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Effects of live rock on removal of dissolved inorganic nitrogen in coral aquaria

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Abstract

Maintaining stable water quality is one of the key processes for recirculating coral aquaculture. Traditional aquarium systems which mainly utilized a nitrification of nitrifying bacteria attached to the surface of massive artificial filter material are difficult to maintain the oligotrophic conditions necessary for coral aquaculture. This study investigated the removal effects of dissolved inorganic nitrogen (ammonia and nitrate) by live rock (LR), a key component in the "Berlin system" coral aquarium. The expression levels of bacterial functional genes, *AOA3*, *amoA* and *nosZ*, were measured on the exterior and interior of LR. The nitrifying and denitrifying bacterial abundance on LR was quantified and the nitrogen nutrient regulatory effects of LR were evaluated. The results demonstrated that LR mainly removed ammonium (NH_4^+) from the water with a mean efficiency of 0.141 mg/(kg·h), while the removal of nitrate (NO_3^-) was not significant. Bacterial diversity analysis showed that ammonia-oxidizing bacteria (AOB) were the most common bacteria on LR, which accounted for 0.5%–1.4% of the total bacterial population, followed by denitrifying bacteria, which accounted for 0.2% of the total population, and the ammonia-oxidizing archaea (AOA) were the least common type (<0.01%). The low abundance of denitrifying bacteria may be responsible for the poor nitrate (NO_3^-) removal of LR. Thus, other biological filtration methods are needed in coral aquaria to control nitrates generated from nitrification or biological metabolism.

Key words: coral, live rock, water purification, dissolved inorganic nitrogen, removal efficiency

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1 Introduction

Along increasing living standards, the market demand for aquatic and especially marine pets is increasing. Among marine ornamental animals, coral has drawn attention due to vibrant color and high ornamental values. It is estimated that about 1.5 to 2 million people have a saltwater aquarium in their house (Delbeek and Sprung, 2005), which does not include commercial ornamental aquacultures in aquaria, clubs and hotels. The high mortality rate of indoor coral cultures also creates a strong market demand for coral (Delbeek and Sprung, 2005). In the recent several decades, the market demand for coral has been growing (Rhyne et al., 2012; Leal et al., 2012; Rocha et al., 2011; Rinkevich, 2005; Shafir et al., 2006). However, according to Wabnitz's research (Wabnitz et al., 2003), wild coral will soon be exhausted if the market demand for coral continues at the growth rate of the previous decade. To reduce the destructive exploitation of wild corals, artificial coral culture technology is being developed vigorously (Heyward et al., 2002).

A highly efficient biological filtration system is one key for successful coral culture (Delbeek and Sprung, 1994). In traditional freshwater or saltwater ornamental fish aquaria, biological filtration occurs mainly through a nitrification effect of nitrifying bacteria attached to the surface of artificial filter material with a large specific surface area (Nagadomi et al., 1999). However, this system may not meet the stringent environmental demands (especially inorganic nutrients) for healthy growth of corals, especially hard corals. These methods alone cannot remove nitrates and phosphates generated from the system nitration, and the accumulation of these substances in the recirculating system will inhibit coral reef calcification (Stambler et al., 1991; Marubini and Davies, 1996; Ferrier-Pagès et al., 2000; Ferrier-Pagès et al., 2001; Renegar and Riegl, 2005). Water eutrophication may lead to algal growth, which will affect coral survival by spatial competition, physical interactions, and allelopathy (Toh et al., 2013).

Live rock (LR) is a key component in "Berlin system" coral aquaria. LRs are mainly collected from healthy coral reefs. LRs are porous loose agglomerated calcium carbonates formed from skeletons of dead corals and other reef organisms. They are rich in pores and tunnels and have a large specific surface area. It is easy to form hypoxic or anoxic environments inside of LR, which is favorable for denitrifying bacterial growth and removal of dissolved inorganic nitrogen (DIN) produced in the aquarium system.

