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Nutritional Profile and Bioremediation of Tropical Marine Aquaculture with an Integrated Microalgae and Bacteria Symbiosis

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Abstract The nutritional profile of tropical mariculture wastewater in Hainan Island and the possibilities of artificial mariculture wastewater (AWW) bioremediation using microalgae and bacteria screened from the South China Sea were studied. A total of 34 sampling sites from field and water were selected to study the current status of mariaquaculture in Wenchang, an important aquaculture production base in Hainan. The mean value of pH, suspended solids (SS), NH_4^+-N, PO_4^{3-}-P, and COD_{Mn} of field samples were 7.52, 229.44 mg/L, 1.44 mg/L, 0.88 mg/L, and 9.5 mg/L, respectively. Monocultures of microalgae (*Chlorella vulgaris*, *Platymonas subcordiformis*, and *Chaetoceros müelleri*) and microalgae-bacteria symbiosis (*C. vulgaris* and *Bacillus* spp.) were also studied in AWW indoor inoculation experiments. The results of monocultures indicated that *C. vulgaris* is the optimal strain for removing NH_4^+-N, NO_2^--N, T_N, and PO_4^{3-}-P, with a removal rate of 97.15%, 99.45%, 95.90%, and 96.62% in 72 h, respectively. By comparison, symbiosis of *C. vulgaris-Bacillus* spp. with an initial concentration of 2.5×10^5 cells/mL microalgae + 5 mL *Bacillus* spp. ($OD_{600}=1.8$) enhanced the removal of NH_4^+-N, PO_4^{3-}-P, and COD_{Mn}, with a removal rate of 100%, 100%, and 35.69% in 72 h, respectively. Combining microalgae and bacteria communities is probably effective in nutrients removal, enhancing microalgal biomass and biofuel production.

Highlights
- Mariculture wastewater pollution was detected in Wenchang, Hainan Province, China.
- High removal efficiency of N and P was achieved by *Chlorella vulgaris*.
- Complete removal of NH_4^+-N and PO_4^{3-}-P was found in the symbiosis of *C. vulgaris* and *Bacillus* spp.

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Keywords *Chlorella vulgaris* · *Bacillus* spp. · Aquaculture wastewater · Nitrogen and phosphorus removal · Bioremediation

1 Introduction

According to Zheng and Shen (2021), the mariculture area of Hainan Province reached 5347 hm^2 in 2018, more than twice that of 2009. Meanwhile, the mariculture output of Hainan Province has increased year by year for the last decade, reaching 124,554 tons in

2018, more than three times that of 2009. The growing food production industry increases high-density tropical mariculture, producing significant mariculture effluent. In general, surplus fishery feeder, excrement, and aquatic remains would build up in the cultivation system, resulting in a high concentration of particulate organic matter and a richness of dissolved nutrients in the wastewater, such as nitrogen and phosphorus, which could eventually cause eutrophication and disrupt the balance of aquaecosystem. Nevertheless, from the perspective of energy, these organic matters and nutrients can be a supply to bacteria and microalgae growth which can be utilized as an economical, efficient, and eco-friendly method to remove various pollutants in mariculture wastewater.

Typical technologies such as physical, chemical, and biological processes, which have been applied in the treatment of mariculture wastewater, include reverse osmosis, ion exchange, and constructed wetland (Santos & Pires, 2018; Sun et al., 2020). Physical methods such as sedimentation, sand filtration, or mechanical filtration are generally applied in the primary stage to clarify large-sized solids (Cai, Park, et al., 2013). Precipitation and ferrous chloride, in terms of chemical treatment processes, are commonly used to remove phosphorous compounds (Guldhe et al., 2017). The conventional biological treatment removes organic matter and nutrients through aerobic or anaerobic activities. Among the three conventional methods, some drawbacks also limit their application in practice. For instance, physical methods are of low efficiency and are not applicable to nutrients removal, chemical methods are costly and may produce some toxic compounds as byproducts, while the conventional biological method is expensive due to the large quantities of sludge production and high cost for drying and great amounts of nutrients would be wasted in the effluent (Guldhe et al., 2017). In contrast, microalgae-assisted bioremediation has the ability to assimilate inorganic nitrogen and phosphorus in a contaminated mariculture environment (Wang et al., 2016). Subsequently, it may offer more efficient and cost-effective alternatives to conventional treatment methods.

Furthermore, microalgae-based wastewater treatment technology can simultaneously remove other pollutants such as refractory organics and heavy metals from sewage. Likewise, it can be used to treat a variety of wastewater, including municipal sewage, aquaculture effluent, and industrial drainage. Especially, it is generally believed that the synergistic function between microalgae and bacteria is responsible for the purification of wastewater (Wang, Jiao, et al., 2020). Currently, higher removal efficiency has been observed based on the monoculture microalgae and microalgae-bacteria symbiosis technology (Chu et al., 2021; Koppel et al., 2019; Li et al., 2020; Liu et al., 2020). However, the differences between the two modes still need to be further investigated to optimize the mariculture wastewater.

This study investigated the nutritional profile of mariculture wastewater in Hainan and aimed to select proper microalgal strain and optimal symbiosis of microalgae and bacteria for the bioremediation of mariculture wastewater. Parameters such as ammonia nitrogen (NH_4^+-N), orthophosphate (PO_4^{3-}-P), nitrite (NO_2^--N), total phosphorus (T_P), total nitrogen (T_N), and chemical oxygen demand (COD_{Mn}) were tested.

