensues (Egan et al. 2001b). There are only few studies regarding CCA allelopathy against seaweed spores, most of them on temperate CCA; our results are similar to those found in temperate studies (Table 4). For instance, Suzuki et al. (1998) found that the CCA *Lithophyllum* spp. contain an allelopathic, nonpolar compound that impedes the settlement of zoospores from the kelp *Laminaria religiosa*. This compound also has the potential to destroy kelp spores as well as dinoflagellate cells (Suzuki et al. 1998). Kim et al. (2004) showed that the CCA *Lithophyllum yessoense* exhibited multiple allelopathic activities against settlement and germination of several seaweed species, and suggested that *L. yessoense* releases water-soluble antifoulants into the environment. The fact that in our study the effect of CCA allelochemicals against macroalgal spores was the same among CCA species ($p = 0.145$; Table 2; Fig. 3), suggest that it might be a common antifouling mechanism in reef-building CCA. Results from previous studies together with our findings on reef-building CCA indicate that allelopathic mechanisms can mediate space competition between CCA and fleshy macroalgae and successfully prevent growth of epiphytic algae.

We demonstrate that the antifouling ability of CCA against seaweed settlement relies not only on the allelopathic compounds produced by the CCA thallus, but also on the microbial biofilms present on the CCA surfaces. The effect of CCA biofilms alone (containing microbes but probably some CCA exudates as well) was clear because of the significantly smaller number of spores settled on dead CCA with biofilms added, compared to the number of spores settled on dead CCA without biofilms (a reduction of ca. 150–100%; Fig. 4; Table 3); this was consistent across CCA species. However, spore settlement was even lower on living CCA treated with antibiotics (but which have naturally occurring allelochemicals) compared to that on CCA with biofilms added (and without allelochemicals); this was observed in two of the studied CCA species, *T. pustulatum* and *Neogoniolithon* sp. (Table 3; Fig. 4). This finding suggests that for *T. pustulatum* and *Neogoniolithon* the addition of the microbial biofilm may have favoured settlement of *Pa. boergesenii* spores to some extent. Facilitation of seaweed spore settlement by bacteria has been observed in the green alga *Ulva* when germination was stimulated by the specific interactions between certain bacteria species (Wichard 2015). However, the potential beneficial effect of biofilms for *T. pustulatum,* and *Neogoniolithon* sp. is much lower than the negative effect of allelochemicals. This also suggests that allelochemicals are the main antifouling mechanism against seaweed settlement, as living CCA with (naturally occurring) allelochemicals but without bacterial biofilms had lower spore settlement than CCA with biofilms but with no allelochemicals. Nonetheless, epiphytic microbial communities contribute to some extent to the antifouling defences of CCA against seaweeds and it is likely that these communities also produce antifouling compounds that work in concert with the CCA-derived compounds to protect the CCA surface. Bacteria living in marine biofilms can produce active compounds against micro- and macro-organisms, including algal spores (Berland et al. 1972; Holmstrom et al. 1996; Lovejoy et al. 1998; Egan et al. 2001a; Harder et al. 2004; Mieszkin et al. 2013). For

instance, from 192 epiphytic bacterial isolates from algae, 63 isolates inhibited spore settlement of the green algae *Ulva lactuca* (Ma et al. 2009). Further, Tebben et al. (2014) showed that *Pseudoalteromonas* isolated from the CCA *Neogoniolithon fosliei*, a genus used in our experiment, produce antibacterial, antifungal and antiprotozoal substances. In summary, our experiment demonstrates that both allelopathy and microbial biofilms are important antifouling defences against seaweed spore settlement for a number of tropical CCA species.

Comparing the different antifouling mechanisms across the studied species identifies several emerging patterns (Table 5). Allelopathic effects were displayed by all species studied and all seem to be of similar intensity. Similarly, microbially mediated interactions against spores were exhibited by all CCA species with comparable strength. There are a number of antifouling mechanical mechanisms that were not tested in our study, including those intrinsic to the CCA, such as removal of epiphytes via epithallial sloughing (Gordon et al. 1976; Masaki et al. 1984; Keats et al. 1993, 1994, 1997), or extrinsic such as dislodgement of epiphytic algae via wave action and grazing by fish and gastropods (Steneck 1983,1986). In our study the effect of CCA epithallial shedding against spore adhesion was not observed, however it is unlikely that this trait influenced our results due to the time span of the assays (24 h). A comparison of epithallial shedding across taxa (Steneck 1983,1986; Keats et al. 1997; Littler and Littler 1997, 1999; pers. obs) demonstrates that this mechanism is particularly important in species of *Neogoniolithon*, followed by *Porolithon* (Table 5). There is no information on *Titanoderma*, although it is likely that this mechanism plays a minor role in antifouling in this species given the thin nature of its thallus. Grazing by herbivorous fish and invertebrates is particularly common for *Neogoniolithon* spp. and *Porolithon* species on healthy reefs (Steneck 1983,1986; Littler et al. 1995; Reyes-Nivia 2013). Based on this analysis, *Neogoniolithon* sp. seems to be more robust to epiphytic colonization due to its

strong allelopathy (Fig. 3), associated microbes (Fig. 4), and considerable epithallial sloughing (Keats et al. 1997; Littler and Littler 1997, 1999). *Porolithon onkodes* and *T. pustulatum* might be slightly more susceptible to epiphyte colonization than *Neogoniolithon* sp., as they mainly depend on allelopathy to keep their surfaces free of macroalgae (Table 5).

CCA play key roles in reef ecosystems, contributing to reef construction and reef resilience, particularly by facilitating the recovery of reefs following disturbances (Matsuda 1989; Harrington et al. 2004; Diaz-Pulido et al. 2010). This is especially critical as many reefs are currently undergoing transitions from coral-dominated to macroalgal (fleshy and algal turfs)-dominated systems (McCook et al. 2001; Diaz-Pulido et al. 2009; Jackson et al. 2014). Here we have demonstrated that CCA actively deter settlement of fleshy macroalgal spores (see also Vermeij et al. 2011) and have identified two mechanisms by which this occurs. The ability of CCA to prevent macroalgal settlement (e.g. Littler and Littler 1997) may potentially enhance reef resilience; therefore increased CCA abundance, together with processes that reduce fleshy macroalgal growth (e.g. improved water quality via reducing sedimentation and nutrient pollution, encouraging herbivore grazing), may contribute to reversing negative phase shifts. A better understanding of the mechanisms by which CCA deter fleshy seaweed recruitment is crucial and timely. It contributes to the ecological knowledge of this important group of reef-building organisms and provides useful information on the processes that influence the dynamics of seaweed proliferation in reefs.

Acknowledgments

Thanks to members of the Coral Reef Algae Research Laboratory Alexandra Ordoñez, Carlos Del Monaco, Emma Kennedy, Patrick Gartrell and Bonnie Lewis for their help with the experiments. Thanks also to Elisa Bayraktarov for advice and discussions. Mark Hay

provided guidance on the allelochemical extractions. The Great Barrier Reef Marine Park Authority provided us with permits to conduct this research (G13/36022.1). This work was supported by the Great Barrier Reef Foundation and an ARC Discovery grant (DP120101778).

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