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Fluctuation of associated microbial with building reef corals *Acropora* sp. from Hang Rai, Ninh Thuan

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ABSTRACT

El Niño and the prolonged warm sea surface temperature significantly impacted coral reefs and caused coral bleaching in some parts of the world. This study evaluated the density of symbiotic algae and bacteria associated with the three coral species, namely *Acropora hyacinthus*, *Acropora muricata*, and *Acropora robusta*, collected in Hang Rai, Ninh Thuan in May, June, August 2016, and June 2017. The number of zooxanthellae with each coral species was statistically significant and correlated with several environmental factors, suggesting that symbiotic algae could play a key role in coral health. The number of associated microbial with the three coral species was significantly different; they tended to depend on sampling time rather than coral species-specific. At the time of ENSO (2016), the difference in the total associated bacteria with all three coral species was statistically significant. While the total number of related bacteria with all three species of coral collected in 2017 did not differ from the total of bacteria in ambient water. In conclusion, symbiotic algae tend to be species-specific, whereas bacteria fluctuate significantly over sampling time. Studying the molecular issues of microalgae, the presence, the role of some groups of bacteria involved in the N, C, P, and S cycles, and the influence of environmental parameters should also be encouraged to understand the relationship of coral holobiont better.

Keywords: Symbiotic microalgae, bacteria, *Acropora* sp., environmental factors, Ninh Thuan.

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INTRODUCTION

The role of symbiotic algae in marine adaptation that can help corals overcome adverse environmental changes has been gathered from numerous studies in the review by Blackall et al., [1]. Symbiotic algae with corals are thought to be highly adaptive to changes in environmental conditions such as rising sea temperatures. In many coral species, symbiotic algae have helped corals cope with environmental changes by changing the population composition to adapt to adverse environmental conditions; however, this mechanism has only been discovered for symbiotic algae that have been present in coral tissues since the time of coral reefs is still a larva. The evidence for the conversion of symbiotic algae from the environment into adult corals is still unclear. Boulotte et al., [2] used molecular biology techniques to study Symbiodinium clades symbiotic with two coral species *Pocillopara damicornis* and *Stylophora pistillata* and show that there is a targeted invasion of some species symbiotic algae from the environment into adult corals after two consecutive bleaching sessions. The genus *Symbiodinium* consists of 9 clades from *A* to *I*. The different clades Symbiodinium have different degrees of physiological adaptation and tolerance to stress (possibly heat stress), most of the new symbiotic algal species was detected at less than 1% in terms of abundance, in contrast, there was a newly introduced clade with an abundance index of over 33%. Intentionally invading *Symbiodinium* was identified as clade *D* thermophilic *Symbiodinium* so it is possible to see population transition driven by two successive bleachings, this finding is particularly important given that the transformation patterns of symbiotic microalgae in the two studied corals that were previously known to have selective symbiosis with algae. When corals with *Symbiodinum* clade *D* were dominant, they were more heat tolerant than corals occupied by other clades [3, 4].

Coral bleaching, a commonly known phenomenon that causes mass coral deaths worldwide, was also observed in the South Asia Pacific and Vietnam waters in 1998. In some places, it was observed to be very severe with more than 30% of corals in the area bleaching. Bleaching was observed in the Gulf of Thailand, Nha Trang bay, Van Phong bay, and others in 2010. The cause was attributed to the influence of the El Niño phenomenon that caused the sea surface temperature to increase [5]. In 2015–2016, El Niño and ocean warming significantly impacted coral reefs and caused coral bleaching in many oceans worldwide. In Indonesia, the first signs of bleaching were reported in April 2016. However, this El Niño has been affecting the Indonesia reef since 2015 through a process other than bleaching caused by temperature. In September 2015, all measured data showed the lowest sea level in the past 12 years, thus affecting the bottom coral reefs. In March 2016. Bunaken island (Northern Sulawesi) is dominated by the coral species *Porites*, *Heliopora* and *Goniastrea* with mortality rates up to 85% according to different coral genera. Most reef flats have the highest mortality rates, and account for 30% of the island's reefs. For reef colonies living near below mean sea level before El Niño, increased coral mortality may be due to corals being exposed to less daily air especially during low tide. All measured data, used to map sea level declines across Indonesia, show widespread coral death to schist reefs in the shallow waters they make up most of the total coral reefs in Indonesia [6].

