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Research article

Changes in soft coral *Sarcophyton* sp. abundance and cytotoxicity at volcanic CO_2 seeps in Indonesia

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Abstract: This study presents the relationship between benthic cover of *Sarcophyton* sp. living on coral reefs and their cytotoxicity (an assumption of soft coral allelochemical levels) along acidification gradients caused by shallow water volcanic vent systems. Stations with moderate acidification (pH 7.87 ± 0.04), low acidification (pH 8.01 ± 0.04), and reference conditions (pH 8.2 ± 0.02) were selected near an Indonesian CO_2 seep (Minahasa, Gunung Api Island, and Mahengetang Island). Cover of the dominant soft coral species (*Sarcophyton* sp.) was assessed and tissue samples were collected at each site. The cytotoxicity tissue extracts were analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinon bromide (MTT) method. Levels of cytotoxicity were strongly correlated with *Sarcophyton* sp. cover ($p < 0.05$; $R^2 = 0.60$ at 30 ppm and 0.56 at 100 ppm), being highest at mean pH 8.01 where the soft corals were most abundant. This finding suggests that *Sarcophyton* sp. can be expected to survive ocean acidification near Indonesia in the coming decades. How the species might be adversely affected by further ocean acidification later in the century unless CO_2 emissions are reduced remains a concern.

Keywords: Soft coral; *Sarcophyton* sp.; cytotoxic metabolites; ocean acidification

1. Introduction

Ocean acidification, or the increasing of anthropogenic CO_2 level in seawater, is currently a marine environmental problem. A number of studies have found that coral reef building organisms

respond negatively to ocean acidification [1-2]. Recent evidences suggest a reduction in seawater pH may lead to lower calcification rate, polyp growth, and zooxanthellae cell density on *Scleractinian* corals [3-5]. Furthermore, observations into marine environments acidified by CO_2 seeps have shown the probable impacts of ocean acidification on the coral reef community [6]. Previous observations at these locations have reported a shift in coral reef composition towards domination of non-coral reef building organisms along acidification gradients caused by shallow water volcanic vent systems [7-9].

Soft coral is one of several non-coral reef building organisms that has been predicted to outcompete hard coral in future ocean acidification scenarios. A study conducted at CO_2 seeps on the seashore of Iwotorishima Island (Japan) showed domination of soft coral *Sarcophyton* sp. in an acidified reef environment [10]. Experimental biology studies have suggested that soft corals are able to mitigate the effects of acidification because their external soft bodies were found to protect the endoskeleton from acidic conditions [11,12]. Moreover, soft corals are also known for their ability to produce toxic allelochemicals. These chemical substances play an important role in soft corals, defensive and invasive capability on benthic environments [13-15]. It has been demonstrated that the amount of allelochemicals present may act as an indicator to predict the level of soft coral invasiveness in coral reef community [16]. Therefore, both biological and chemical ecology factors may have a significant influence on soft coral invasiveness patterns under future acidification pressures.

However, there has been little discussion about the allelochemicals of a particular coral reef organism under acidification pressures. This study presents benthic cover of *Sarcophyton* sp. living on coral reefs and their cytotoxicity along acidification gradients caused by shallow water volcanic vent systems in Indonesia. The aim of this study was to evaluate the influence of allelochemical factors on soft coral invasiveness patterns under ocean acidification pressures. This study used a high throughput screening cytotoxic assay, on the assumption that cytotoxicity is an indicator for allelochemical levels in soft corals. This rapid non-ecological assay approach was chosen because allelochemicals have been shown to be the major cytotoxic compounds present in soft corals. Previous studies have reported allelochemical activity in soft corals is caused by the production of terpenoid-type compounds [17-19]. Terpenoids from soft corals can cause mortality or growth inhibition in *Scleractinian* corals through direct contact or water column mediation [20]. Meanwhile, natural marine product research commonly found this type of compound, such as sarcophytoxide or sarcophytol, as a major cytotoxic compound in soft corals [21,22]. The percent composition of this particular compound class was found to be up to 61% of all of secondary metabolites in soft corals [23].

