Evidence against a mechanism of allelopathy in the green alga *Chlorodesmis fastigiata*

William E Kumler Corresp. [1, 2]

[1] Environmental Science Policy and Management, University of California, Berkeley, Berkeley, CA, United States
[2] Earth and Planetary Science, University of California, Berkeley, Berkeley, CA, United States

Corresponding Author: William E Kumler
Email address: wkumler@berkeley.edu

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Evidence against a mechanism of allelopathy in the green alga *Chlorodesmis fastigiata*

William E Kumler [1,2]

[1]Department of Earth and Planetary Science, University of California, Berkeley, CA 94720 USA

[2]Department of Environmental Science, Policy, and Management, University of California, Berkeley, CA 94720 USA

Abstract. Allelopathic macroalgae have been shown to have significant negative effects on corals via the transfer of toxic compounds. The interaction that takes place between allelopathic macroalgae and other algae, however, has not been studied in detail. Here, the effects of the allelopathic *Chlorodesmis fastigiata* on other macroalgae were analyzed. These effects were first tested on complete individuals on multi-day time scales, then on small samples of the macroalgal species exposed to isolated toxins over the scale of minutes. However, neither experiment found significant negative effects on either *Sargassum mangarevense* or *Boodlea kaeneana* due to the interaction between these algae and the toxin produced by *C. fastigiata*. Distribution and abundance of *C. fastigiata* were also assessed around the island of Mo'orea in French Polynesia.

Introduction

Coral reef health and conservation have become topics of much conversation in recent years. For the most part, these discussions center around large-scale environmental changes such as climate change and ocean acidification (Hoegh-Guldberg *et al.,* 2007), increased sedimentation rates (Rogers 1990), nutrient fluxes (Fabricius, 2005), and a rise in fishing pressure (Jackson *et al.* 2001). These negatively affect reef health and create an increased risk of colonization by macroalgae (McCook, 1999). Some algae facilitate this colonization process via the transfer of toxic nonpolar compounds directly onto the coral (Rasher and Hay, 2010; Rasher *et al*, 2011; Bonaldo and Hay, 2014). *Chlorodesmis fastigiata*, commonly known as turtleweed, exemplifies this allelopathic interaction and its diterpene toxins causes appreciable bleaching of sensitive corals in merely a few days (Rasher and Hay, 2010; Rasher *et al*. 2011; Bonaldo and Hay 2014).

It is not known exactly how the algal toxins function, but they may act by blocking photosynthesis. Previous studies on aquatic and marine algae have shown that toxins produced by algae can inhibit the light-dependent reactions of photosynthesis. (Patterson *et al*, 1979; Patterson and Harris, 1983) This is especially true if the toxin is nonpolar and has a low molecular weight (Leflaive and Ten-Hage, 2007; Smith and Thanh, 2007) such as is the case for the toxins produced by *Chlorodesmis fastigiata*. (Rasher *et al*, 2011). Further, Warner *et al*. (1999) found photosynthetic efficiency in zooxanthellae to be a strong indicator of bleaching. This evidence cumulatively suggests that the toxins act upon the chloroplasts of the symbionts, either blocking their function entirely or reducing their effectiveness. This in turn causes the symbionts to abandon the coral in search of more hospitable environments.

Despite being a green alga, and thus reliant upon photosynthesis for its energy, *Chlorodesmis fastigiata* produces toxins that may attack the chloroplasts of the photosynthetic zooxanthellae in corals. One possible mechanism for this could be that the chloroplasts are derived from separate endosymbiotic events. Green algae, such as *Chlorodesmis* and *Boodlea*, as well as all land plants, are the products of hundreds of millions of years of evolution following a single endosymbiotic event. Current research (Dorrell and Smith, 2011; Keeling, 2010) suggests that a cyanobacteria was ingested via endocytosis but was not digested, and over time became an integral part of the eukaryote's function. Chloroplasts in brown algae such as *Sargassum* and *Symbiodinium* are the products of a secondary endosymbiotic event, this one featuring the ingestion of a red alga already containing chloroplasts (Cavalier-Smith, 2002; McFadden, 2001). This difference could be what *C. fastigiata* exploits when it, as a green alga, produces toxins that cause bleaching in the symbiotic brown algae of corals.

This study examines the possibility that the diterpene toxins produced by *Chlorodesmis fastigiata* are able to attack corals without damaging the alga itself because the corals have different chloroplasts, derived from a secondary endosymbiotic event. Thus, brown algae and symbionts should undergo reduced photosynthetic efficiency in the presence of the toxins, while green algae are unaffected by it.