Co-living microorganisms have a certain role for the coral host, they are the suppliers of nutrients for the coral [7], participating in the natural defense mechanism against pathogenic microorganisms through the production of substances that are resistant to microorganisms, such as peptides and antibiotics [8, 9], and competitive regulation of microorganisms within the same host [10].

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Over the past 30 years, the coral disease has been considered a severe threat to global coral reefs; however, its causes are primarily due to changes in habitat leading to changes in composition and raising abnormal proliferation of bacteria that cause coral disease. Many studies have shown the causative agents of coral diseases; for example, coral bleaching disease *Oculina patagonica* is caused by opportunistic bacteria *Vibrio shiloi* [11], and *Vibrio coralliilyticus* causes bleaching disease for coral *Pocillopora damicornis* [12]. White pox disease in coral *Acropora palmate* was identified as caused by Gram-negative bacteria *Serratia marcescens* [13]. Research on microorganisms living with 18 coral species belonging to 10 genera in 6 families of block corals and reef-forming corals in the East Vietnam Sea shows that bacteria living with coral species have a species-selective ability to adapt to cold and heat tolerant; however, known coral pathogens were found in almost all coral samples [14]. Ocean acidification does not affect corals without symbiotic *Leptopsammia pruvoti* of the Mediterranean Sea [15]. Recent evidence shows that coral bleaching is most likely closely related to coral symbiotic plankton and groups of nitrogen-fixing bacteria [16].

METHODS

Materials and research methods

Three coral species, *Acropora hyacinthus*, *Acropora muricata*, and *Acropora robusta*, were used as research materials in this study. Live coral samples showing no bleaching and showing no signs of bleaching at the time of collection were collected as healthy and diseased coral samples, respectively. Coral samples were collected in May, June, August 2016, and June 2017 by skilled divers (SCUBA) at a depth of 5–7 m at the location at coordinates 109°18’28.1”E, 11°67’71.7”N, at Hang Rai-Ninh Thuan. The method of sample collection and pretreatment of samples, and analysis of samples and environmental factors are detailed in our previous study [17]. The symbiotic algae, bacteria in healthy coral samples, and bleached coral samples were treated with 1% potassium citrate solution to remove the fluorescent colorants available in corals, then filtered through a 0.02 µm filter (AnodiscTM Whatman) and stained with SYBR Gold (Invitrogen) [18] and examined under a fluorescence optical microscope. The total number of bacteria and bacterial shapes (spheres, rods, bacilli, etc.) will be counted with an Olympus Provis AX70 fluorescence microscope and image processing with digital imaging software (Olympus-DP71).

Data processing

The principal coordinate analysis (PCoA) graph was applied to identify and cluster sample groups. PCoA is a method used to analyze and represent the similarities or differences between data sets. First, each data combination will establish a similarity or dissimilarity matrix (based on the distance matrix). This report selects the Gower distance [19] to construct the similarity/dissimilarity matrix.

The formula for calculating the Gower coefficient between two elements i and j is calculated as follows (d_{ij}):

$$d_{ij} = \frac{\sum_{f=1}^{p} \delta_{ij}^{(f)} \cdot d_{ij}^{(f)}}{\sum_{f=1}^{p} \delta_{ij}^{(f)}}$$

where: $\delta_{ij}^{(f)} = 1$ if x_{ij} and x_{jf} or the f variable are present, if not, it equals 0; $\delta_{ij}^{(f)} = 0$ if f is an asymmetric binary variable and elements i and j both have the value 0–0. If the variable f is binary or identifier then $d_{ij}^{(f)} = 0$ with $x_{ij} = x_{if}$ and $d_{ij}^{(f)} = 1$ with $x_{ij} \neq x_{if}$

The similarity/dissimilarity matrix through Gower distance was calculated and set up on R 3.0.2 software with package ade4 and Vegan R-Development Core Team [20]. After being created, the similarity/dissimilarity matrix will be used to represent and group on the PCoA chart [21].