2. Materials and Methods

2.1. Study sites

The research was conducted at three Indonesian coral reef locations that were acidified by shallow water volcanic vent systems. The first location was the reef at Minahasa Seashore, North Sulawesi Province. The second location was a reef acidified by CO_2 from a seeps of an active volcano near Gunung Api Island, Banda-Neira region, in Maluku Province. The last location was selected at the reef of Mahengetang Island near large CO_2 vents of an active underwater volcano called Banua Wuhu, in the North Sulawesi Province. Three specific sites were selected at each location. The first and second sites were selected near CO_2 seeps. The first site was a "moderate acidification" site and selected with pH environment of 7.87 ± 0.04. The value was chosen based on prediction of pH level

within the next 100 years [24]. The second site was a "low acidification" site and selected near the same area as the first site, but moved away from the main vents until the surrounding pH was 8.01 ± 0.04. The last site was the "reference" site at a reef remote from the other two sites with a normal tropical pH level of 8.2 ± 0.02. The longitude and latitude of each site was recorded by a Garmin eTrex 10 GPS (Table 1). Preliminary research on seawater carbonate chemistry was conducted before sampling activities in order to select reef sites at each location (Table 2).

Table 1. Sampling locations and sites.

Location	Acidification Site	Latitude and Longitude
Minahasa Seashore	Moderate and Low	1°40.424'N, 125°7.088'E
	Reference	1°44.662'N, 125°8.406'E
Gunung Api Island	Moderate and Low	4°30.371'S, 129°53.067'E
	Reference	4°29.883'S, 129°56.113'E
Mahengetang Island	Moderate and Low	3°8.813'N, 125°26.818'E
	Reference	3°9.484'N, 125°31.620'E

2.2. Animal material, coral cover observation, and cytotoxic testing

Sarcophyton sp. samples were collected on the basis of their morphological similarities according to Fabricius & Alderslade [26]. Three 30 m line intercept transects (LIT) were laid at 4–6 m depth on each site. Underwater photographs with a 0.5 m^2 frame were taken along the lines to estimate the individual *Sarcophyton* sp. cover at each site (60 frames per LIT). Manta tow observations along the transect lines (three replicates at each site) were conducted, to gain information about dominant benthic cover (categorized as "hard coral" for coral building organisms, "other biota" for non-coral building organisms, or "abiotic") at each site. Five random *Sarcophyton* sp. samples were selected and the size (oral disk diameter in cm) of each sample was recorded *in situ.* Fresh 20 g of each sample was immediately preserved in 50 mL brown vial with 20 mL ethanol. Samples were placed in a cool box containing ice packs and transported to the laboratory. All samples were exhaustively extracted with ethanol at the laboratory, to yield the extracts used for cytotoxic study. The cytotoxic analysis was conducted in triplicate at low (30 mg/L) and high (100 mg/L) concentration to a single human MCF-7 cancer cell line. The analysis was conducted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinon bromide (MTT) assay [27].

2.3. Data processing and Statistical analysis

Calculation of individual *Sarcophyton* sp. cover at each site was undertaken through the employment of CPCe 3.6 software [28]. Prior to the statistical analyses, variables of cytotoxic activities, cover, and size (oral disk diameter), were log (x+1) transformed to satisfy the requirement of normally distributed data. All data normality was assessed using the Shapiro-Wilk W test. An ANOVA with Duncan Post-Hoc analysis was employed to analyze coral cover variation among locations and acidification sites. The same test was conducted to analyze variation of *Sarcophyton* sp. individual cover, size, and cytotoxic activity. Kruskall-Wallis correlation and linear regression analysis were conducted to analyze the relationship and determination level between cover, size, and

cytotoxic activity in *Sarcophyton* sp.. Statistical analyses were conducted with Past Statistical Software v3.08 [29].