Materials and Methods

Study site

This study was carried out on the island of Mo'orea, one of the Society Islands in French Polynesia (S 17° 32' 20", W 149° 49' 46", WGS84). This volcanic island is surrounded by a barrier reef that separates the open ocean from a shallow and calm lagoon next to the shore. Field studies were carried out at various locations throughout this lagoon, and *Chlorodesmis fastigiata* collections occurred at Temae Public Beach (S 17° 32' 29", W 149° 45' 14", WGS84) (Fig. 1). Lab studies were carried out at the Richard B. Gump South Pacific Research Station.

Effects of pairing *Chlorodesmis fastigiata* with corals and algae

A controlled lab experiment was designed to assess the effects of *C. fastigiata* on various coral and algae. Complete individuals of *Acropora millepora* and *Porites lutea* were collected from the forereef outside of Cook's Bay (S 17° 28' 17", W 149° 49' 2", WGS84; Fig. 1). *Sargassum mangarevense* and *Boodlea kaeneana* were collected from Motu Tiahura (S 17° 29' 11", W 149° 54' 46", WGS84; Fig. 1). These individuals were then placed in aquaria for one week to allow acclimation. After that time, half of the individuals from each species were paired with *Chlorodesmis fastigiata*. Pairing consisted of attaching a healthy individual of *C. fastigiata* to the surface of the coral with monofilament fishing line to ensure continued physical contact. Algae were paired using zip ties with the same goal (Fig. 2). Photos were taken of the corals before and after the pairing, then every 24 hours for the next 7 days. The bleached area of each individual was assessed via ImageJ using an in-frame scale and expressed as a ratio between the bleached surface area and the surface area in contact with *C. fastigiata*.

Damage to algal tissues was measured via photosynthetic efficiency, since algae do not bleach and a fluorometer was unavailable. Dissolved O_2 measurements were taken via respirometer, the PreSens Sensor Dish Reader with Oxodish® Optode Plate (PreSens Precision Sensing GmbH, Germany; SDR software v38). This respirometer measures the oxygen concentration of 750µL of water in each of its 24 wells. Temperature was kept constant throughout each trial with a recirculating water bath because the respirometer was found to be highly sensitive to small changes in temperature. A 28W 10,000K dual compact fluorescent/actinic aquarium light was used to provide light favorable for photosynthesis. The rate of photosynthesis of each alga was sampled initially and once every 24 hours for 5 days. This rate was measured by collecting a portion of the alga from the point of contact between the alga and *C. fastigiata*. Control samples were obtained from a separate individual that had not been paired with *C. fastigiata.* These samples were shaken to remove excess water then weighed before being placed in the wells of the respirometer closest to the light source. Oxygen concentration was measured every minute for 20 minutes.

Toxicity Assay

A second experiment tested the effects of the toxins on the algae on a shorter time scale. The toxins responsible for coral bleaching were extracted from live *Chlorodesmis fastigiata* using the procedure described in Rasher and Hay (2010). This portion of the study tested the response of algae to the toxins on the scale of minutes rather than days.

Respiration was again measured using the PreSens respirometer. Trials were run on small samples of photosynthetic material obtained from each species using the same procedure as detailed above. These samples were then distributed among the different treatment wells of the respirometer. The respirometry chamber was broken into two portions, one which was illuminated by the CFL/actinic aquarium light and the other which was kept dark. This setup is detailed in Figure 3.

The blank wells were filled with 10% methanol/seawater solution but did not contain any photosynthetic tissue. The positive control wells were filled with the methanol solution and included the algal tissue. The negative controls were also filled with the methanol solution but were kept in the dark portion of the respirometer to prevent photosynthesis and provide a baseline respiration rate. The treatment wells were filled with a 10% methanol/seawater solution in which the toxins previously extracted was resuspended. These assays were carried out in the same way as above, with oxygen concentration data collected once every minute for 20 minutes.

Field abundance survey

A field abundance survey was carried out because little is known about the distribution of *Chlorodesmis fastigiata* on the island of Mo'orea and thus the magnitude of its effects on local reef health. Six sites were sampled at points across the island (Fig. 1).

At each location, a qualitative assessment of water quality and flow as well as precise GPS coordinates were taken before entering. In the water, a 30-minute visual survey was performed to check for the presence of *Chlorodesmis fastigiata*. If *C. fastigiata* was found, a 50 meter transect tape was laid parallel to the reef crest 5-15 meters from shore, starting from a random point determined prior to entering the water. *C. fastigiata* abundance was assessed by visual survey along the tape, and when an individual was found, its location along the tape was recorded along with its depth. Finally, a picture was taken for later verification.