However, studies on the effect and underlying mechanism of

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LR are sparse despite its long widespread usage (Yuen et al., 2009). Our knowledge on the water purification effect of LR and the underlying mechanism is limited, because there are only few reports and limited perceptual experiences from researchers and hobbyists during coral aquaculture. Fully understanding the water purification efficiency of LR can help to optimize the biological nutrient processing system of indoor coral aquaculture systems and improve survival rate of cultured corals. Thus, this study selected LRs harvested from Hainan Houhai coral reefs and studied removal efficiency of ammonium and nitrate due to either artificial addition or biological metabolism. This study also measured expression levels of several functional genes (*AamoA*, *BamoA* and *nosZ*), which was widely detected in coral samples contribute to nitrification and denitrification processes (Rusch and Gaidos, 2013; Yang et al., 2013; Siboni et al., 2008), on the interior and exterior of LR. The archaeal ammonia monooxygenase gene (*AamoA*) was used as a target gene for nitration of nitrifying ammonia-oxidizing archaea (AOA) (Stephen et al., 1999), bacterial ammonia monooxygenase gene (*BamoA*) was used as a target gene for nitration of ammonia-oxidizing bacteria (AOB) (Könneke et al., 2005), and nitrous oxide reductase gene (*nosZ*) was used as a target gene for denitrification (Nogales et al., 2002; Rich et al., 2003; Dandie et al., 2008). These three genes, as functional markers in nitrification and denitrification processes, could evaluate the removal ability of inorganic nitrogen via enumeration of these genes in the LR. Quantification analysis was performed to detect nitrifying and denitrifying bacterial abundance in LR. This study aimed to understand the effects of LR in coral aquaculture and to help determine a reasonable amount of LR in coral aquaria. This study can also provide data for future research on artificial LR, which may reduce the usage of natural LR and the destruction of coral reefs.

2 Materials and methods

2.1 *Experimental materials*

All live rocks were collected from Houhai coral reefs in Sanya, Hainan Province (Fig. 1a). After collection, LRs were temporarily cultured in a pool in the laboratory for at least three months. The seawater recirculating system in the pool mainly utilized a "Berlin System" combined with seaweed treatment for water purification. The inorganic nitrogen levels in the system were NH_4^+<0.03 mg/L, NO_2^-<0.03 mg/L and NO_3^-<0.3 mg/L. LRs of similar quality and similar size, without a large cover of coralline algae or visible biofouling, were selected for experiments.

Turbo argyrostomus are common herbivorous snails seen in coral reefs, and are also commonly used in indoor coral aquaculture to control algae growth in the aquarium system. Thus, *T. argyrostomus* was selected as a nitrogen source due to biological excretion for the biogenic ammonia removal experiment. *T. argyrostomus* used in this experiment were purchased from Xiamen seafood market (Fig. 1b). Snails of equal sizes (body length of ~3 cm) were selected for the experiments. The snails were temporarily kept in a recirculating seawater system and mainly fed on algae in the pool. Before experiments, the snails were transferred to a bucket without food, aerated and allowed to empty for 24 h.

2.2 *Removal of inorganic nitrogen nutrients by LR*

2.2.1 *Experiments with artificially added ammonium*

Artificial sea salt (Blue Treasure, Qingdao) was diluted with deionized water to create artificial seawater with a salinity of 34. The prepared seawater was allowed to sit at room temperature for 24 h before use. Five liters of seawater were added to the experimental tank (20 cm×20 cm×20 cm), which was covered with clean plastic film to prevent contamination from air dust and then aerated. The tank was surrounded by thick canvas to protect from light. Three experimental treatments were used for a total of six tanks. Baseline nitrogen concentration was created by adding 0.5 mL of 1 mol/L NH_4Cl (equivalent to 18 g/L NH_4^+) stock solution to the tank. LR was added to the experimental treatments 4 h later. During the first 24 h, samples were collected every 3 h; after 24 h, samples were collected every 4 h for 72 h. An amount of 50 mL of water sample was collected each time, then filtered through a 0.45 μm microporous membrane and stored at –20°C.

2.2.2 *Experiments with biologically-derived ammonium*

The preparation of seawater and tanks were as described in the previous section. In each experimental tank, three *T. argyrostomus* were added when the experiments started. No treatment was added to any tank during the first 40 h. At the 40th hour, one LR was added to each tank; at the 80th hour, LRs were removed from one treatment group and snails were removed from the other treatment group; measurements continued for another 28 h. During the first 24 h, samples were collected every 3 h; after 24 h, samples were collected every 4 h for 108 h. An amount of 50 mL of water sample was collected each time, then filtered

