# DETERMINING THE OPTIMUM RATIO OF POLYCULTURED SHELLFISH AND SEAWEED: A MICROCOSM STUDY

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(Received 16th Sep 2021; accepted 15th Feb 2022)

**Abstract.** The polyculture of shellfish and seaweed together is an effective way to solve the current bottleneck in developing the shellfish culture industry, and determining the optimal ratio is an important prerequisite for its application. Large-scale oyster monocultures in the Beibu Gulf (South China) have led to environmental degradation and exceeded the aquaculture carrying capacity. In order to cope with the problem, this study designed a microcosm of polyculture with shellfish and seaweed, trying to explore the optimal ratio with both environmental protection and economic benefits. Different densities of *Gracilaria tenuistipitata* (0.33–4.17 kg/m$^3$) combined with a fixed density (83.3 ind/m$^3$) of *Crassostrea hongkongensis* were investigated to measure the growth rate and nutrient removal effects. The oyster growth rates in the polyculture groups were 6–14-fold that of the oysters in the monoculture group. The highest oyster and seaweed growth rates were in the medium-density seaweed group (0.83 kg/m$^3$). Compared with the low-density seaweed groups, a high density of seaweed effectively removed the nutrients released by the oysters. A response surface analysis showed that a seaweed density of 1.567 kg/m$^3$ was the optimal proportion with 83.3 ind/m$^3$ (10 individuals) of *C. hongkongensis*, with a predicted growth rate of 0.110%/day for the oyster and 0.564%/day for the seaweed. This research indicates that combining *G. tenuistipitata* in a polyculture system could increase oyster production and provide the exciting prospect of improving seawater quality in the Beibu Gulf.
**Keywords:** *marine ecological restoration, sustainable development, seawater quality, Gracilaria tenuistipitata, Crassostrea hongkongensis, Beibu Gulf*

## Introduction

Mariculture wastewater increases marine pollution and aggravates eutrophication in the Beibu Gulf. This decline in water quality affects public health in coastal areas and directly threatens the development of the mariculture industry. In addition, the increasing monoculture has resulted in decreased yield and frequent disease outbreaks (Neori, 2007), and the culture capacity is approaching its limit (Nunes et al., 2003).

The solution to the problem of monoculture and environmental pollution is to develop a polyculture system (McVey et al., 2002; Neori et al., 2004; Wartenberg et al., 2017). Macroalgae are important species for mixed cultivation and the environmental


http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2002_12411250

restoration of bays (Neori et al., 2004; McGlathery et al., 2007; Yang et al., 2015; Buschmann et al., 2017). The seaweed uses the nutrients in aquaculture wastewater to form new tissues (Qian et al., 1996; Sandoval-Gil et al., 2016), increases dissolved oxygen for the animals via photosynthesis (Yang et al., 2015), and improves the culture environment. Macroalgae have been widely used as a biofilter in treating aquacultural wastewater from shellfish farming (Mao et al., 2009; Sandoval-Gil et al., 2016), shrimp ponds (Jones et al., 2001; Mai et al., 2010), and fish ponds (Hayashi et al., 2008), as well as for the ecological restoration of bays and estuaries (Yang et al., 2006; McGlathery et al., 2007). Several studies have shown that seaweed and animals in mixed cultures have higher growth rates than those in monoculture (Qian et al., 1996; Mao et al., 2009). In addition, many kinds of seaweeds have economic value and can be used as raw materials for such things as agar and edible and medicinal products (Neori et al., 2004; Yang et al., 2015).

Determining the optimal ratio of seaweed and animals is the key premise of polyculture, as a proper ratio is an important condition for the balance and stability of a polyculture system. Excessive seaweed could reduce the seaweed and animal growth rates (Yang et al., 2006), while too little seaweed may be ineffective at improving the system environment and promoting growth (Mao et al., 2009). The response surface methodology (RSM) is an effective tool for studying optimal ratios under the influence of multiple factors. Li et al. (2015) used RSM to study the optimal ratios for *Gafrarium tumidum* (tropical clam) and *Eucheuma gelatinae* (macroalga) and selected two optimal ratios for field validation tests. The results showed that both combinations could significantly decrease the microalga density and dissolved nutrients in a sea mesocosm.