Results

Effects of pairing *Chlorodesmis fastigiata* with corals and algae

Both corals, *Porites lutea* and *Acropora millepora*, responded strongly to the pairing. Each species showed significant bleaching by the end of the experiment, and after only 24 hours each coral was noticeably affected. Since the toxins produced by *C. fastigiata* are nonpolar and thus transferred by direct contact, only the portion of the coral that was exposed to the algae was assessed for bleaching. The bleached area increased linearly each day until the end of the experiment, at which time 40% of the exposed area of *P. lutea* and 27% of the exposed area of *A. millepora* was bleached. (Fig. 4)

Algae, however, showed no significant change in photosynthetic efficiency. The rate of oxygen concentration change for each trial was found via best-fit linear regression lines matched to each 20-minute set of data. Any data with an R^2 value less than 0.8 were not used to calculate average rates. The average photosynthetic efficiency rates of the paired algae were then subtracted from the average rates of the control algae to obtain a final relative photosynthetic efficiency rate. This normalized rate is shown for each day in Figure 4.

Spearman's Rank Correlation tests on both species showed no significant correlation between days in contact with *C. fastigiata* and photosynthetic efficiency (*S. mangarevense* = -0.5, B. *kaeneana* = -0.7). Following those tests with a linear regression fit supported this conclusion, as *S. mangarevense* had an R^2 of -0.064 and p-value equal to 0.447 and *B. kaeneana* had an R^2 of -0.012 and a p-value equal to 0.622.

Effect of isolated toxins on photosynthetic efficiency

As above, the rate of oxygen concentration change for each trial was found via best-fit linear regression lines matched to each 20-minute set of data. Any data with an R^2 value less than 0.8 was again removed from average rate calculations. These data were collected and differences between means were calculated via nested ANOVA followed by Tukey's HSD. No significant difference was found between the negative controls of each algae ($p > 0.95$, n=16), the positive control (p=0.86, n=16), or the toxin assay (p=0.60, n=16). These results are shown in Figure 5.

Chlorodesmis distribution on Mo'orea

Chlorodesmis fastigiata was found at two locations; Temae Public Beach, found at the northeast corner of the island, and Motu Tiahura, at the northwest corner of the island. At Temae, *C. fastigiata* was highly abundant, with an individual found, on average, every $4m^2$. 35 total individuals were found across three independent 50 meter linear transects. These algae tended to be on the side of coral bommies with higher flow and avoided exposed, flat areas of rubble. They were usually found on surfaces exposed to sunlight during the time of the transect (10am). They are found most commonly on dead *P. lutea* coral heads and often on coral rubble that was sheltered and secured to the lagoon floor (Fig. 6).

On Motu Tiahura, *Chlorodesmis fastigiata* was found only on the north side in small, sheltered bays. Here, it was much less abundant than at Temae, and was found clustered within specific bays, which would either have many individuals or none. Transects here were run on the shore rather than 5-15m out because of this distribution. Eighteen small bays fell within the three independent 50 meter transects, four of which contained *C. fastigiata*. In these bays, clusters of 4-10 individuals were found, and solitary individuals were rarely discovered. *C. fastigiata* here was found on coral rubble most often, but without coral heads to sample this is expected, since coral rubble was the second most common substrate at Temae.

Discussion

Allelopathy is just one of the many interactions that occur on reefs. While its importance is in aquatic systems is debated, the transfer of harmful chemicals has been shown to be a powerful tool for algae as they claim space on reefs and defend against herbivory (Bonaldo and Hay, 2014). *Chlorodesmis fastigiata* has been previously studied because it interacts with other organisms via allelopathy, but a mechanism has yet to be proposed for its allelopathic effects. One study noted that "little is known regarding the interactions of enol-acetate functionalities with biological molecules" (Paul and Fenical, 1986). The toxins produced by *C. fastigiata* are examples of such enol-acetate molecules, and when this information is coupled with evidence that *C. fastigiata* secretions are neither strongly antibacterial or antifungal (Paul and Fenical, 1986), it is clear that more study is needed to propose a mechanism by which these toxins cause damage. This has not yet been completed due to the difficulty inherent in demonstrating mechanisms in the field (Rodriguez-Ramos, Lorenzo, and Gonzalez, 2007).

Effects of pairing *Chlorodesmis fastigiata* with corals and algae

As previously found by Rasher and Hay (2010), transplanting *Chlorodesmis fastigiata* onto hard corals has a negative effect upon the health of the portion in contact with the alga. In this experiment, *Acropora millepora* responded as expected, with bleaching induced across the entire surface that was in contact with the alga over the 5-day period. *Porites lutea* bleached more quickly than expected given its documented resilience (Loya *et al.*, 2001), possibly because the coral was stressed in the aquarium and thus more sensitive than it would have been in the field.