RESULTS AND DISCUSSION

Environmental parameters at the time of sample collection

The environmental parameters measured at the sampling site in May, June, August 2016,

and June 2017 are calculated and presented as the mean and standard deviation in Table 1.

The temperature fluctuated wildly between the surveys; the highest temperature was in May 2016 with an average value of 30.55 ± 0.05°C and the lowest in June 2016 with an average value of 24 ± 0.5°C. Especially in June 2016, the study area had the largest turbidity of 4.5, whereas, in June 2017, the lowest measured turbidity was 2.50. TSS, POM, and DO parameters measured in June 2016 and 2017 all have higher values than the other two surveys. The dissolved oxygen content in the study area is relatively abundant, ranging from 5.79–7.00 mgO_2/L, which is a suitable range for the growth and development of marine organisms. Generally, the study area's water quality is still good; all survey factors satisfy QCVN 10/2015 (Table 1).

Table 1. Results of environmental parameters

Parameters	5/2016	6/2016	8/2016	6/2017	QCVN
pH	8.10 ± 0.00	7.69 ± 0.02	7.61 ± 0.03	8.03 ± 0.005	6.5–8.5
Temperature (°C)	30.55 ± 0.05	24.00 ± 0.50	27.95 ± 0.05	27.10 ± 0.10	-
Salinity (‰)	34.30 ± 0.00	34.80 ± 0.10	34.60 ± 0.00	33.45 ± 0.15	-
Turbidity (NTU)	3.50 ± 0.50	4.50 ± 0.50	4.00 ± 0.00	2.50 ± 0.50	-
TSS (mg/L)	0.87 ± 0.17	1.27 ± 0.33	0.80 ± 0.20	2.50 ± 0.05	50
POM (mg/L)	0.47 ± 0.10	0.78 ± 0.22	0.37 ± 0.10	1.44 ± 0.04	-
DO (mgO_2/L)	6.46 ± 0.02	6.65 ± 0.22	5.79 ± 0.20	7.00 ± 0.13	≥ 5
BOD_5 (mgO_2/L)	0.60 ± 0.07	0.95 ± 0.07	0.51 ± 0.15	0.63 ± 0.06	-
NO_2 (µgN/L)	1.80 ± 0.18	8.61 ± 4.89	0.79 ± 0.59	1.61 ± 0.58	-
NO_3 (µgN/L)	73.49 ± 6.03	89.99 ± 16.04	58.59 ± 10.14	49.67 ± 8.14	-
NH_4 (µgN/L)	59.63 ± 2.64	69.27 ± 14.78	48.12 ± 3.22	55.60 ± 4.37	100
PO_4 (µgP/L)	15.89 ± 2.30	9.50 ± 2.58	4.77 ± 1.29	9.57 ± 2.19	200
Chl-a (µg/L)	0.90 ± 0.08	1.52 ± 0.31	0.71 ± 0.12	0.73 ± 0.85	-

Notes: QCVN: Vietnamese standards 10/2015; "-": not yet regulated.

Symbiotic algae

The number of algae (cells/g of fresh coral) presented in Figure 1 represents the mean and standard deviation (Figure 2).