The Beibu Gulf is a primary oyster culture center in southern China as well as an important natural oyster seedling center. Studies have shown that as marine suspended filter-feeders, oysters can reduce suspended matters (e.g., phytoplankton, inorganic matter, and particulate organic matter) through water filtration, which purifies the seawater (Schröder et al., 2014; Petersen et al., 2016) and reduces the risk of red tide. However, oysters also release dissolved nutrients through metabolism (Newell et al., 2005; Hoellein and Zarnoch, 2014), especially ammonia ($NH_4^+$), which can increase seawater eutrophication. The increasing scale of oyster cultivation in recent years has aggravated eutrophication in the Gulf, especially increasing the concentration of inorganic nitrogen (Wang et al., 2016). Moreover, it has resulted in negative impacts on oyster growth such as a decrease in the individual weight of commercial oysters and frequent mass deaths in the spring (Yu et al., 2016). These negative phenomena indicate there may be a bottleneck in the oyster carrying capacity of the Beibu Gulf, making it necessary to adjust the cultivation patterns to achieve sustainable economic growth and environmental protection.

To examine how to deal with this oyster cultivation bottleneck and mitigate eutrophication in the Beibu Gulf, this study attempted to construct a microcosm of polyculture system with *Gracilaria tenuistipitata* (edible red seaweed) and *Crassostrea hongkongensis* (Hong Kong oyster). The growth performance and nutrient removal effects of *G. tenuistipitata* and *C. hongkongensis* were measured to determine their optimal ratio, which would be conducive for the further implementation of a shellfish and seaweed polyculture program in the Gulf and improve seawater quality. *Gracilaria tenuistipitata* is the dominant species of macroalgae in the mangrove conservation area of the Gulf, and it has great economic value as a raw material for agar. Our hypothesis was that *G. tenuistipitata* could promote the growth of *C. hongkongensis*.


http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2002_12411250

## Materials and Methods

### Experimental design

The experiment was designed with 7 groups, which included a seaweed-monoculture, an oyster-monoculture, 4 groups with different levels of seaweed and oysters mixed, and one group as a blank control. In addition to the control group, each of the other groups had three replicates. To determine the optimal amount of *G. tenuistipitata* biomass in combination with one string of *C. hongkongensis*, 10 oysters of equal size were used in the oyster-monoculture and mixed groups, while 200 g (wet weight) of *G. tenuistipitata* was used in the seaweed-monoculture group. The 4 mixed groups included 40 g (0.33 kg/m$^3$), 100 g (0.83 kg/m$^3$), 200 g (1.67 kg/m$^3$), and 500 g (4.17 kg/m$^3$) of *G. tenuistipitata*, respectively. Details of the experimental design are shown in *Table 1*. The experiment lasted 7 days, between 21–28 March 2017. To study the growth rates of the oyster and seaweed, the shell lengths of *C. hongkongensis* and wet weights of *G. tenuistipitata* were measured before and after the experiment. The concentrations of ammonia ($NH_4^+$), nitrate ($NO_3^-$), nitrite ($NO_2^-$), and phosphate ($PO_4^{3-}$) were determined daily during the experiment to observe the effect of *G. tenuistipitata* on removing dissolved nutrients.

***Table 1.*** *Experimental polyculture design with Crassostrea hongkongensis and Gracilaria tenuistipitata*

| Group | ***G. tenuistipitata*** **(kg/m$^3$)** | ***C. hongkongensis*** **(ind/m$^3$)** |
|---|---|---|
| Control | – | – |
| *Monoculture* | | |
| Oyster-monoculture | – | 83.3 |
| Seaweed-monoculture | 1.67 | – |
| *Polyculture* | | |
| Low-seaweed | 0.33 | 83.3 |
| Medium-seaweed | 0.83 | 83.3 |
| High-seaweed | 1.67 | 83.3 |
| Ultra-seaweed | 4.17 | 83.3 |

### Experimental materials and environmental conditions

*Crassostrea hongkongensis* was obtained from the oyster raft culture area at Longmen Port (21°40'41" N, 108°36'46" E) in Qinzhou, China. The experimental oysters were all 2-year-old individuals with an average shell length of 98.47 ± 4.61 mm and a soft body dry weight of 1.63 ± 0.27 g, which acclimated for one week before the experiment. *Gracilaria tenuistipitata* was obtained from the Golden Bay Mangrove area near Beihai (21°25'15" N, 109°12'02" E) in Guangxi province, which provided one week of training using Provasoli enriched seawater culture medium before the experiment. Several high-density polyethylene cylindrical tanks (44 cm diameter, 80 cm height, 120 L capacity) were used as the containers for this experiment. The filtered experimental seawater (water temperature 21–23 °C, salinity 26–28 ppt, dissolved oxygen 7.49–7.54 mg/L) was obtained from the Zhulin area of Tieshan Port in Beihai (Beibu Gulf). The experiment was performed outdoors under a semi-transparent sunshade in the Shellfish Comprehensive Experimental Station of the Guangxi Academy of Fishery Sciences in Beihai with an illumination intensity of 3300–6000 lux (light:dark was 14 hr:10 hr). The oysters were placed evenly at the bottom of the tanks,