2 Materials and Methods

2.1 Sampling of Tropical Mariculture Wastewater

A total of 34 sampling sites were selected surrounding the mariculture facility based on mariculture output, area, and mariculture wastewater receiving points at the sea site (as shown in Fig. 1).

Sampling was carried out on three consecutive sunny days (from October 30th to November 1st, 2017). All the mariculture wastewater samples were collected using 1-L polyethylene bottles at locations about 100 m away from drainage outlets and labeled as the sequence of sampling sites (see Supplementary Table S1). The samples were immediately delivered to the laboratory and stored at 4 ℃ in a refrigerator until further treatment and analysis; the parameters such as pH, suspended substance (SS), COD_{Mn}, PO_4^{3-}-P, and NH_4^+-N content were measured.

2.2 Preparation of the Artificial Tropical Mariculture Wastewater

Artificial mariculture wastewater was prepared on the basis of the concentrations of pollutants in practical wastewater sampled from the 34 sites in

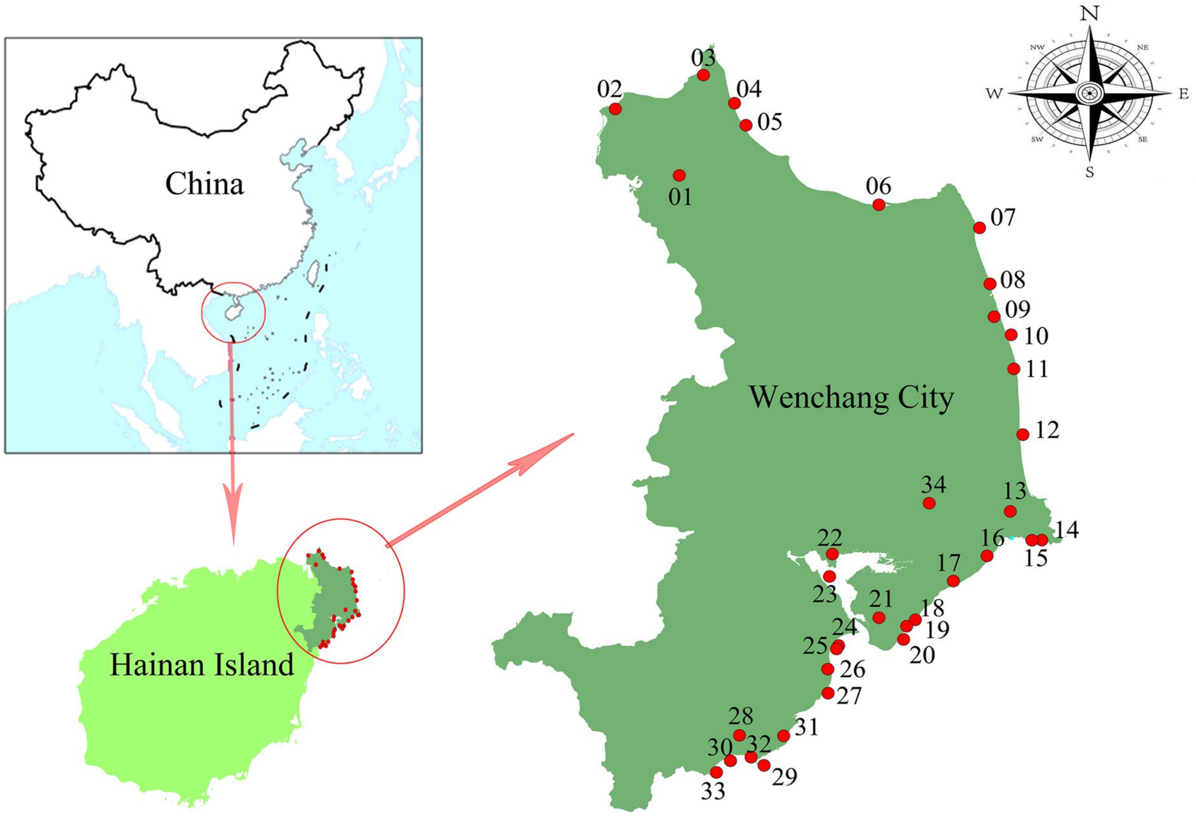

Fig. 1 Sampling sites in the study area

Hainan. For details, the AWW was composed of glucose (200 mg/L), NH_4Cl (5 mg/L), NaH_2PO_4 (1.5 mg/L), $NaNO_2$ (3 mg/L), $NaNO_3$ (3 mg/L), and stock of trace elements, as the same as that of *f/2* medium in "Microalgal strain, medium, and cultivation condition." As reported, a molar ratio of N:P lower than 5:1 would result in nitrogen limitation, whereas phosphorus limitation would occur at a ratio of higher than 30:1 (Larsdotter, 2006). In this study, the N:P molar ratio was 16.24, indicating that neither the nitrogen nor phosphorus limitation was reached. However, it was higher than that of the recommended medium of *C. vulgaris* (e.g., *f/2* medium), in which N of 12.36 mg/L, P of 1.12 mg/L with an N:P ratio of 11.