Table 2. Carbonate chemistry (Mean ± SD, n = 18) calculated by CO_2SYS v2.1 [25], at sites with moderate acidification (pH 7.87 ± 0.04), low acidification (pH 8.01 ± 0.04), and reference pH (pH 8.2 ± 0.02) at Minahasa Seashore, Gunung Api, and Mahengetang Island.

Location	Acidification Sites	TA (mol/kg SW)	pCO_2 (atm)	CO_2 (mol/kg SW)	HCO_3 (mol/kg SW)	CO_3 (mol/kg SW)	Ω Aragonite	Ω Calcite
Minahasa	Moderate	2190.57± 0.74	545.26± 61.56	14.21 ± 1.87	1758.49 ± 42.89	173.29 ± 16.86	2.83 ± 0.29	4.26 ± 0.42
	Low	2189.64 ± 0.72	378.45 ± 44.54	9.88 ± 1.30	1646.48 ± 46.57	217.82 ± 18.83	3.56 ± 0.32	5.36 ±0.47
	Reference	2190.33 ± 0.13	214.24 ± 7.39	5.59 ± 0.30	1449.50 ± 24.46	296.49 ± 9.62	4.85 ± 0.17	7.29 ±0.25
Gunung Api	Moderate	2195.61 ± 1.86	643.99 ± 87.14	16.76 ± 2.39	1805.97 ± 39.17	155.38 ± 16.34	2.54 ± 0.27	3.82 ±0.40
	Low	2189.50 ± 1.39	411.76 ± 47.46	10.70 ± 1.34	1670.65 ± 41.68	207.78 ± 17.15	3.40 ± 0.29	5.11 ±0.42
	Reference	2188.34 ± 1.39	256.69 ± 16.20	6.65 ± 0.48	1509.19 ± 28.15	271.78 ± 11.66	4.45 ± 0.20	6.69 ±0.29
Mahengetang	Moderate	2187.79 ± 0.52	659.72± 71.99	17.19 ± 2.12	1810.93 ± 35.63	151.73 ± 14.41	2.48 ± 0.24	3.73 ±0.36
	Low	2187.54 ± 0.75	436.23 ± 50.10	11.39 ± 1.44	1691.50 ± 42.32	199.30 ± 17.14	3.26 ± 0.29	4.90 ±0.43
	Reference	2189.91 ± 0.64	226.96 ± 15.03	5.91 ± 0.48	1468.13 ± 33.26	288.64 ± 13.31	4.72 ± 0.23	7.10 ±0.33

3. Results and Discussion

It is apparent that coral cover was significantly different ($p < 0.05$) among locations and acidification sites (Figure 1). Significantly lower coral building organisms and higher abiotic cover were observed at Minahasa sites. Visual observation showed the reefs at Minahasa Seashore may also be negatively impacted by anthropogenic pressures. Numerous inorganic wastes lay on the benthic area, particularly at the moderate acidification site, located in the subtidal zone of human domesticated area. Therefore, higher abiotic factors at this site may suggest the impact of both anthropogenic and acidification pressures. Similar results have been shown by other studies at CO_2 vents in Papua New Guinea, which found the cumulative impacts of both pressures were more severely damaging to the reef structure than a single pressure alone [24].