Algae, however, were not found to respond significantly to the pairing. Neither *Sargassum mangarevense* nor *Boodlea kaeneana* showed any significant decrease in photosynthetic efficiency when paired with *Chlorodesmis fastigiata* for 5 days. This is surprising because the *S. mangarevense* appeared visually damaged – it was greenish and notably more brittle at the point of contact. The time scale of a few days was long enough to bleach a significant portion of the corals in contact with *C. fastigiata,* as noted above. These results imply that the toxins do not affect other macroalgae on the same time scale as corals. It is also possible that the deleterious effect caused by the transfer of allelopathic chemicals on this time scale was small enough that it was within the large signal variation produced by the respirometer. This portion of the study could be improved by using a fluorometer to measure algal stress directly.

Effects of isolated toxins on photosynthetic efficiency

Chlorodesmin is considered a strongly potent compound (bioactive at 0.032–0.12 μg/g of algal dry mass, Rasher *et al.* 2011). Despite this, the toxin assays showed no significant difference between the photosynthetic efficiency of algae samples exposed and those kept as controls. Thus, this experiment provides some evidence supporting the hypothesis that the toxins do not act on the chloroplasts of the corals, and that the loss of coral vitality is due to another process. There have been many such alternatives proposed, including microbial activity (Smith *et al.*, 2006) and cytotoxicity applied to the polyps themselves (Birrel *et al.,* 2008). It is also possible that the toxins act via oxidative stress. This hypothesis would explain both the greenish color of the paired *S. mangarevense* and the apparently higher oxygen production of the toxin assays. Thus, more study is needed to determine if this is the mechanism by which *C. fastigiata* causes bleaching.

Field surveys

The rarity of *Chlorodesmis fastigiata* around the island was unexpected. Its abundance at Temae Beach is surprising given the lack of representation anywhere else on the main island. Even at

Motu Tiahura, the only other site *C. fastigiata* was found at, individual density remained far lower than those found at the public beach. Although described as a "common" reef algae (Payri, 2000), it was found much more rarely than other common algae such as *Sargassum mangarevense* and *Turbinaria ornata*. It is possible that a unique combination of abiotic factors created a significant advantage at Temae, but similar environmental conditions were observed at every site around the island. *Chlorodesmis fastigiata*'s relationship to herbivores such as the gobies *Gobiodon histrio* and *Paragobiodon echinocephalus* (Dixson and Hay, 2012; Rasher, Hoey, and Hay; 2013) is a possible cause of this discrepancy, but these species were noted at Temae beach as well as the other locations. The distribution at Motu Tiahura was also highly variable. Its tendency to be found in high density in occasional, sheltered bays created a challenge for sampling via linear transect and resulted in the methodology used for sampling the other sites. Environmental factors here mirrored those at Temae except that these individuals were found in much shallower water, almost in the intertidal zone. At all other sites, *C. fastigiata* was unable to be found despite extensive visual surveys across the lagoon and back reef.

Conclusion

Chlorodesmis fastigiata is an archetype of allelopathy and its interactions with hard corals have been examined extensively. Here, a possible mechanism for this allelopathy was examined, inspired by evidence that suggested an interruption in the photosynthetic pathways of the coral. The results of these experiments provide evidence against this mechanism, and suggest that future research should focus on mechanisms such as oxidative stress or microbial-layer disruption.

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Figure 1

Map of Mo’orea and its reefs.

Blue dots denote locations where *Chlorodesmis fastigiata* was surveyed and yellow dots denote collection locations.