http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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and the seaweeds were hung 20 cm under the water surface with a nylon rope below a float pipe. During the experiment, 600 mL water samples were siphoned from 30 cm under the water surface at 10:00 am every day to determine the concentration of dissolved nutrients, and a 300 mL algae culture solution of *Chlorella vulgaris* (about $5 \times 10^8$ cells/L) was fed to the oysters. Each tank was equipped with two air stones, which provided continuous aeration to ensure sufficient dissolved oxygen.

### *Sample determination and data analysis*

To determine the dissolved nutrient concentrations, the water samples were filtered through 0.7 μm GF/F filters (Whatman, England), and the filtrates were stored at −20 °C until the analysis (within one month). The $NH_4^+$, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$ concentrations were determined using an AutoAnalyzer 3 (SEAL, Germany).

The growth rates for *C. hongkongensis* and *G. tenuistipitata* were determined by the following equation 1 and 2, respectively:

$$LGR\ (\%/\text{day}) = (\ln L_t - \ln L_0) / t \times 100 \qquad \text{(Eq.1)}$$

$$WGR\ (\%/\text{day}) = (\ln W_t - \ln W_0) / t \times 100 \qquad \text{(Eq.2)}$$

where *LGR* is the shell length growth rate of the oysters, $L_t$ is the shell length of the oysters at the end of the experiment, and $L_0$ is the shell length of the oysters at the beginning of the experiment; *WGR* is the biomass growth rate of the seaweed, $W_t$ is the wet weight of the seaweed at the end of the experiment, and $W_0$ is the wet weight of the seaweed at the start of the experiment; *t* is the days of culture.

The response surface analysis was performed using Design Expert 12 to estimate the seaweed density at the organics' maximum growth and nutrient removal rates. A one-way analysis of variance (ANOVA) was performed using SPSS 19.0 to test the significance between different treatment groups with the significance level set at 0.05. Sigma 14.0 and Origin 9.0 were used for visualizations.

## Results

### *Growth of the oyster and seaweed under monoculture and polyculture conditions*

During the experiment, *C. hongkongensis* grew well in all groups, with a 100% survival rate. The growth status of *G. tenuistipitata* in the polyculture groups was good, appearing glossy and a healthy brown color. However, in the seaweed-monoculture group, some of the leaf tips were whitening by Day 6. *Figure 1* shows the growth performance of the oyster and seaweed. The results show that the growth rates of the oysters (LGR) in the polyculture were higher ($p < 0.05$) than those in the monoculture group. The LGRs were ranked as follows: medium-seaweed group (0.11%/day) > high-seaweed group (0.10%/day) > ultra-seaweed group (0.08%/day) > low-seaweed group (0.05%/day) > oyster-monoculture group (0.01%/day). With the exception of the algae in the ultra-seaweed group, the growth rates of *G. tenuistipitata* (WGR) in polyculture were significantly higher than that in the seaweed-monoculture group ($p < 0.05$). The WGR ranking for the seaweed was medium-seaweed group (0.81%/day) > high-seaweed group (0.48%/day) = low-seaweed group (0.48%/day) > seaweed-monoculture group (0.28%/day) > ultra-seaweed group (0.09%/day).


http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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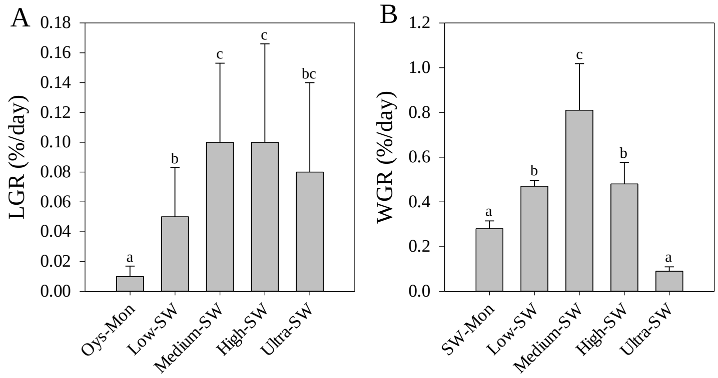