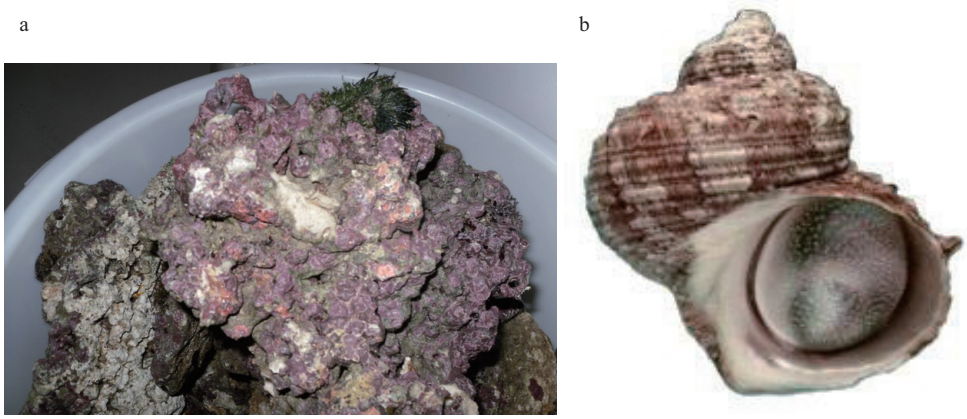

Fig. 1. The experimental materials: live rock collected from Houhai coral reefs in Sanya, Hainan Province (a); and turbo snail (*Turbo argyrostomus*) (b).

through a 0.45 μm microporous membrane and stored at –20°C.

2.2.3 *Experiment with nitrate addition*

The preparation of seawater and tanks were as described in the previous section. Three experimental treatments were used for a total of six tanks. The baseline nitrogen concentration was created by adding 0.5 mL of 1 mol/L KNO_3 (equivalent to 62 g/L NO_3^-) stock solution to the tank and LR was added to the experimental treatments 4 h later. Samples were collected every 4 h for 96 h. Water samples were filtered through a 0.45 μm microporous membrane and stored at –20°C.

All water samples were analyzed using a flow analyzer (SKALAR SAN ++ Flow Injection Analyzer, Netherlands) after thawing.

2.3 *Bacteria analysis in LR*

2.3.1 *Experimental materials*

LR samples harvested from the culturing tanks were temporarily cultured in coral aquaria for more than three months. Sampling locations are shown in Fig. 2; samples were collected from both the interior and exterior of LR.

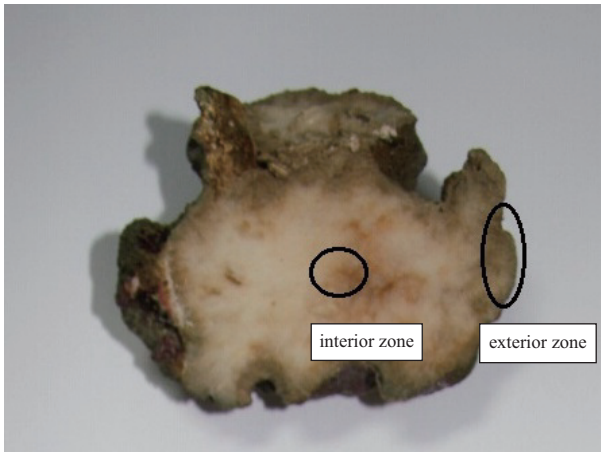

Fig. 2. Sampling location of the live rock (interior and exterior).

2.3.2 *Experimental methods*

(1) Harvest of LR

LR samples with loose textures were selected. Samples were collected from both the interior and exterior of the rocks. After the samples were weighed individually, total DNA of each sample was extracted with an MP fast DNA spin kit for soil.

(2) Construction of real-time fluorescent quantification PCR standard curve

Standard DNA of *BamoA*, *nosZ* genes and 16S rDNA gene fragments was amplified respectively from standard bacterial strain *Nitrosomonas europaea,* environmental samples, and *Marinomonas pollencensis* (primers sequences are shown in Table 1 and the program settings are shown in Fig. 3).