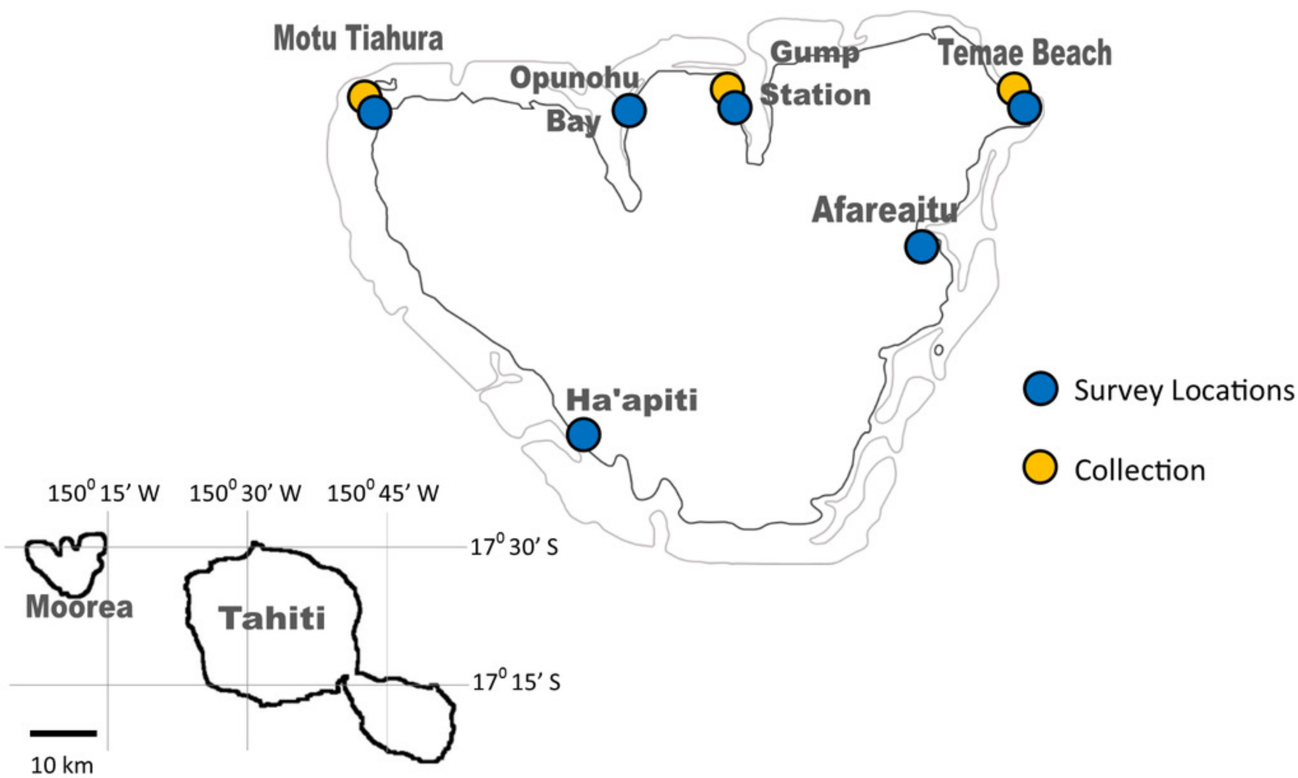


Figure 2

Demonstration of the pairing methods for each coral and algae.

Acropora millepora and *Porites lutea* were collected from the reef crest outside Gump Station, and *Boodlea kaeneana* and *Sargassum mangarevense* were collected from Motu Tiahura.

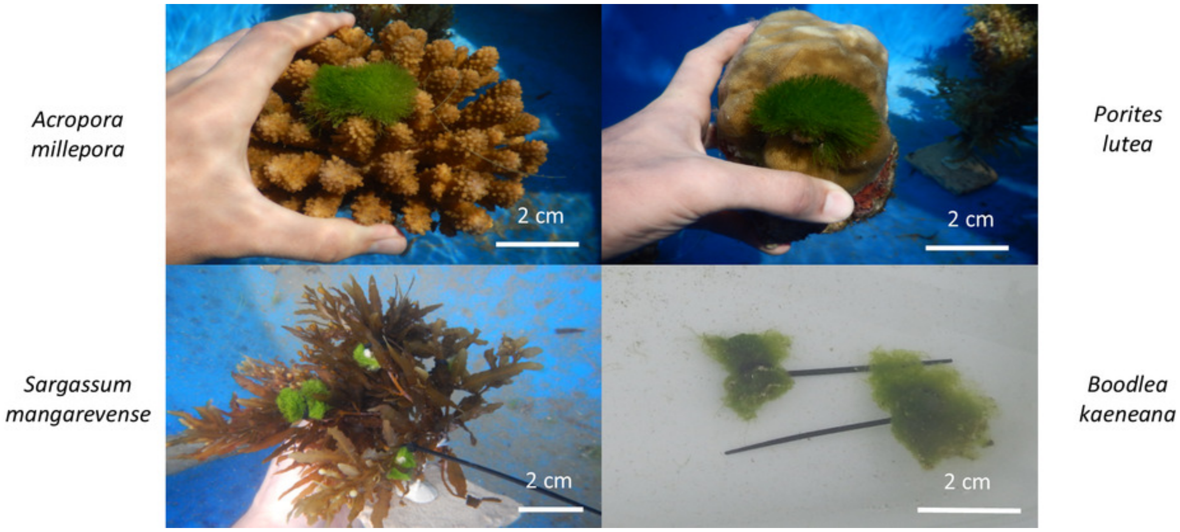


Figure 3

Setup for the PreSens respirometer.

Only the wells used are shown.