***Figure 1.*** *The length growth rate (LGR) of Crassostrea hongkongensis (n = 10) and biomass growth rate (WGR) of Gracilaria tenuistipitata (n = 3). Different letters (a–c) indicate a significant difference (p < 0.05). Ys, oyster; SW, seaweed; Mon, monoculture*

### *Effect on nutrients of the oyster and seaweed in monoculture and polyculture*

The effect of the seaweed on the dissolved nutrient concentrations is shown in *Figure 2*. First, dissolved nutrients were continuously released in the monoculture oyster group during the experiment, with the final $NH_4^+$, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$ concentrations at 4.2-, 1.6-, 13.3-, and 8.0-fold the initial concentrations, respectively. Second, the monoculture seaweed group greatly affected nutrient removal (except for the $NO_2^-$ index for the high-seaweed group and the $PO_4^{3-}$ index for the ultra-seaweed group). Third, compared with the control group, the dissolved nutrient concentrations for the high- and ultra-seaweed groups were significantly lower from the third day forward ($p < 0.05$), indicating these seaweed densities could effectively remove nutrients. The highest $NH_4^+$, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$ removal rates were 89.24%, 76.89%, 93.89%, and 90.97%, respectively. The $NH_4^+$ and $PO_4^{3-}$ concentrations in the medium- and low-seaweed groups were higher than those in the control group after the second day, indicating the seaweed density of these two groups was insufficient to remove nutrients.

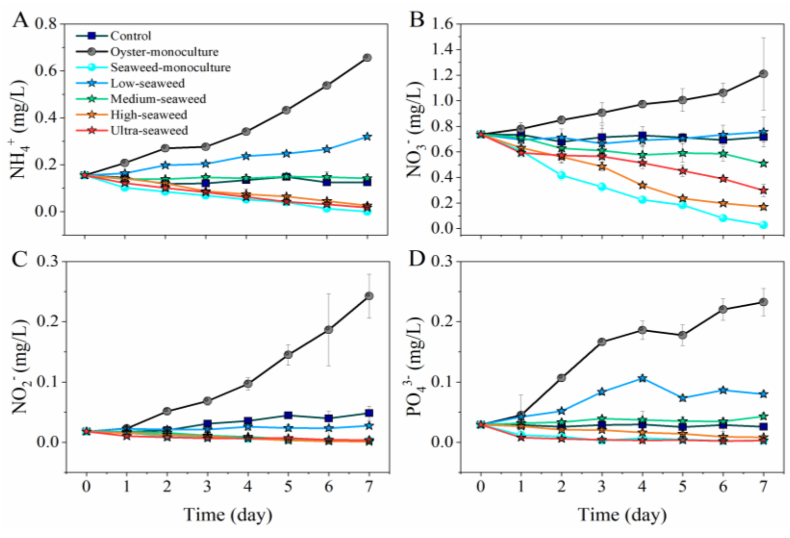


***Figure 2.*** *Variation in dissolved-nutrient concentrations changed with time under the different treatments. All values are the mean ± SD (n = 3)*


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### *Optimal proportion of oysters and seaweed in a polyculture system*

To simulate the optimal proportion of oysters and seaweed for the highest growth and nutrient removal rates in a polyculture system, the constraints in the response surface analysis were set to “maximize” for the growth rates of the oyster (LGR) and seaweed (WGR), and to “minimize” for $NH_4^+$, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$. The variance analysis (ANOVA) showed that the confidence levels for LGR, WGR, $NH_4^+$, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$ in the response surface model were high ($p < 0.01$). The simulation results showed that when the seaweed density was 1.567 kg/m$^3$, the growth and nutrient removal rates reached their highest expectation (0.824). At this level, the LGR of the oyster was 0.11%/day, the WGR of the seaweed was 0.564%/day, and the concentrations of $NH_4^+$, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$ were 0.030 mg/L, 0.207 mg/L, 0.001 mg/L, and 0.011 mg/L, respectively (*Figure 3*).