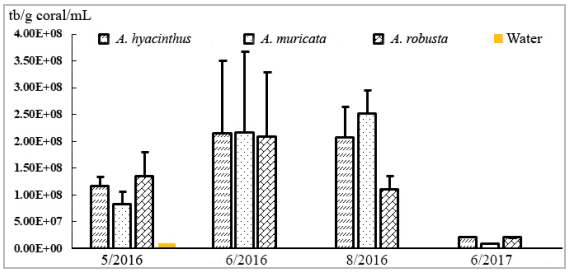

Figure 1. Total number of heterotrophic bacteria by EFM fluorescene counting

In general, at three months, the number of algae in all three coral species was within the normal range calculated per 1 cm^2 of unbleached living coral tissue surface of 1–5 × 106 cells [22]. However, the number of algae in *A. hyacinthus* in August 2016 was about 100 times higher than usual. Algae symbiosis positively correlates with the total number of bacteria, salinity, turbidity, and NO_3 inversely with TSS and pH. The symbiotic algae had neither a positive nor negative correlation with the temperature factor (Table 2). Microbiological studies of 34 *Acropora millepora* colonies living on the Great Barrier Reef (Australia) from October 2000 to March 2003, including coral bleaching time, have shown that during bleaching, the density of algae symbiosis decreased up to 64% and was negatively correlated with temperature, whereas the percentage of degenerate zoox-algae was positively correlated [23]. The symbiotic algae *Symbiodinium* clade *C* with three coral species *A. hyacinthus*, *A. japonica*, and *Cyphastrea chalcidicum* in Tanabe bay, Japan, is thought to be the dominant group at low temperatures [24]. *Symbiodinium* clade *D* is the dominant group found at higher

temperatures in an experiment with *Porites* coral in Palau [25]. However, Bellantuono et al., [26] suggested that temperature did not affect the symbiotic algae, nor the microbiota living with the coral and indicated that it is the flexibility of the physiological relationship of the corals. The new coral and microbiota that live together help corals overcome the adverse effects of increased environmental temperatures. In this paper, only the number of algae was studied, so it is impossible to indicate the specific algae clade in the coral.

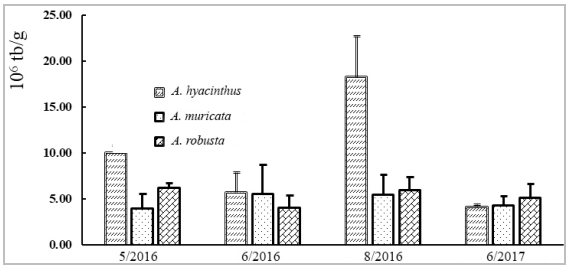

Figure 2. Total number of algae symbiotically with three coral species

Total number of heterotrophic bacteria

The total bacteria in all three coral species in June 2017 was lower than in the 2016 samples. Total bacteria were positively correlated with the total number of microalgae, which was strongly associated with environmental factors such as salinity, turbidity, and NO_3 and negatively correlated with pH (Table 2). Bacteria in corals were about 200 times higher than bacteria in seawater when counted directly. This result is similar to similar studies by Nguyen et al., [27].

The composition of bacteria in *A. hyacinthus*, *A. robusta*, and in water samples in May 2016 was almost the same, with the majority of components being cocci, comma and rod (rod-shaped bacillus) at least, whereas, in *A. muricata*, the bacterial component accounted for the highest percentage.

The bacteria that produce and use nitrogen found in coral tissue are related to the host, which is actually very closely related in terms of nutrition; they are the bacilli *Roseobacter*, *Spongiobacter*, *Vibrio*, and *Alteromonas* [28, 29]. Bacilli increased gradually in June and August in water samples, coral *A. muricata*, and *A. robusta*. Compared with the water sample, the bacterial composition of the three coral species changed over time; when in the water sample, cocci always accounted for the highest percentage, followed by bacteria and bacilli, except for the water sample in June 2017, bacteria accounted for the highest rate. Bacterial composition in June 2017 in coral *A. hyacinthus* and *A. robusta* had similar proportions of bacilli and bacilli while cocci and filaments accounted for an equally small proportion.

In August, with the lowest pH compared to the other months, bacilli in *A. muricata* and *A. robusta* increased, while bacilli predominated in *A. hyacinthus*. Total bacteria, bacilli, were negatively correlated with pH (Table 3). The results of this paper are similar to the studies by Meron et al., [30, 31] on coral *Acropora eurystoma* in Eilat bay, the Red Sea, which showed that at pH = 7.3, the bacterial flora lived with the same corals were more diverse in composition as well as in number than the living bacteria when at pH = 8.2, *Vibrionaceae* and *Alteromonadaceae* were the most dominant. Especially when most antibiotic-resistant bacteria were also isolated from coral at pH = 7.3 out of 54 antibiotic strains, up to 50% of strains belonged to *Vibrionaceae*, and 29% belonged to *Rhodobacteraceae*. It is clear that when corals are cultured at low pH conditions, the microbiota associated with disease and stress (possibly Vibrio) for the coral increases. On the other hand, bacteria with antibacterial potential (probably *Vibrio*, *Rhodobacteraceae*) also increased when corals were at low pH.