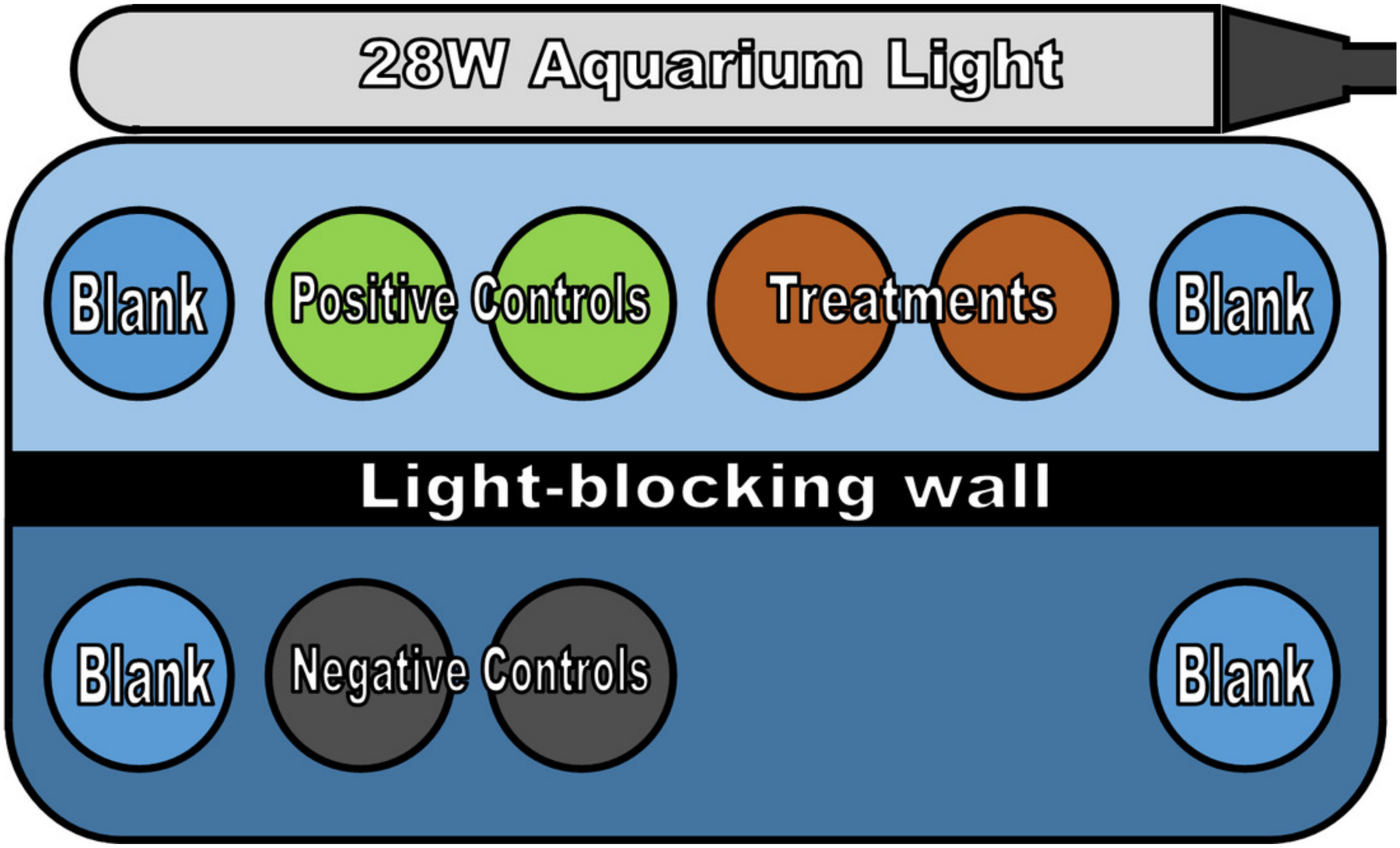


Figure 4

Results obtained by pairing *Chlorodesmis fastigiata* with corals and algae.

n=1 for each coral and n=2 for each alga. On the left is the diagram of coral bleaching over the eight days of the coral pairing experiment, and on the right is the diagram of algal photosynthetic efficiency over the five days of the algal pairing experiment