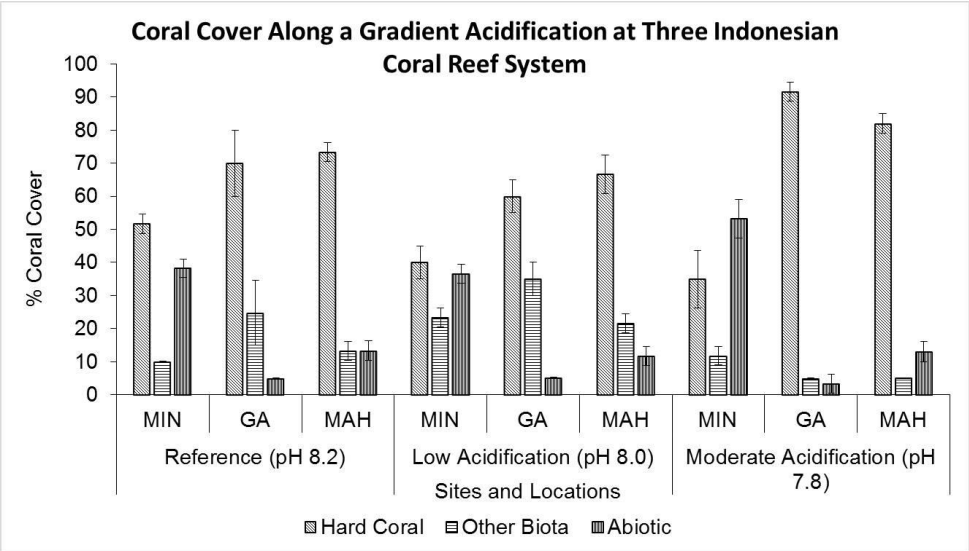
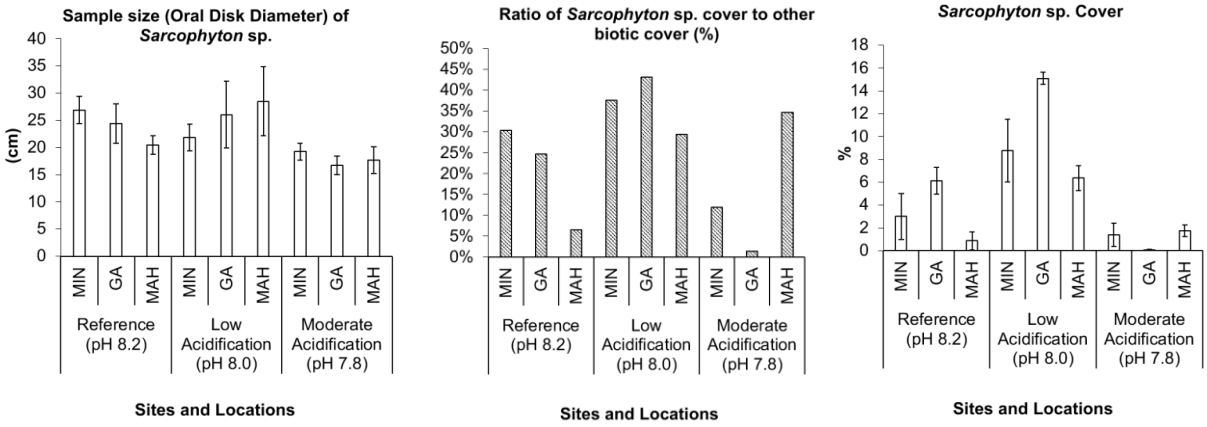


Figure 1. Coral cover along an acidification gradient of reference sites (pH 8.2 ± 0.02), low acidification (pH 8.01±0.04), and moderate acidification (pH 7.87 ± 0.04) at three Indonesian coral reef systems near CO_2 vents; Minahasa seashore (MIN), Gunung Api Island (GA), and Mahengetang Island (MAH).

Meanwhile, higher cover of "other biota" was observed at low acidification sites and significantly ($p < 0.05$) differed from other acidification sites. Even "other biota" cover was observed to increase at low acidification levels, but overall, hard coral organisms were detected to dominate the observed reefs. In contrast to other findings, this study found no evidence for predictions that suggest non-coral reef building organisms will outcompete hard coral organisms as the seawater becomes more acidic. Visual observations detected that *Acroporidae* dominated the reef at moderate acidification site at Gunung Api, while *Poritidae* dominated the live cover at moderate acidification sites at Mahengetang and Minahasa Seashore. Both of these corals are known for their capability to mitigate the effects of acidification [30-32]. These results support Doney et al. [33], who suggested the impacts of ocean acidification on coral reef structures will be more varied than previously thought, with many possible

combinations. Coral community composition may shift with much variance and without significantly reducing the live cover as the seawater becomes more acidic with a pH near 7.8.