In August 2016, with the lowest PO_4 concentration, bacteria and bacilli increased to overwhelm cocci. Bacteria living on corals of bacilli and bacilli group were negatively correlated [17] with PO_4 content. In contrast, the proportion of cocci and bacilli in water samples over the months did not change significantly, and Anova's test showed that this difference is also not statistically significant ($p > 0.05$). However, when comparing all the data obtained in 2016 and June 2017, bacilli have a statistically significant inverse correlation with PO_4 content, but the bacteriophage group has no correlation with this factor (Table 2). Moreover,

bacilli have a statistically significant negative correlation with bacilli and cocci. In June 2017, the PO_4 content was equivalent to May and June 2016; the proportion of bacilli in *A. hyacinthus* and *A. robusta* was higher than that of other bacterial components, while the proportion of bacterial components in *A. muricata* had similarity with the bacterial composition in water (Figure 3). In May 2016, when the temperature measured at the sampling area was the highest in 4 collection times, the bacilli had the lowest percentage in all samples, including water samples, analyzing the correlation between bacilli and temperature. The levels show a statistically significant negative correlation (Table 2).

Table 2. Results of the Pearson correlation coefficient test

Compare	*r* (*n* = 36)	*p*
Bacteria and zooxanthellae	0.369	0.05
Zooxanthellae pH	-0.402	0.05
Zooxanthellae and turbidity	0.507	0.01
Zooxanthellae and salinity	0.566	0.01
Zooxanthellae and NO_3	0.393	0.05
Zooxanthellae TSS	-0.424	0.01
Total bacteria and pH	-0.690	0.01
Total bacteria and turbidity	0.681	0.01
Total bacteria and salinity	0.735	0.01
Total bacteria and NO_3	0.411	0.01
Total bacteria and PO_4	-0.352	0.05
Total bacteria and temperature	-0.431	0.01
Rod and pH	-0.531	0.01
Rod and PO_4	-0.448	0.01
Comma and temperature	0.609	0.01
Comma and pH	0.326	0.05
Comma and NO_3	-0.445	0.01
Comma and Chl-a	-0.488	0.01
Comma and BOD_5	-0.632	0.01
Rod and comma	-0.535	0.01
Rod and coccus	-0.835	0.01

To our knowledge, there are many studies on the antibiotic-producing ability of *Bacillus* and research on detecting pathogenic microorganisms from corals; however, there are no studies on the impact of heat degree of variation in bacilli in corals. Indeed, bacilli are a group with a relatively wide ecological range of temperatures ranging from 0–45 degrees; however, when they live in the coral component, they are known to be species-specific and can vary by species host regulation [32]. Recently, many findings have shown that even bacteria are commonly thought to be involved in causing diseases such as *Vibrio*, *Shewanella*, etc. Still, when harvested from invertebrates, for example, *Shewanella algae*-Gram-negative bacilli isolated from the sponge, *Callyspongia diffusa* of the Indian sea is a strain resistant to many bacteria and also resistant pathogenic fungi [33]. In this study, the May coral samples at the sampling site were bleached, and bacilli accounted for the lowest percentage of the bacterial composition in coral and water.