2.3 Microalgal Strain, Medium, and Cultivation Condition

In this study, three microalgal species such as *C. vulgaris*, *Platymonas subcordiformis*, and *Chaetoceros müelleri* were selected to cultivate in mariculture wastewater. The strains were obtained from the microalgae culturing collection center at the College of Oceanology, Hainan University in China. The *f/2* medium was based on Guillard(1975), which composed (per liter seawater, all analytical grade) $NaNO_3$ (8.83×10^{-4} mol/L), $NaH_2PO_4\bullet2H_2O$ (3.63×10^{-5} mol/L), $Na_2SiO_3\bullet9H_2O$ (1.07×10^{-4} mol/L), $CuSO_4\bullet5H_2O$ (4×10^{-8} mol/L), $ZnSO_4\bullet7H_2O$ (8×10^{-8} mol/L), $CoCl_2\bullet6H_2O$ (5×10^{-8} mol/L), $MnCl_2\bullet4H_2O$ (9×10^{-7} mol/L), $Na_2MoO_4\bullet2H_2O$ (3×10^{-8} mol/L), Na_2EDTA (1×10^{-5} mol/L), $FeCl_3\bullet6H_2O$ (1×10^{-5} mol/L), vitamin B_1 (3×10^{-7} mol/L), biotin (1×10^{-6} mol/L), and vitamin B_{12} (1×10^{-10} mol/L). The inoculum of microalgae was prepared in 250-mL flasks containing 120 mL fresh *f/2* medium. The flasks containing fresh medium were sterilized at 121 ℃ and 0.22 MPa pressure conditions for 15 min. After cooling down to room temperature, the medium was inoculated to reach an initial cell density of 2.5×10^5 cells/mL. The

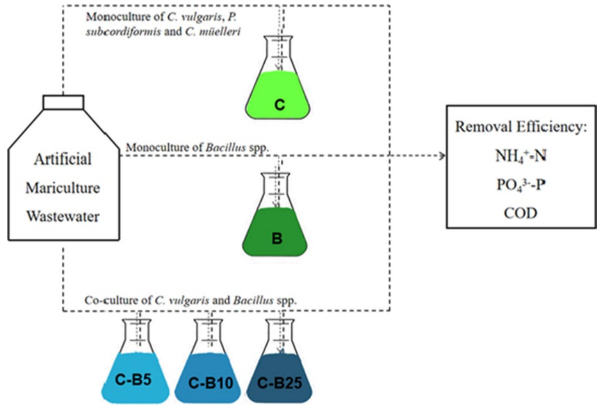

Fig. 2 Sketch of experimental design

microalgae were cultivated in an illumination incubator (MGC-100, Blue Pard) at 28 ± 1℃, with the illumination of 660 μmol photons/(m^2•s) at 12:12 light: dark cycle under an array of fluorescence lights (DULUX L 36 W/840, OSRAM).

2.4 Bacteria strain, Medium, and Cultivation

Bacteria *Bacillus* spp. was the dominant strain in seawater around Hainan Island, screened by the Eco-toxicology Laboratory, Hainan University in China. Saline Lactose Broth (SLB) medium (per liter seawater, analytical grade) is composed of beef extract (5 g/L) and peptone (10 g/L) with a pH of 7.8 was used for the cultivation. After being sterilized and cooled down, the medium was inoculated with 2 mL *Bacillus* spp. with an initial optical density of 1.89 measured at a 600 nm (OD_{600}) wavelength and kept at 30 ℃ and 160 rpm for 16 h in a shaker (ZQP-75D, Laboratory).

2.5 Nutrients Removal from Mariculture Wastewater Using Microalgae

Monoculture microalgae and microalgal-bacteria symbiosis were cultivated in AWW to evaluate their nutrient removal efficiencies from mariculture wastewater. For the monoculture experiment, three microalgal species of *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* were separately inoculated into sterilized AWW, to reach an initial cell density of 2.5×10^5 cells/mL. The microalgal-bacteria symbiosis experiment was conducted by inoculating the aforementioned optimal microalgae and bacteria *Bacillus* spp. with different inoculation ratios to determine the optimal symbiosis for nutrients removal from AWW. An experimental sketch contained information on two monocultures (C and B) and three symbiosis (C-B5, C-B10, and C-B25) shown in Fig. 2.

In this study, each experiment was carried out with three replicates. Shake the flasks at least three times each day in the morning, noon, and afternoon of cultivation days, respectively. Besides, parameters such as optical density (OD), concentrations of NH_4^+-N, NO_2^--N, PO_4^{3-}-P, T_N, and COD_{Mn} were determined daily. Other cultivation conditions were kept as the same as that in "Preparation of the artificial tropical mariculture wastewater."

2.6 Analytical Methods

The biomass concentration of microalgae and bacteria was represented by the index of absorbance (OD), which was by the absorbance of microbial medium suspension in optical density at the wavelength of 680 nm (OD_{680}) and 600 nm (OD_{600}), respectively. The pH was determined by a Mettler Toledo Seven2Go pH meter, and SS was measured using the gravimetric method. Amount of PO_4^{3-}-P was determined by the spectrophotometric determination of a blue phosphomolybdic complex that specifically absorbs at 882 nm compared with the deionized water as the blank solution (Strickland & Parsons, 1972). Nitrites were determined by a colorimetric chemical reaction forming an azo dye. While the concentration of NH_4^+-N and TN content follows the Phenate Method and Potassium Persulfate Oxidation Method compared with the ultrapure water as blank solution and NO_2^--N content used deionized water as the blank solution. Additionally, the content of COD_{Mn} was determined by the method from Ansari et al. (2017).