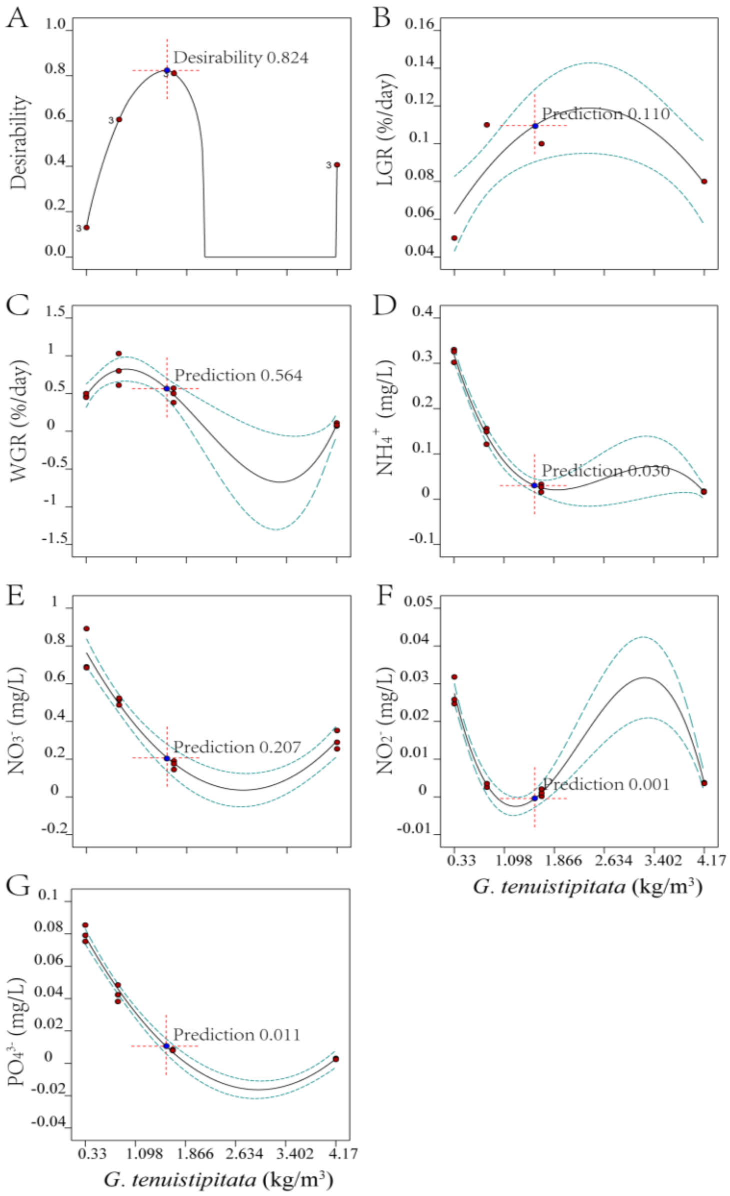


***Figure 3.*** *Optimal solution for Crassostrea hongkongensis and Gracilaria tenuistipitata in polyculture as calculated by the response surface analysis*


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DOI: http://dx.doi.org/10.15666/aeer/2002_12411250

## Discussion

As a biofilter, the red seaweed *G. tenuistipitata* can significantly reduce the inorganic nutrients in an integrated culture system. Studies have reported the use of *G. tenuistipitata* in a variety of polyculture systems such as those for shrimp (Anh et al., 2019), tilapia (An and Anh, 2020), and rainbow trout (Haglund and Pedersén, 1993). The $NH_4^+$, $NO_3^-$, $NO_2^-$, and $PO_4^{3-}$ concentrations were effectively reduced in these polyculture systems, while $NH_4^+$ and $PO_4^{3-}$ concentrations were reduced by 95.71% and 95.74%, respectively, under the polyculture conditions used with 3.5 g/L of the shrimp *Penaeus vannamei* (Sarkar et al., 2019). In the mixed culture system, *G. tenuistipitata* synthesizes its own tissue, obtaining nutrients from the aquatic animals. The growth rate of *G. tenuistipitata* can reach 4%/day under the optimal conditions of the three summer months (Haglund and Pedersén, 1993) and reach 2.4%/day under natural pond conditions over a year (Wu et al., 1994). In this study, the daily growth rate of *G. tenuistipitata* was less than 1%, which might be related to the short culture period (one week), when it may still be in the environmental adaptation stage. However, *G. tenuistipitata* significantly reduced the amount of nutrients (76.89–93.89%) in polyculture with the oyster, indicating that it could play a role in improving water quality in a mixed-culture system.