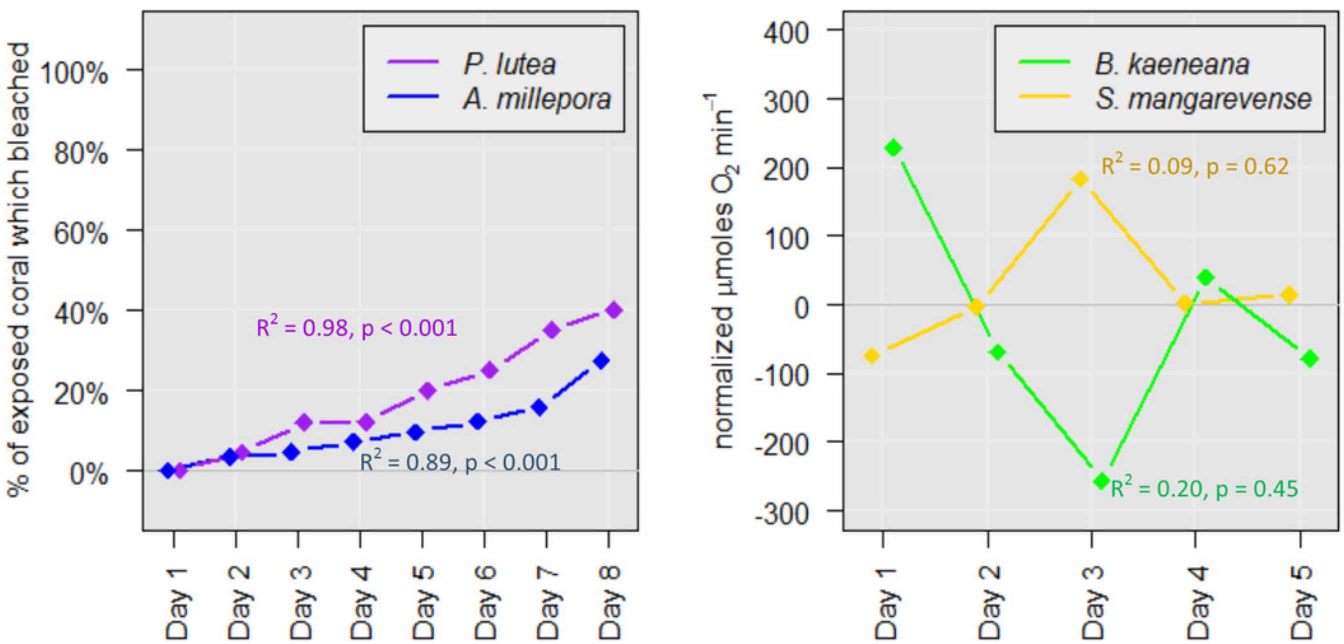


Figure 5

Results of the isolated toxin assays

n=16 for each treatment.