2.7 Data Analysis

Data were presented as the average by recording in Microsoft Office Excel™. Correlational analysis was performed using the SPSS 23 software (IBM Corporation, Armonk, New York, USA). Graphical analyses were performed using Originlab Origin Pro 8.0™. The removal efficiencies and removal rates of nutrients were calculated according to Eqs. (1) and Eq. (2).

$$RE_i(\%) = (1 - \frac{S_{t,i}}{S_{0,i}}) \times 100 \quad (1)$$

$$R_i\left(\frac{\text{mg}}{L \cdot \text{h}}\right) = \frac{S_{0,i} - S_{t,i}}{t_0 - t} \quad (2)$$

where RE_i ($i = PO_4^{3-}$-P, NO_2^--N, NH_4^+-N, T_N, or COD_{Mn}) is the removal efficiencies of nutrients,R_i ($i = PO_4^{3-}$-P, NO_2^--N, NH_4^+-N, T_N, or COD_{Mn}) is the removal rate of nutrients, $S_{0,i}$ is the initial concentration value of substrate, and $S_{t,i}$ shows corresponding substrate concentration at time t.

3 Results

3.1 Current Status of Tropical Mariculture Wastewater in Hainan

3.1.1 Mariculture Wastewater Discharge in Hainan

The field investigation showed that four main mariculture systems such as a high-level pond, low-level pond, common cage, and industrial factory are widely applied in Hainan, with an area of 692 hm^2, 2489 hm^2, 8 hm^2, and 789,000 m^3, respectively (2017). The main mariculture species include grouper (*Epinephelus* sp.), *Penaeus* sp., and high economic profits shellfish species including *Babylonia lutosa* and *Scapharca inflate*, with yields of 14,424 tons, 20,507 tons, and 11,930 tons, respectively. As investigated, the water exchange methods varied with the types of aquatic species, culturing period, and mariculture method (see Supplementary Table S2). It can be seen that large quantities of mariculture wastewater are directly discharged, resulting in adverse effects on the quality of the surrounding sea.

The current status of investigated sites is shown in Fig. 3. Dense aquaculture wastewater pipes are randomly distributed along the beachside (Fig. 3a,b). Moreover, some large-scale drainage ditches are also randomly distributed (Fig. 3c). Since many substances such as residual baits, excrement, and other culturing residues are produced in cultivation, most residues accumulate and decompose in the culturing system. Thus, the water quality would be affected significantly. As investigated, the seawater turns green, turbid, and stinky due to the accumulation of pollutants (Fig. 3d). Besides, the plastic sewage pipes are potential contributors of plastic debris and even micro-/nano-plastics to either beaches or seawater (Fig. 3e). In some places, the sands have become hardened and blacked due to the long time receiving the mariculture wastewater (Fig. 3f). The study also found many large-scaled culturing systems (e.g., industrial factories and high-level ponds) in Wenchang (Fig. 3g, h).

3.1.2 Current Status of Mariculture Wastewater

For the 34 sampling sites, the temperature varied between 33 ℃ and 35 ℃ without drastic fluctuation (Table 1). The pH values of the samples ranged from 6.89 to 8.25, while the SS content ranged from 16.77 to 653.33 mg/L. The site with the highest SS content (653.33 mg/L) was site 21, which receives water samples from a high-level shrimp pond (Table 1).

The mean value of NH_4^+-N, PO_4^{3-}-P, or SS was calculated on the basis of all 34 sampling sites (Table 1). The concentration of NH_4^+-N ranged from 0.33 mg/L (at site 10) to 3.15 mg/L (at site 21). Higher concentrations of NH_4^+-N were observed at sites 02, 09, 18, and 21, where *Babylonia*, fishes, or shrimps have been cultivated for a long period. Furthermore, the maximum NH_4^+-N value (i.e., 3.15 mg/L) was observed at site 21. For PO_4^{3-}-P content of 34 sites ranged from 0.07 to 2.35 mg/L, in which three samplings showed a high level of PO_4^{3-}-P from site 02 (1.94 mg/L), site 21 (1.89 mg/L), and site 27 (2.35 mg/L). Among them, site 27 was a fish culturing draining outlet, while site 02 and site 21 were close to the port. The range of COD_{Mn} was 0.43–18.44 mg/L for the 34 sampling locations. For COD_{Mn} with the maximum value of