Table 3. Fisher's test results on microalgae, bacteria

Symbiotic algae	Group					Bacteria	Group			
AH-Aug16	*A*					AR-Aug16	*A*			
AH-May16		*B*				AM-Aug16	*A*	*B*		
AR-May16		*B*	*C*			AM-Jun16	*A*	*B*	*C*	
AR-Aug16			*C*			AH-Jun16	*A*	*B*	*C*	
AH-Jun16			*C*			AR-Jun16	*A*	*B*	*C*	
AM-Jun16			*C*	*D*		AH-Aug16	*A*	*B*	*C*	
AM-Aug16			*C*	*D*		AR-May16		*B*	*C*	*D*
AR-Jun17			*C*	*D*		AH-May16		*B*	*C*	*D*
AH-Jun17			*C*	*D*	*E*	AM-May16			*C*	*D*
AM-Jun17			*C*	*D*	*E*	AH-Jun17				*D*
AR-Jun16			*C*	*D*	*E*	AR-Jun17				*D*
AM-May16			*C*	*D*	*E*	AM-Jun17				*D*
						Water-Aug16				*D*
						Water-May16				*D*
						Water-Jun16				*D*

Notes: A. robusta (AR), *A. muricata* (AM), *A. hyacinthus* (AH) according to the time. Where: 5/2016 (May16), 6/2016 (Jun16), 8/2016 (Aug16) and 6/2017 (Jun17), water-water.

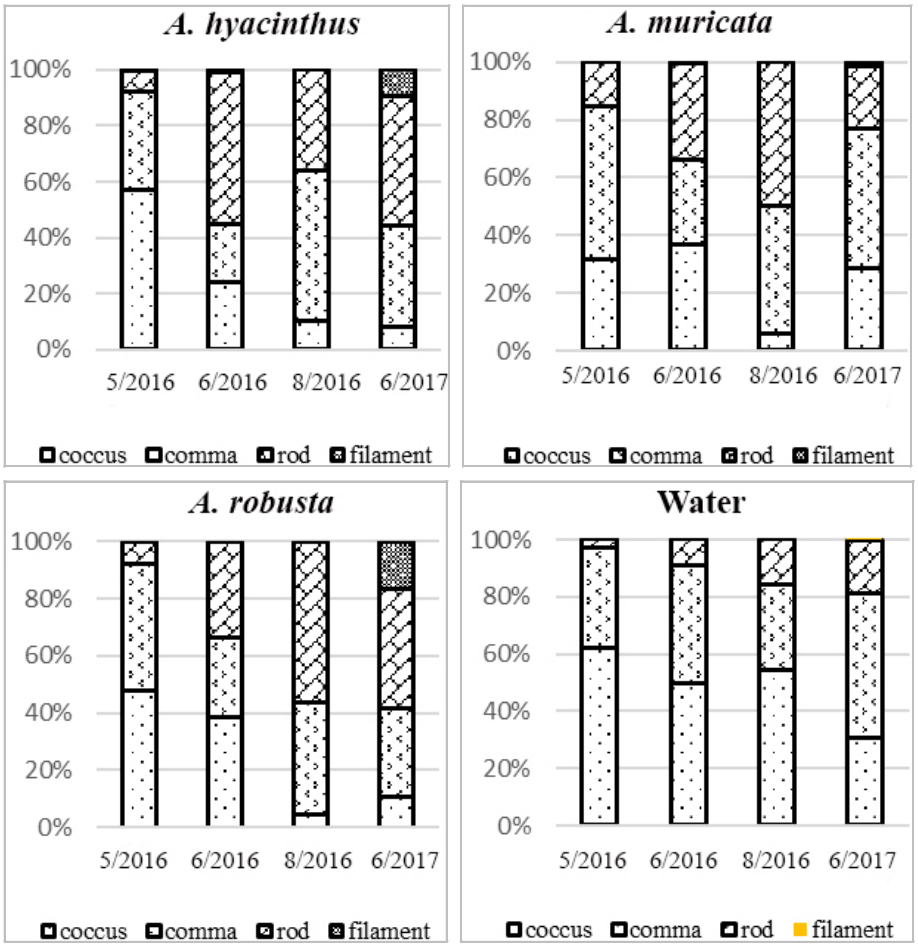

Figure 3. Composition of bacteria that live with coral

The subgroup of symbiotic microalgae, bacteria that live with corals

Figure 4 shows the degree of subgrouping of bacteria, microalgae, and bacterial composition in three coral species and water samples, excluding environmental factors. The vertical axis PC2 (21.4%) and the horizontal axis PC1 (48.8%) show a total of 70.2% of the research samples distributed according to the trend shown in Figure 4.