Table 1. Primers used in the study

Primers name	Sequence (5′–3′)	Product length/bp	Reference
BamoAf	STAATGGTCTGGCTTAGACG	490	Rotthauwe et al. (1997)
BamoAr	GCGGCCATCCATCTGTATGT		
AamoAf	STAATGGTCTGGCTTAGACG	637	Park et al. (2006)
AamoAr	GCGGCCATCCATCTGTATGT		
nosZf	ATGTCGATCARCTGVKCRTTYTC	254	Dandie et al. (2011)
nosZr	WCSYTGTTCMTCGACAGCCAG		
Bacf	GTGCCAGCAGCCGCGG	378	Nunoura et al. (2012)
Bacr	CCGYCAATTCMTTTRAGTTT		

Gene molecular weights were calculated by the cloning sequencing technique, and the DNA absorbance measured from Nanodrop was converted into concentration (ng/μL) to compute the copy number of standard genes.

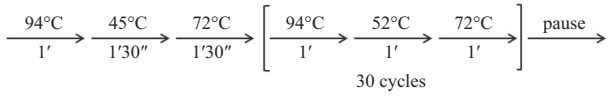

Fig. 3. PCR program settings of the experiment.

Standard DNA samples were prepared in serial dilution, and quantitative standard curves were then constructed by using the logarithm of gene copy number and the Ct value derived from a Real-time PCR instrument (Eppendorf Realplex). The amplification condition is shown in Fig. 4. The amount of the corresponding target genes in the samples were then quantified accordingly.

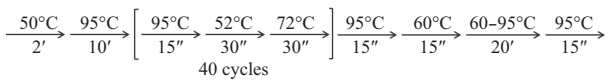

Fig. 4. Real-time PCR program.

(3) Real-time fluorescent quantification PCR

Measurement of *amoA* and *nosZ* gene abundance: Specific primers for real-time fluorescent PCR were used to amplify *amoA* and *nosZ* genes on the interior and exterior of LR. Melting curves were analyzed, and the quantification standard curve and sample amplification Ct values were used to quantify the two metabolism genes (amplification program is shown in Fig. 4). Sample bacteria 16S rDNA was quantified to determine the proportion of AOB and denitrifying bacteria of the total bacteria.

2.4 *Data analysis*

(1) Ammonium removal efficiency of LR: Ammonium removal efficiency of live rock per unit mass per unit time was used in this study to express the ammonium removal ability of LR. The following formula was used:

$$\alpha = \frac{c_o v_o - cv}{mt} = \frac{\Delta c}{mt}, \quad (1)$$

where c_o is the baseline ammonium concentration (mg/L), c is the final concentration of NH_4^+ (mg/L), v_o is the baseline volume of the water body, v is the final volume of the water body, m is the

quality of LR (g), and t is the treatment time.

(2) NH_4^+ removal efficiency of LR at different time points: Linear regression was performed on ammonium concentrations at different time points (0–40 h, 40–80 h and 80–108 h) and the nitrogen nutrients removal efficiency of LR was compared.

(3) Nitrate removal efficiency of LR: Variation of nitrate concentration with time was graphed.

(4) Sigmaplot 10.0 was used for graphing and SPSS 16 was used for statistical analysis. Independent-Samples t tests were used in compare the differences between the treatment and control in both experiments on removal effect of LR on artificially added ammonium and nitrate, respectively. The significant level is $p<0.05$. Pierre's correlation analysis was performed on ammonium concentrations at different time points (0–40 h, 40–80 h and 80–108 h).

3 Results

3.1 *Removal effect of LR on artificially added ammonium*

Figure 5 shows the removal effect of LR on artificially added ammonium.

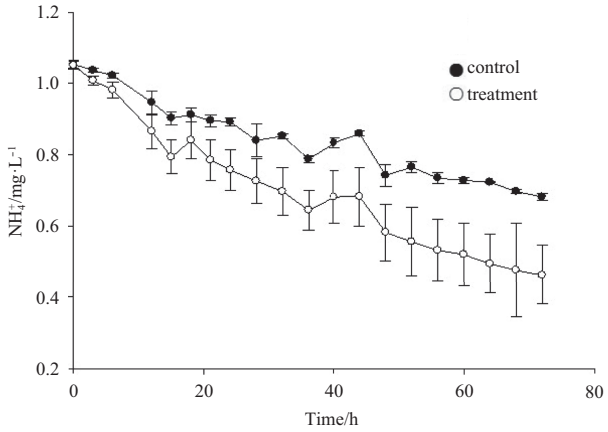

Fig. 5. Removal effect of LR on artificially added NH_4^+ in seawater.