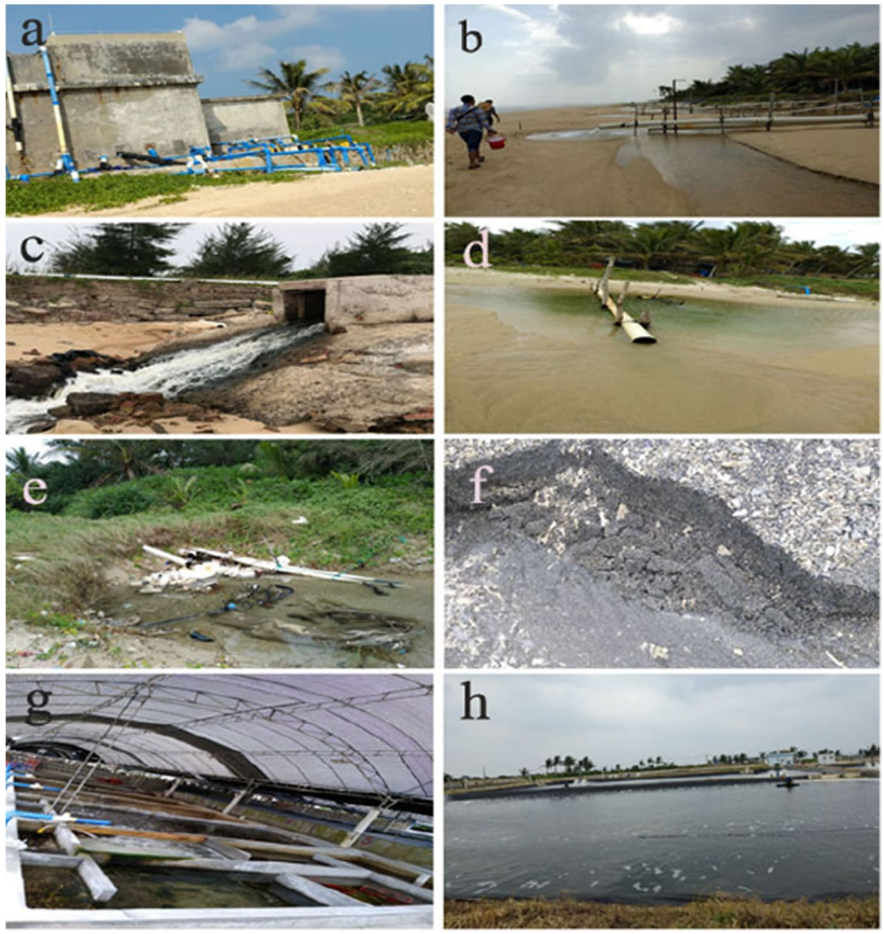

Fig. 3 Representative present situation of mariculture sites in Wenchang City: (**a**) intake and drainage pipes on the beach, (**b**) coastal drainage pipes, (**c**) a drainage ditch, (**d**) water body color around the drainage outlet becomes green, (**e**) wasted pipe plastic garbage, (**f**) blackened and harden sands, (**g**) an industrial aquaculture pond, and (**h**) a high-level pond

18.44 mg/L was observed close to downtown and around under construction Shuxiang Park at site 25.

3.2 Effects of Free-celled Microalgae on Nutrients Removal from AWW

According to the above field investigation, the main pollutants included SS, NH_4^+-N, PO_4^{3-}-P, and COD_{Mn} and the following experiments focused on the removal of these components.

3.2.1 Cell Growth of Microalgae in AWW

As shown in Fig. 4, an initial cell density of 1×10^6 cells/mL was inoculated with 25% (v/v) microalgal inoculum of *C. vulgaris*, *P. subcordiformis*, and *C. müelleri*. The growth profiles presented a slight increase in OD_{680} biomass(0.13–0.30) at the end of the cultivation period ($P < 0.05$). The maximum biomass concentration of *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* with OD_{680} values of 0.18, 0.14, and 0.33 were obtained in AWW at 240 h of cultivation, respectively.

3.2.2 Phosphorus Removal by Microalgae from AWW

As a fundamental material of life module in DNA, RNA, ATP, proteins, and amino acid, phosphorus is regularly existing in wastewater as inorganic anionic types, including $H_2PO_4^-/HPO_4^{2-}$ which is highly relative to microalgae metabolisms (Salama et al., 2017). In this experiment, three microalgal species exhibited high removal efficiency of PO_4^{3-}-P in 24 h or 48 h of cultivation (Fig. 5). For example, near-zero residual PO_4^{3-}-P level was achieved in monoculture *C. vulgaris* and *P. subcordiformis* in 24 h, while a remaining concentration of 0.86 mg/L PO_4^{3-}-P in the treatment of *C. müelleri*, but it

Table 1 Water quality of sampling sites and the national standard values[a]

Site codes	pH value	SS (mg/L)	NH_4^+-N (mg/L)	PO_4^{3-}-P (mg/L)	COD_{Mn} (mg/L)
01	8.03	136.67	0.85	0.38	7.28
02	7.63	246.67	3.07	1.94	12.77
03	7.66	127.78	1.72	0.64	0.43
04	7.81	203.33	2.55	1.21	16.57
05	6.89	346.67	1.46	0.07	5.14
06	7.83	76.67	1.33	0.90	9.85
07	8.2	116.67	2.37	0.33	13.71
08	7.02	458.33	1.37	0.38	15.42
09	7.65	425.56	2.37	0.69	7.34
10	\[b]	25.00	0.33	0.85	14.73
11	7.85	186.67	0.89	1.26	8.08
12	6.96	188.89	1.11	0.74	12.16
13	7.82	523.33	1.42	1.14	12.89
14	7.53	257.78	0.98	\[b]	17.46
15	7.75	160.00	0.89	0.43	6.28
16	7.83	54.44	1.46	0.69	13.48
17	7.27	385.56	0.76	1.21	12.08
18	7.48	445.56	2.37	1.06	4.65
19	7.5	485.56	1.16	0.74	7.10
20	7.49	653.33	1.27	0.54	9.87
21	7.35	148.33	3.15	1.89	3.84
22	7.55	66.67	1.07	0.48	5.39
23	7.92	28.89	1.72	0.26	11.02
24	7.74	33.33	1.76	0.64	12.89
25	7.94	16.67	1.94	1.11	18.44
26	7.71	115.56	1.37	1.37	14.08
27	7.71	192.22	2.37	2.35	8.15
28	7.68	158.33	0.98	0.54	12.97
29	7.62	150.00	1.03	1.26	5.26
30	7.36	148.33	0.42	0.80	0.47
31	7.65	173.33	0.87	1.47	5.69
32	7.51	63.33	0.42	0.59	7.75
33	7.47	525.00	0.81	0.85	4.71
34	7.39	476.67	1.16	0.38	5.18
Mean value	7.52	229.44	1.44	0.88	9.5
National standard [a]	6.5–9	≤100	1	0.1	20