In this study, both the oyster and seaweed grew better in the polyculture system than in monoculture. Other studies have reported similar results. Qian et al. (1996) found higher growth rates for the red algae *Kappaphycus alvarezii* and pearl oyster *Pinctada martensi* in a field co-culture. The mechanisms operating during the polyculture of shellfish and seaweed can be explained by the following aspects. On the one hand, the great filtration skill of the oysters increases the transparency of seawater (McVey et al., 2002; Schröder et al., 2014; Petersen et al., 2016), which may improve the photosynthetic efficiency of the seaweed (Newell et al., 2005). In addition, the rich dissolved nutrients released by the oyster (Newell et al., 2005; Hoellein and Zarnoch, 2014), especially $NH_4^+$, could benefit the seaweed's growth (Sandoval-Gil et al., 2016). On the other hand, seaweed photosynthesis releases oxygen, providing more dissolved oxygen for the oyster, which would be conducive to its growth (Yang et al., 2015). The seaweed also removes $NH_4^+$ excreted by the oyster, which reduces ammonia toxicity for the oysters, helping them maintain healthy growth. In this polyculture system, the oyster and seaweed each obtain what they need, forming a mutually beneficial and stable symbiotic system.

In the polyculture system, a moderate seaweed density is the key to promoting the growth of the organisms and improving the culture environment. Studies have shown that at a medium density, seaweed greatly effects nitrogen and phosphorus removal in a mixed-culture system (Huang et al., 2010). In this study, the appropriate seaweed density promoted the growth rate of both oyster and seaweed, and the nutrient removal rate was high. However, the growth and nutrient removal rates were low under the low-density and high-density seaweed conditions. For instance, in the medium-density seaweed group, the growth rates of the oysters and seaweed were roughly 14 and 3 times higher than that in their respective monoculture groups. Under the high-density seaweed conditions, the nutrient removal rates reached 77–94%. Therefore, while a medium seaweed density can promote growth, high seaweed density is conducive to improving water quality. To balance the economic benefits and environmental protection, it is necessary to find the optimal oyster to seaweed ratio.


http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Polyculture is defined as putting two or more species with different ecological niches into a culture environment that combines their nutritional resources and living space, improves nutrient utilization, minimizes cost and maximizes economic value, and benefits the environment. To maximize the economic and environmental benefits, the optimum proportion of the different species in the polyculture need to be studied. Huang et al. (2010) found that better nitrogen and phosphorus removal was achieved when 6 $kg/m^3$ of the red alga *Kappaphycus striatum* was combined with 60 $ind/m^3$ of the bivalve *Paphia exarata*, while the effect was poor when the densities were too high or too low. Qian et al. (1996) found that 200 g of *K. alvarezii* could effectively absorb the total amount of nitrogen released by 80 *P. martensi* individuals within 6 hr. In our study, the optimal densities for *G. tenuistipitata* and *C. hongkongensis* were 1.567 $kg/m^3$ and 83.3 $ind/m^3$ (10 individuals), respectively, which resulted in a balance of the biological growth rate and the nutrient removal rate.

## Conclusions

Both *C. hongkongensis* and *G. tenuistipitata* grew better in a polyculture system than in a monoculture system. The appropriate seaweed density removed significant amounts of the dissolved nutrients released by the oysters. Considering both the economic benefits and environmental protection, the best combination was 10 oysters (83.3 $ind/m^3$) with 1.567 $kg/m^3$ seaweed. Our results indicate that seaweed in co-culture could effectively reduce dissolved nutrients, improve water quality, and increase oyster production. *Gracilaria tenuistipitata* could be used in the ecological restoration of the bay to mitigate eutrophication and improve seawater quality, especially in the Beibu Gulf, where the oyster aquaculture center is in South China. In recent years, eutrophication has intensified, the risk of red tide has increased, aquaculture diseases have frequently appeared, and the size of commercial oysters has decreased. In this case, combining seaweed with the oyster culture system would be expected to extend the carrying capacity barrier and achieve a balance of economic growth and environmental protection. The optimal ratio in this study was obtained under semi-outdoors condition. Future studies will verify the actual effect of this ratio in the field, as well as explore the optimal ratio of other seaweeds and the oysters.

**Acknowledgments.** We are very grateful to Dr. Ke Pan (Shenzhen University) for his language modification of the manuscript. This study was supported by the Science and Technology Project of Guangxi (GuikeAB20297018, GuikeAB20297037, GuikeAD19110140), the Open Research Fund Program of Guangxi Key Lab of Mangrove Conservation and Utilization (GKLMC-201901), China Postdoctoral Science Foundation (014420).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2002_12411250

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