The results showed that the ammonium rate of decline in the LR treatment was significantly higher than in the control treatment ($p<0.05$) during the experimental period (72 h). The ammonium concentration was reduced by 80% of the baseline concentration in control treatment, while it was only reduced by 50% in the LR treatment. The qualities of the four LRs were different, but the variation of ammonium removal efficiency was small, which averaged 0.141 mg/(kg·h) (Table 2).

Table 2. Removal efficiency of LR on artificially added NH_4^+ in seawater

	LR1	LR2	LR3	LR4	Mean
m/g	328.7	275.6	298.4	300.1	300.7
Δcv/mg	3.510	2.516	2.918	3.262	3.052
α/mg·kg^{-1}·h^{-1}	0.148	0.127	0.136	0.151	0.141

3.2 *Removal effect of LR on biologically derived ammonium*

Figure 6 shows the removal effect of LR on biologically-derived ammonium. During the first 40 h without LR, ammonium concentration increased in all tanks; from 40 h to 80 h, ammonium concentration slightly decreased in the LR-removal treatment after the addition of LR and slightly increased in the snail-removal treatment, but the difference between the two treatments was not significant ($p>0.05$). After 80 h, the ammonium concentration of the snail-removal treatment continued to decline and approached 0 mg/L with LR after snail removal, while the ammonium concentration of the LR-removal treatment continued to increase due to continuous release of ammonium from the snails.

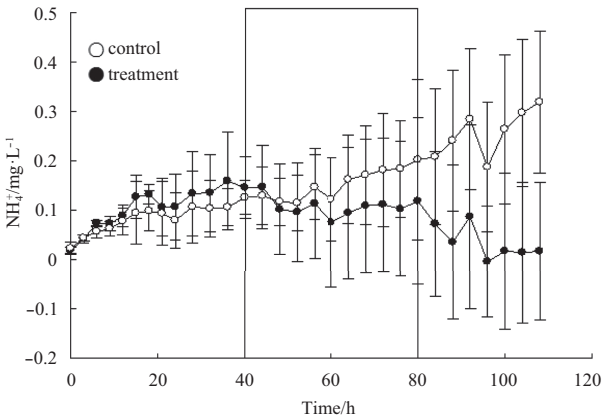

Fig. 6. Removal effect of LR on biologically derived NH_4^+ in seawater.

To better illustrate the removal effects of LR on ammonium, a linear regression was performed on ammonium concentrations at different time points (0–40 h, 40–80 h and 80–108 h) and the nitrogen nutrient removal efficiency of LR was compared (Table 3). The results showed that, from 80 to 108 h, the LRs in Tanks A, B and C of the snail-removal treatment continued to process ammonium after the snails were removed. Results showed that $\alpha<0$, demonstrating that ammonium concentration decreased significantly in all three tanks of the snail-removal treatment. For the three tanks in the LR-removal treatment, the slope $\alpha>0$ and significantly increased from 80 h to 108 h, indicating that ammonium concentration increased significantly due to the continuous nitrogen release from snails after LRs were removed.

3.3 *Removal efficiency of LR on added nitrate*

Figure 7 shows the removal effect of LR on artificially added nitrate. During the 96 h experimental period, the presence of LR had no significant impact on nitrate concentration ($p>0.05$), and the nitrate concentration did not differ significantly between the control and LR treatments.

3.4 *Bacterial variation in LR*

As shown in Table 4, the copy number concentration of 16S rDNA from all bacteria was about 5.62 times higher on the exterior than on the interior of LR. Inside LR, the total number of bacteria colonies decreased as depth increased.

The ammonia-oxidizing archaea (AOA) DNA copy number concentration on the exterior was 2.75–4.17 times more than that on the interior of LR (Table 5). Since AOA only accounted for less than 0.01% of the total bacteria, even though AOA number was much higher on the surface than inside of LR, when compared to the DNA copy number of total bacteria, AOA still only accounted for a very small portion.