[a]The Chinese national standards of pH, NH_4^+-N, and SS refer to SC/T 9103–2007

[b]The symbol "\" indicates not detectable

also reached a near-zero residual by 48 h of cultivation. The results indicated that PO_4^{3-}-P could be removed entirely via cultivating such monoculture microalgae as *C. vulgaris*, *P. subcordiformis*, and *C. müelleri*.

3.2.3 Nitrogen Removal by Microalgae from AWW

Ammonium could be absorbed by microalgae cells through active transport and directly utilized for amino acids synthesis (Han et al., 2019). Figure 6 shows nitrogen removal in terms of NH_4^+-N, NO_2^--N, and T_N in AWW by monoculture *C. vulgaris*, *P. subcordiformis*, and *C. müelleri*, respectively. The removal efficiencies of NH_4^+-N achieved by cultivating *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* reached 88.79%, 61.42%, and 50.25% in 24 h, respectively (Fig. 6a). A near-zero residual NH_4^+-N level (0.01 ± 0.005 mg/L) was achieved by

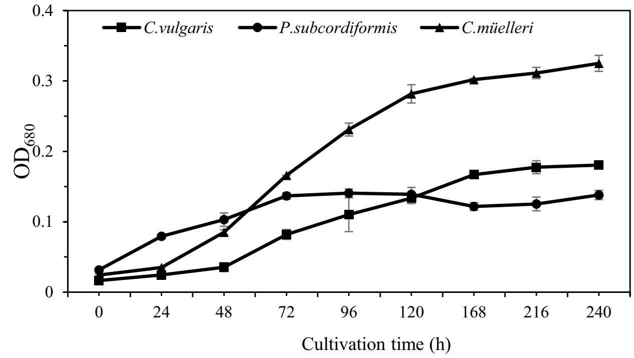

Fig. 4 Changes in *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* under OD_{680} ($n = 3; \bar{x} = SD$)

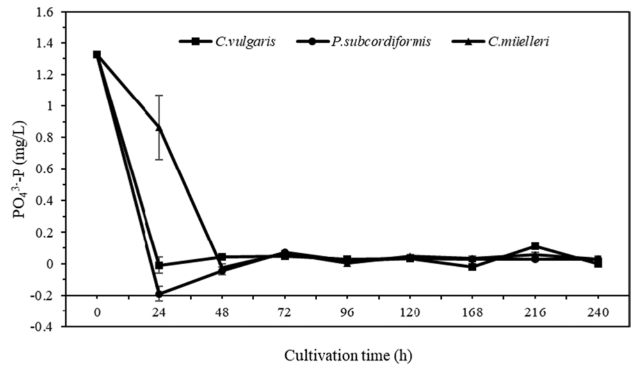

Fig. 5 Removal of PO_4^{3-}-P in AWW by *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* ($n = 3; \bar{x} = SD$)

cultivating monoculture *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* within 48 h. By comparison, *C. vulgaris* showed a relatively faster removal rate than *P. subcordiformis* and followed by *C. müelleri*.

NO_2^--N can be eventually removed by monoculture either *C. vulgaris*, *P. subcordiformis*, or *C. müelleri* (Fig. 6b). For instance, NO_2^--N was completely removed by *C. vulgaris* in a short cultivation time (i.e., 72 h), while by *C. müelleri* and *P. subcordiformis* in almost 96 h and 216 h, respectively. In the current study, *C. vulgaris* presented the optimal bioremediation in consideration of advantages such as shorter treatment time, higher removal efficiency, and vigorous cultures.

T_N concentration in monoculture of *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* declined in the first 48 h or 72 h. It then remained lower for the rest of the cultivation time (Fig. 6c). Microalgae *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* can uptake almost all the T_N from AWW, with the residue of 0.49 mg/L, 0.73 mg/L, and 0.55 mg/L in 48 h, respectively, corresponding to removal efficiencies of 90.24%, 85.36%, and 88.99%.

Overall, among the three monocultures of microalgal species, *C. müelleri* showed faster cell growth. While *C. vulgaris* exhibited a more rapid removal rate of NH_4^+-N, PO_4^{3-}-P, T_N, and NO_2^--N than other microalgal species in AWW. Thus, the optimal microalgae *C. vulgaris* was selected for the combination of *Bacillus* spp. in the following tests of AWW biotreatment.