The individual cover of *Sarcophyton* sp. ranged between 0.07–15.10% and was up to 43% of the overall "other biota" cover (Figure 2a and 2b). Furthermore, *Sarcophyton* sp. cover was significantly ($p < 0.05$) different among locations and acidification levels. The lower cover of *Sarcophyton* sp. at Mahengetang Islands was significantly different than other locations. Meanwhile, size of the samples varied between 14.4–28.5 cm (Figure 2c). Size was not significantly ($p > 0.05$) different among locations, but significantly ($p < 0.05$) different among acidification levels. Smaller samples were observed at moderate acidification sites.



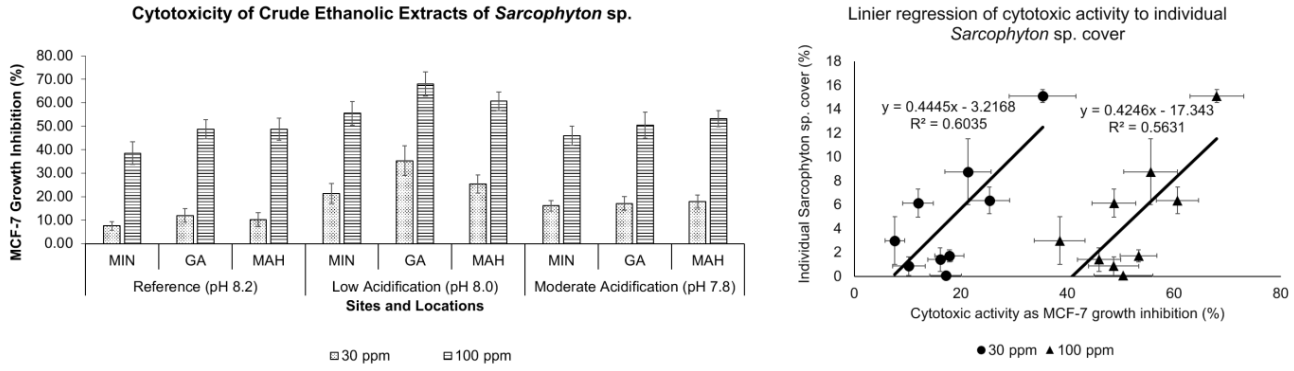
(a) (b) (c)

Figure 2. Individual cover (a), ratio of cover to overall "other biota" cover (b), and samples size by oral disk diameter measurement (c), of *Sarcophyton* sp. samples from moderate acidification (pH 7.87 ± 0.04), low acidification (pH 8.01 ± 0.04), and reference (pH 8.2 ± 0.02) sites at three Indonesian coral reef systems near CO_2 vents; Minahasa seashore (MIN), Gunung Api Island (GA), and Mahengetang Island (MAH).

Results of this study indicates that *Sarcophyton* sp. is a major component in "other biota" composition at all sites and locations. Soft corals domination is commonly observed on many near-shore coral reefs areas (4–6 m) in Pacific region, as an effect of their wide range of adaptation ability under various environmental pressures [34]. However, the size variation findings suggest soft corals may struggle to grow in moderate acidification environment. As a consequence, it appears that soft corals will not invade the reef as the seawater becomes more acidic through high pCO_2 levels. Similar patterns were also detected in a study at CO_2 seeps on the seashore of Iwotorishima Island, Japan. Higher cover of *Sarcophyton* sp. was only detected in the medium level of pCO_2 environment [10]. Therefore, despite soft coral possessing the capability to mitigate the effects of acidification pressures, this biological characteristic does not enhance their invasiveness in highly acidic environments.