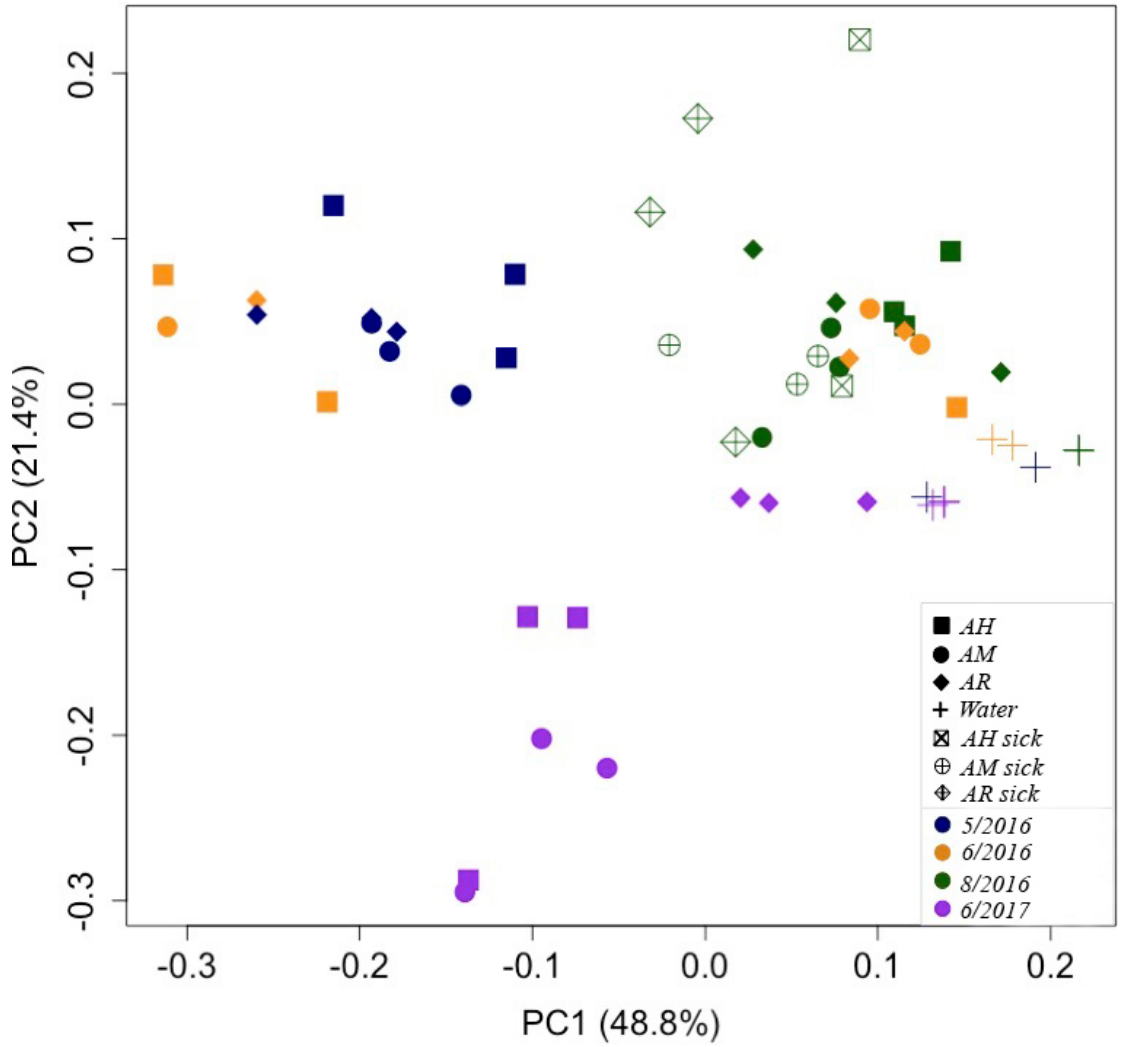

Figure 4. Distribution of microbial samples that live with coral

Bacteria and bacterial composition are distributed into three groups, of which the most obvious is the subgroup of bleached coral collected in May 2016 and healthy coral collected in May 2016, similar to the samples collected in 2017 tended to form a separate group, and the water samples from all four samplings formed a different group. However, it is unclear whether they are influenced by coral species or tend to be distributed over sampling time. One-way ANOVA test allows determining the frequency of difference of the experimental samples showing that $R^2_{bacteria}$ = 0.659, F_{test} = 3.556, $R^2_{symbiotic\ algae}$ = 0.852, F_{test} = 10.887 compared to $F_{standard}$ = 2.82; this difference is significant, and for further clarification, Fisher's pairwise test is presented in Table 3.

Table 3 shows that the number of symbiotic algae with coral *A. hyacinthus* in August 2016 (AH-Aug16 group *A*) compared with all other samples is statistically significant. Algae symbiotic with *A. hyacinthus* in May 2016 (AH-May16, group *B*) significantly differed from all samples. In June 2016, symbiotic algae with coral *A. hyacinthus* belonged to group *C*, while in June 2017 belonged to groups *C*, *D*, and *E*. Therefore, the number of symbiotic algae in *A. hyacinthus* can be seen between the two groups. The months are meaningful. Similar to the symbiotic algae with *A. hyacinthus*, the difference in the number of symbiotic algae with *A. robusta* was also significant between months. While symbiotic algae with *A. muricata* in June and August 2016 were not statistically significant (both in groups *C* and *D*). The difference in symbiotic algae with *A. muricata* in May 2016 and June 2017 was also not statistically significant (same group *C*, *D*, *E*).

In general, the variation of symbiotic algae among coral species tended to differ by coral species rather than by sampling time. Many works have reported that algae play a crucial role for coral hosts in resisting adverse changes in habitat, especially coral bleaching [2].

The differences between the non-statistically significant samples grouped into a group and denoted *A* to *D* for bacterial comparisons are presented in Table 3.