The AOB *amoA* gene DNA copy number was consistent on the exterior and interior of LR and was ~2.3 (Table 6). In areas near the surface of LR, AOB accounted for about 0.5% of the total

Table 3. Slope variation of the NH_4^+ concentration curves of each treatment

Group 1	α (0–40 h)	R	P	α (40–80 h)	R	P	α (80–108 h)	R	P
a	0.010 3	0.666 6	<0.05	0.002 8	0.942 4	<0.05	0.019	0.853 2	<0.05
b	0.002 6	0.269 9	>0.05	0.001 4	0.125 0	>0.05	0.032 3	0.958 7	<0.05
c	0.006 5	0.704 5	<0.05	0.003 9	0.499 6	>0.05	0.017 8	0.714 7	<0.05
Group 2	α (0–40 h)	R	P	α (40–80 h)	R	P	α (80–108 h)	R	P
A	0.001 7	0.451 6	>0.05	0.003 2	0.554 8	<0.05	–0.021 3	0.790 2	<0.05
B	0.015 8	0.981 9	<0.05	–0.010 6	0.774 4	<0.05	–0.030 6	0.839	<0.05
C	0.019 6	0.992 3	<0.05	0.013 4	0.820 6	<0.05	–0.017 2	0.752 2	<0.05

Note: Slope α>0 represents concentration increase; Slope α<0 indicates concentration decrease. P>0.05 indicates the variation between treatments is not significant; the larger the R value, the better the linear fit.

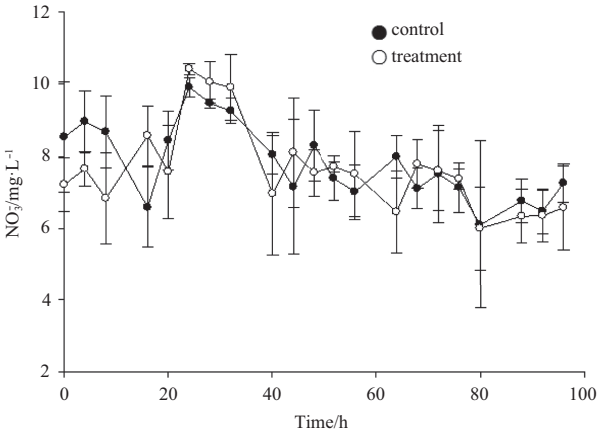

Fig. 7. Removal effect of LR on artificially added NO_3^- in seawater.

Table 4. 16S rDNA copy concentration of the total bacteria in LR

LR ID	LR area	16S rDNA copies/μL^{-1}	Exterior/interior
A	exterior	1.463×10^{10}	4.38
	interior	3.336×10^{9}	
B	exterior	1.721×10^{10}	5.80
	interior	2.965×10^{9}	
C	exterior	2.149×10^{10}	4.76
	interior	4.515×10^{9}	
D	exterior	2.260×10^{10}	7.55
	interior	2.993×10^{9}	

bacteria, while inside LR, AOB concentration increased to about 1% of the total bacteria, with the highest value reaching 1.494%. This relatively high proportion indicates that the ammonia oxidation reaction of LR may be primarily derived from AOB. Compared to the total number of bacterial colonies, the AOB ratio inside of LR was relatively high.

Table 7 shows the result of *nosZ* gene expressed by denitrifying bacteria. The denitrifying bacteria concentration on the interior and exterior of LR differed significantly. The *nosZ* gene concentration was much higher in areas near the surface than inside of LR, where the highest value was 15 times that of inside. In LRs A, B and C, the percentage of *nosZ* to total 16S rDNA was high in areas near the surface, while in LR D, the percentage of *nosZ* gene was high inside the rock.

4 Discussion

4.1 *Removal effect of LR on inorganic nitrogen*

Through the ammonium removal experiments, it was demonstrated that LR can significantly improve water quality by effectively removing ammonium from the water body. High ammonium concentration is harmful for organisms (Camargo and Alonso, 2006). Our results showed that the mean ammonium removal efficiency of the four LR treatments was 0.141 mg/(kg·h). Thus, in a 500 L large coral aquarium, 50 kg of LRs is enough to reduce the ammonium concentration from a relatively high level of 1 mg/L to a relatively low threshold (i.e., <0.03 mg/L) in 72 h. From previous experience, 0.25–0.5 kg of LRs is needed for every liter of water (Delbeek and Sprung, 2005), indicating that LR in the aquarium can provide sufficient ammonium removal activity.