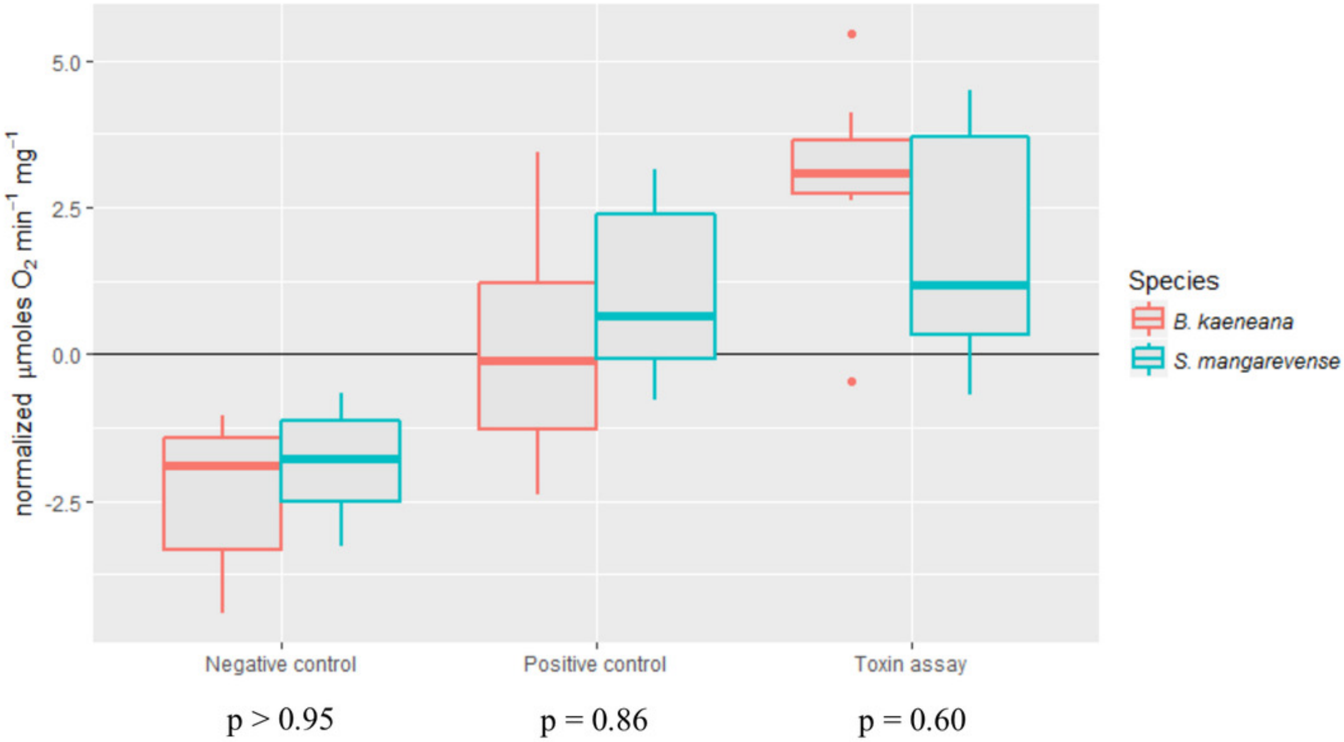
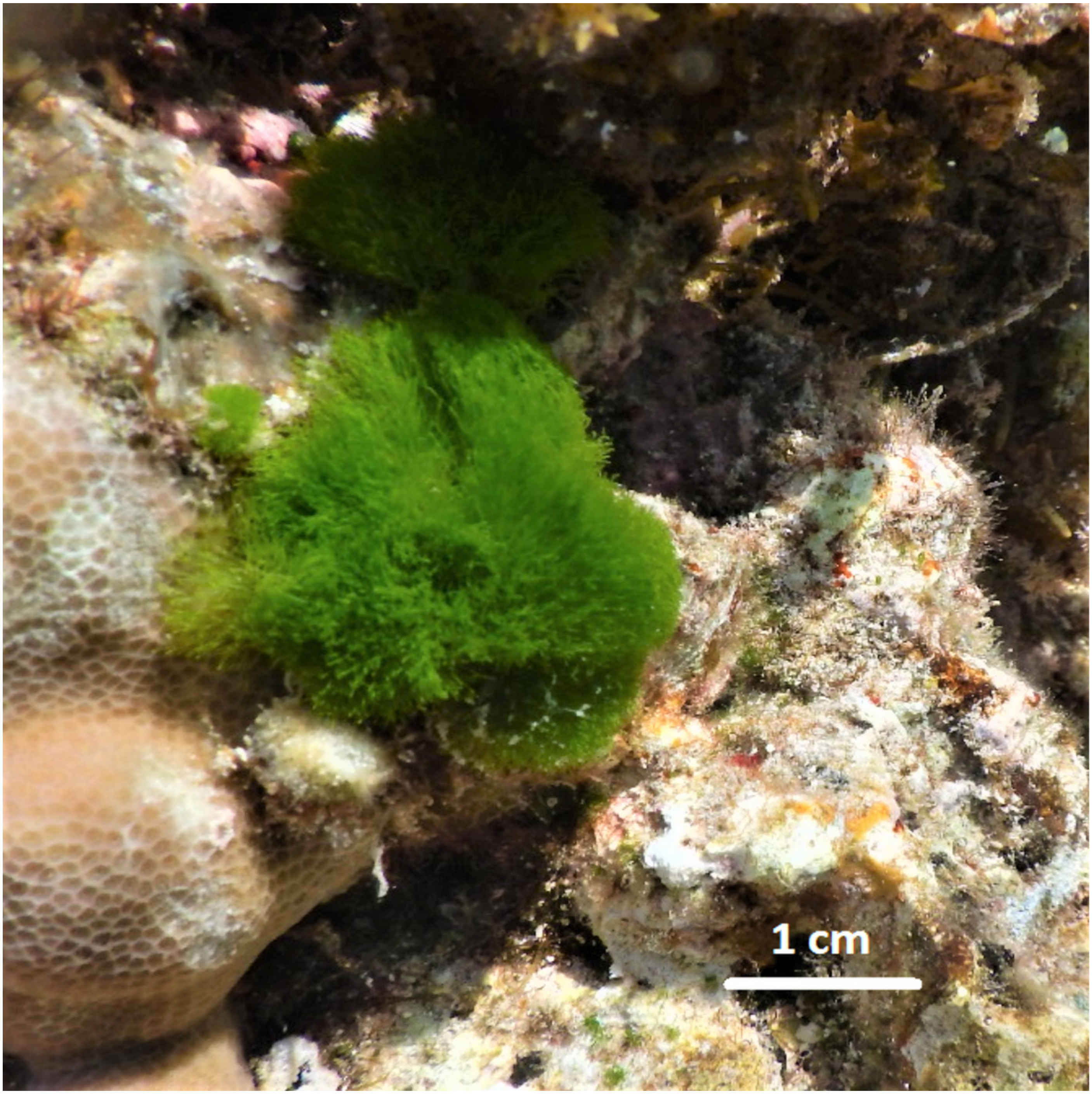


Figure 6

Chlorodesmis fastigiata in the field

Chlorodesmis fastigiata in the lagoon of Mo’orea, as seen during one of the field surveys carried out at Temae Public Beach. Note the damaged coral nearby.



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