3.3 Effects of Microalgal-bacteria Symbiosis on Nutrients Removal in AWW

As aforementioned, the microalgal-bacteria symbiosis was based on *C. vulgaris* inoculation of 2.5×10^5 cells/mL with 5 mL, 10 mL, and 25 mL inoculum of *Bacillus* spp. ($OD_{600} = 1.89$), which are labeled as C-B5, C-B10, and C-B25, respectively. More specifically, we summarized and compared the microbial growth performance of both *C. vulgaris* and *Bacillus* spp. and the removal of NH_4^+-N, PO_4^{3-}-P, and COD_{Mn} from AWW in bioremediation between monoculture and symbiosis system in Table 2. As for the pollutants

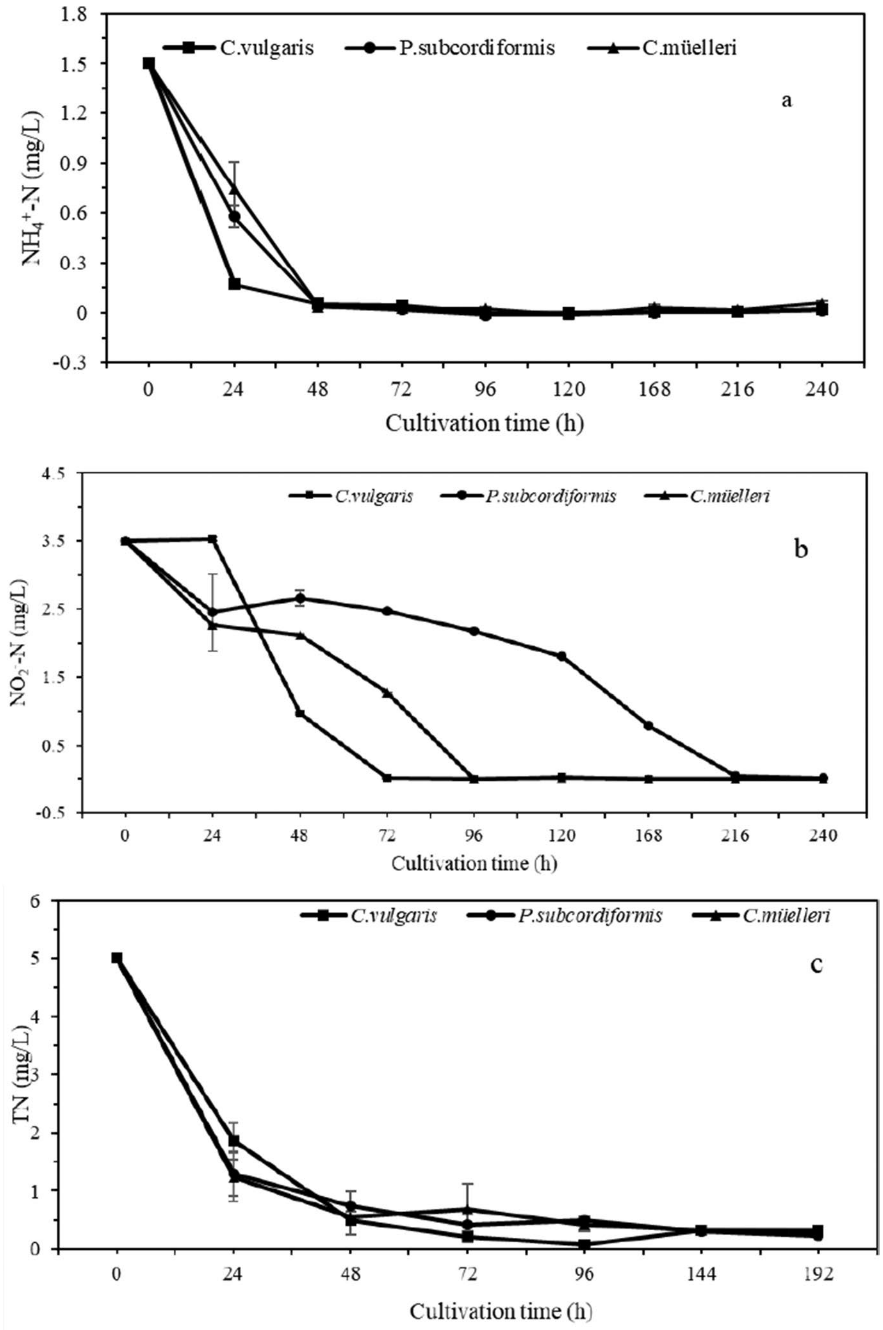

Fig. 6 Removal of NH_4^+-N (**a**), NO_2^--N (**b**), and T_N (**c**) removal in AWW by monoculture *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* ($n=3;\bar{x} = SD$)

removal rates, in the same treatment period, all of them were higher in three consortium groups than that in the pure cultures of microalgae or bacteria groups. It should be noted that only NH_4^+-N was measured in this test in consideration of the main pollutants in AWW.