Yuen et al. (2009) found that LR can effectively control ammonium, nitrite and nitrate in water. In a system with LR, the inorganic nitrogen released by sea cucumbers can be removed by LR effectively. LR can also effectively maintain the pH of the water. However, no significant nitrate removal was detected in our study. The possible reason may be that the concentration of the added nitrates was too high. In the nitrate addition experiments, the amount of added nitrates was equivalent to 6 mg/L, which is much higher than either the nutrient level in the temporary culture tank (NO_3^-<0.3 mg/L), or the required level for coral reef culture (Delbeek and Sprung, 2005), or the average level in common reef water (Wang et al., 2002; Peng et al., 2002), or the peak nitrate level in Yuen's experiment. At such a high substrate concentration, the degradation rate of denitrification may not be observed; therefore, further analysis is warranted in future experi-

Table 5. AOA bacteria located on the interior and exterior of LR

LR ID	LR area	16S rDNA copies/μL^{-1}	AOA3 copies/μL^{-1}	AOA exterior/interior	AOA3/16S/%
A	exterior	1.463×10^{10}	6.566×10^{5}	3.92	0.004 49
	interior	3.336×10^{9}	1.676×10^{5}		0.005 02
B	exterior	1.721×10^{10}	6.486×10^{5}	4.17	0.003 77
	interior	2.965×10^{9}	1.556×10^{5}		0.005 25
C	exterior	2.149×10^{10}	5.150×10^{5}	2.75	0.002 40
	interior	4.515×10^{9}	1.873×10^{5}		0.004 15
D	exterior	2.260×10^{10}	9.999×10^{5}	3.89	0.004 42
	interior	2.993×10^{9}	2.571×10^{5}		0.008 59

Table 6. AOB bacteria located on the interior and exterior of LR

LR ID	LR area	16S rDNA copies/μL^{-1}	AOB copies/μL^{-1}	AOB exterior/interior	AOB/16S/%
A	exterior	1.463×10^{10}	7.910×10^{7}	2.36	0.541
	interior	3.336×10^{9}	3.354×10^{7}		1.005
B	exterior	1.721×10^{10}	9.822×10^{7}	2.43	0.571
	interior	2.965×10^{9}	4.036×10^{7}		1.361
C	exterior	2.149×10^{10}	9.762×10^{7}	2.04	0.454
	interior	4.515×10^{9}	4.777×10^{7}		1.058
D	exterior	2.260×10^{10}	1.167×10^{8}	2.61	0.517
	interior	2.993×10^{9}	4.473×10^{7}		1.494

Table 7. *NosZ* denitrifying bacteria located on the interior and exterior of LR

LR ID	LR area	16S rDNA copies/μL^{-1}	*nosZ* copies/μL^{-1}	*nosZ* exterior/interior	*nosZ*/16S/%
A	exterior	1.463×10^{10}	5.282×10^{7}	15.16	0.361
	interior	3.336×10^{9}	3.483×10^{6}		0.104
B	exterior	1.721×10^{10}	6.499×10^{7}	9.62	0.378
	interior	2.965×10^{9}	6.759×10^{6}		0.228
C	exterior	2.149×10^{10}	6.075×10^{7}	6.89	0.283
	interior	4.515×10^{9}	8.812×10^{6}		0.195
D	exterior	2.260×10^{10}	6.876×10^{7}	5.15	0.304
	interior	2.993×10^{9}	1.334×10^{7}		0.446

ments. The denitrifying bacteria that play key roles in water treatment are mostly heterotrophic (Cai, 2008). In this experiment, the reason why LR lacks the ability to degrade nitrate may be due to the lack of dissolved organic carbon (DOC) as a reaction substrate and energy source. In Yuen's study, other organic waste excreted by sea cucumbers could be used as reaction substrates and energy sources for denitrifying bacteria, thus enabling the denitrification reaction. The LRs selected in their experiments were covered by a large amount of crustose coralline algae (CCA), and the experiments were not kept from light. The absorption of nutrients through CCA photosynthesis was not considered; thus, nutrient decline may be due to CCA absorption instead of metabolism of bacteria inside LR.

4.2 *Bacterial composition and distributionin LR*

Our results showed that the total copy number of 16S rDNA from bacteria on the exterior of LR was 5.62 times that of the interior. Thus, the number of bacteria was much less on the interior than on the surface of LR. Even though LR is rich in porous structure and the calcium carbonate skeleton of coral is loose, the interior areas of LR are normally hypoxic due to poor water flow. In a water body with a saturated oxygen environment, the exterior areas of LR will have rapid oxygen exchange with the water body. Therefore, the number of live bacteria on the exterior of LR is relatively high, while the number on the interior is significantly lower due to the relatively hypoxic environment. Nevertheless, the presence of AOA, AOB and denitrifying bacteria indicated that LR has the potential to process nutrients.