Bacteria living with *A. robusta* in August 2016 differed significantly from those collected in May 2016 (AR-May16, group *B*, *C*, *D*) and those contained in June 2016 (AR-Jun16, group). *A*, *B*, *C*), and the June 2017 collection (AR-Jun17, group *D*). Bacterial coexistence with all three coral species collected in May 2016 significantly differed from all samples collected in other months of the study. Bacteria living with three coral species collected in June 2016 significantly differed from those collected in June 2017. There is a significant difference between the number of bacteria residing with *A. murica* and the other two coral species during the same sampling period in May 2016.

Bacteria in all three coral species in the samples collected in June 2017 (including AH-Jun 17, AR-Jun 17, and AM-Jun 17 of group *D*, the same group as the water samples collected in May, June, and August 2016, and June 2017) showed that bacteria living with the three coral species in June 2017 had statistically significant differences for all bacteria living with the three coral species studied in 2016 sample collection. The number of bacteria residing with corals significantly depended more on the time parameter than the coral species. 2017 is the year without the ENSO phenomenon; the difference in the number of bacteria living with corals compared to the difference in bacteria in seawater is not statistically significant (same group *D*). The results of this study show that the number of bacteria in all three coral species significantly differs according to the time of sampling, especially at times of adverse environmental conditions such as May and August 2016, compared with the time of sampling in June 2017. This result tends to coincide with previously published studies; when there are adverse environmental effects, the coral holobiont system has mechanisms to regulate the coexistence of the community to overcome the disadvantages of the environment [32].

CONCLUSIONS AND RECOMMENDATIONS

Algae and bacteria living with corals are positively correlated with each other and with salinity, turbidity, NO_3 factors, and negatively correlated with pH. The bacteria cohabiting with corals varied significantly over sampling, suggesting that the coral microbiota is co-regulated with the host to adapt to environmental conditions. Symbiotic algae tend to be species-specific, so they can play a crucial role in coral biota and coral health. To better understand the clade (branches) of algae that symbiotically with corals, further studies such as studying genes are needed. Some groups of bacteria participating in the *N*, *C*, *P*, and *S* cycle should be prioritized for research on their presence, their role in corals, and the influence of environmental parameters.

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