Growth inhibition to MCF-7 cell lines was observed in the range between 8.07–36.73% for 30 mg/L samples and 38.76–71.01% for 100 mg/L samples (Figure 3a). Cytotoxic activity was

significantly ($p < 0.05$) different among samples that were taken from different locations and acidification levels. Lower cytotoxicity was found in the samples from Minahasa location and higher cytotoxicity was detected in the samples from low acidification sites. Moderate (R = 0.78 for the samples at 30 mg/L and 0.75 for the samples at 100 mg/L) and significant ($p < 0.05$) correlation was found between individual cover and cytotoxicity of *Sarcophyton* sp. Meanwhile, low (R < 0.4) and insignificant ($p > 0.05$) correlation was detected between cytotoxicity and size of the samples. Furthermore, linear regression analysis revealed cytotoxic activity moderately (R^2 approximately 0.6) determined the level of *Sarcophyton* sp. individual cover (Figure 3b).



(a) (b)

Figure 3. Cytotoxic activity of ethanol extracts (a) and linear regression of cytotoxic activity on individual cover (b) from all *Sarcophyton* sp. samples taken from moderate acidification (pH 7.87 ± 0.04), low acidification (pH 8.01 ± 0.04), and reference (pH 8.2 ± 0.02) sites at three Indonesian coral reef systems near CO_2 vents; Minahasa seashore (MIN), Gunung Api Island (GA), and Mahengetang Island (MAH).

A possible relationship between abundance and allelochemicals of soft coral *Sarcophyton* sp. under acidification pressures may be inferred if cytotoxicity is assumed to be an indicator of allelochemical levels. First, the finding of insignificant correlation between cytotoxicity and size of the samples (size approximately relates with age and other biological stages) may indicate that biological stage has an insignificant relationship with the capability of soft coral to produce allelochemicals. The production of allelochemicals depends on the need of competition for space. Similar patterns have also been shown in two marine sponges *Hyrtios erecta* and *Ianthella basta*; in both, the size of the sponges was not detected to relate with their cytotoxicity level [35]. Secondly, the finding of strong and significant correlation between cytotoxic activity and individual cover of soft coral in a particular reef sites may suggest allelochemicals play an important role in soft corals' invasiveness in an acidic environment.

The hypothetical relationships described above may mirror the allelochemical production and abundance of *Sarcophyton* sp. at each location and acidification site. Lower cytotoxic/allelochemical metabolites production in the samples from Minahasa seashore sites may suggest the effects of low competition for space. Higher abiotic elements may mean lower competition for space and *Sarcophyton* sp. may respond by decreasing allelochemical production. Previous studies have shown similar results, as lower cytotoxicity was observed in soft coral and sponges from higher abiotic reef

environments that were pressured by anthropogenic activities [36-38]. Meanwhile, cytotoxicity was highest at pH 8.01 where the *Sarcophyton* sp. was most abundant, which may indicate higher amounts of allelochemicals enhance the ecological competitiveness of soft coral. However, further acidic conditions might disturb the metabolic system of allelochemical production, thus affecting the ecological competitiveness and as a consequence, lower cover of soft coral may be observed.

Similar patterns have been shown in other ocean acidification studies. The lower ecological competitiveness of sea grass in the presence of acidification pressures was predicted, as experimental study has shown a reduction of their phenolic substances (chemical defensive metabolites) production in increasing pCO_2 [39]. Meanwhile, a higher ecological competitiveness level of anemones in acidification pressures was predicted, as field observation has shown their adaptation in acidic environments via elevated productivity and their use of allelochemicals to outcompete algae species [40]. Therefore, this study suggests that the defense/allelochemical system is an important factor for soft corals survival and invasiveness patterns in future ocean acidification.

4. Conclusion

Even having biological characteristics to mitigate the effects of ocean acidification, the finding of elevated cytotoxicity suggests that allelochemicals may have an important role in *Sarcophyton* sp. invasiveness pattern under ocean acidification pressures. However as this study used non-ecological assays, which assumed cytotoxicity is an indicator of allelochemical activity, how the species might be adversely affected by ocean acidification later in the century unless CO_2 emissions are reduced remains a concern. Further study using ecologically relevant bioassays to examine the described findings will be insightful.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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