Generally speaking, AOA plays an important role in the marine nitrogen cycle and the abundance of the *AamoA* gene, a key functional gene of AOA, is higher than the relative abundance of *BamoA* gene in bacteria (Francis et al., 2007; Mincer et al., 2007). For example, in a marine environment, crenarchaeota can account for 39% of the total planktonic prokaryotes (Karner et al., 2001), while the proportion of AOB in general microbial communities is as low as less than 0.1% (Ward et al., 2000). AOA is generally dominant in number, diversity and habitat range (He and Zhang, 2009). However, the proportion of AOA in the total bacteria of LR is extremely low, at only ~0.01%. Conversely, relatively higher AOB abundance showed that most of the ammonia oxidation in LR is derived from AOB. In fact, AOB will form a microaerophilic environment when AOB consumes O_2 during ammonia oxidation and facilitate a nitrification bacterial structure with more AOB than AOA.

Coral aquaria commonly usually display a certain degree of low eutrophication. NH_4^+ increases due to biological excretion, and the substrates for AOB metabolism increases. The surface of LR is close to the oxygen-rich water body, which makes LR a suitable growing place for aerobic AOB. As shown in Table 8, the *amoA* gene of AOB was 2.04–2.61 times higher on the exterior than on the interior of LR, while *nosZ* was 5.15–15.16 times higher. The ratio of *BamoA*/*nosZ* was about 1.5, while in areas deep inside LR, the ratio increased to 3.353–9.630, with a varying value, indicating great spatial heterogeneity. The outside of LR is gener-

Table 8. AOB and *nosZ* gene concentrations on the interior and exterior of LR

LR ID	LR area	AOB concentration	*nosZ* concentration	AOB/*nosZ*
A	exterior	7.91×10^{7}	5.28×10^{7}	1.498
	interior	3.35×10^{7}	3.48×10^{6}	9.630
B	exterior	9.82×10^{7}	6.50×10^{7}	1.511
	interior	4.04×10^{7}	6.76×10^{6}	5.971
C	exterior	9.76×10^{7}	6.08×10^{7}	1.607
	interior	4.78×10^{7}	8.81×10^{6}	5.422
D	exterior	1.17×10^{8}	6.88×10^{7}	1.698
	interior	4.47×10^{7}	1.33×10^{7}	3.353

ally more abundant in oxygen and ammonia nitrogen supply compared to the inside of LR, which provides abundant metabolic substrates for AOB. AOB metabolism consumes O_2. When O_2 is not replenished in a timely fashion, the local region will be hypoxic, which will provide abundant metabolic substrates and a relatively hypoxic environment for denitrifying bacteria. The NO_3^- generated from nitrification can also meet the needs of denitrifying bacteria. Thus, the number of denitrifying bacteria on the surface will be higher than that on the inside of LR. Song et al. (2012) reported that the copy number of denitrifying bacteria in topsoil was 1.58 times higher than that in the root layer of soil upon nitrogen fertilizer treatment.

Compared to other sediments which are capable of denitrification, denitrifying bacteria content is very low in LR. Nitrifying bacteria are dominant in the total bacteria of LR compared to the denitrifying bacteria. Inside LR, the number of nitrifying bacteria can be ten times higher than the number of denitrifying bacteria. Compared to other sediments that are capable of denitrification, the denitrification of LR is not strong. For example, 10%–90% of the bacteria in the activated sludge system have a denitrification effect; *nosZ* accounted for 6.59% of the bacteria in artificial wetland Iris rhizosphere soil and 2.63% in landscape river sediment (Wang et al., 2012). The concentration is 0.17%–19.8% in soil for bacteria that can metabolize inorganic nitrogen into N_2 (Chèneby et al., 2000). Thus, other biological filtration methods are needed in coral aquaria to control the nitrates generated from nitrification or biological metabolism.

In most coral aquaria, it was very difficult to maintain water body as oligotrophic as *situ* healthy coral reefs. Slight eutrophication (i.e., 1–5 mg/L of nitrate) is normally observed in indoor coral aquaria. Only LR cannot lower the concentration of nitrate in coral aquaria as was found in our case. Based on our experiments, therefore, other biological filtration methods are needed in coral aquaria to control nitrates generated from nitrification or biological metabolism.

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