3.3.1 Cell Growth of Microalgal-bacteria Symbiosis

Cell growth of both *C. vulgaris* and *Bacillus* spp. increased during the period of treatment ($P<0.05$), and the maximum DCW was achieved in 168 h. For symbiosis C-B5, C-B10, and C-B25, higher

Table 2 NH_4^+-N, PO_4^{3-}-P, and COD_{Mn} removal capacity and efficiency with *C. vulgaris* and various inoculation rates of *Bacillus* spp. from AWW

Trial code	Biomass accumulation		Treatment period (h)			Residue (mg/L)			Removal rate (mg/L•d)			Removal efficiency (%)		
	MA (mg/L)	BS (OD_{600})	NH_4^+-N	PO_4^{3-}-P	COD	NH_4^+-N	PO_4^{3-}-P	COD	NH_4^+-N	PO_4^{3-}-P	COD_{Mn}	NH_4^+-N	PO_4^{3-}-P	COD
C	318 ± 24	-	72	12	168	0.023 ± 0.062	0.206 ± 0.003	72.736 ± 1.02	0.462	2.587	3.954	98.36	86.24	27.56
B	-	0.148 ± 0.021	72	12	168	0.069 ± 0.024	0.145 ± 0.008	64.575 ± 7.36	0.447	2.709	5.120	95.12	90.31	35.69
C-B5	344 ± 11	0.191 ± 0.01	72	12	168	0 ± 0.005	0.091 ± 0.005	64.575 ± 3.93	0.481	2.818	5.120	100	93.94	35.69
C-B10	457 ± 12	0.256 ± 0.007	72	12	168	0 ± 0.009	0.112 ± 0.004	61.587 ± 5.47	0.475	2.777	5.546	100	92.55	38.67
C-B25	1,039 ± 7	0.567 ± 0.004	72	12	168	0.071 ± 0.006	0.146 ± 0.013	55.292 ± 3.04	0.446	2.707	6.446	94.97	90.24	44.93

The initial inoculation density of *C. vulgaris* is 2.5×10^5 cells•mL^{-1}; the OD_{600} value of *Bacillus* spp. is 1.89 ($n = 3$; $\bar{x} = SD$). Symbols C and B indicate monoculture *C. vulgaris* and monoculture *Bacillus* spp. respectively; while C-B5, C-B10, and C-B25 represent a symbiosis of *C. vulgaris* with 5 mL, 10 mL, and 25 mL *Bacillus* spp., respectively

microalgal biomass concentrations of 344 mg/L, 457 mg/L, and 1,039 mg/L were obtained than monoculture *C. vulgaris* value of 318 mg/L. The supply of carbon dioxide and inorganic substances from bacteria benefitted the microalgal biomass accumulation. The DCW was significantly increased by C-B10 and C-B25 ($P < 0.05$), while no significant difference was observed in C-B5 ($P > 0.05$) when comparing the cell growth in microalgal-bacteria system with monoculture *C. vulgaris*. This indicated that *Bacillus* spp. contributed to the higher cell density of *C. vulgaris*, and C-B25 achieved the best cell growth. Additionally, the cell density of *Bacillus* spp. also exhibited the same trend as *C. vulgaris*.

3.3.2 NH_4^+-N Removal by Microalgal-bacteria Symbiosis

As shown in Table 2, NH_4^+-N contents decreased from 1.5 to 0.023 mg/L and 0.069 mg/L by cultivating monoculture of *C. vulgaris* and *Bacillus* spp. within 72 h, with the removal efficiency of 98.36% and 95.12%, respectively. By comparison, higher NH_4^+-N removal efficiency (up to approximate 98%) was achieved by microalgal-bacteria symbiosis in a shorter time (i.e., the first 48 h of cultivation time). It could be attributed to the cooperative interaction in microalgal-bacteria symbiosis.

Among the three symbioses of microalgal-bacteria, complete removal of NH_4^+-N (corresponds to 0 mg/L in the effluent) was achieved in the trial of C-B5 and C-B10 in 72 h, while 0.071 mg/L NH_4^+-N remained in the trial of C-B25 in 72 h, respectively. The results indicated that optimal bacteria was added and a relatively higher removal efficiency of NH_4^+-N was achieved ($P < 0.05$).

3.3.3 PO_4^{3-}-P Removal by Microalgal-bacteria Symbiosis

High removal efficiency of PO_4^{3-}-P was achieved by either monoculture or symbiosis of *C. vulgaris* and *Bacillus* spp. by 48 h of cultivation ($P < 0.05$). For instance, PO_4^{3-}-P in almost all treatments was completely removed from 1.5 to 0–0.003 mg/L (corresponds to the rates of up to 1.490–1.497 mg/(L•d)). Zero residual of PO_4^{3-}-P was achieved by C-B5 and C-B25 within 48 h. The fastest and highest PO_4^{3-}-P removal was found in C-B5 (93.94% reduction within 12 h), followed by C-B10 (92.55% in 12 h) and

C-B25 (90.24% in 12 h), while the lowest removal efficiency of PO_4^{3-}-P (86.24%) was obtained in the monoculture of microalgae, which may be due to the lack of bacteria involvement and relatively poor PO_4^{3-}-P removal capability.

3.3.4 *COD_{Mn} Reduction by Microalgal-bacteria Symbiosis*

As shown in Table 2, the COD_{Mn} removal was similar in the treatments with the removal efficiencies of 27.56–44.93%, which indicated that there was no strong relationship between COD_{Mn} removal and initial inoculum ratios of monoculture or symbiosis microalgae or bacteria. Nevertheless, the COD_{Mn} removal efficiencies achieved by symbiotic bioremediation of microalgae and bacteria (35.69–44.93%) were slightly higher than monoculture *C. vulgaris* (27.56% in 168 h) and *Bacillus* spp (35.69% in 168 h). Moreover, it can be seen that the optimal efficiency of COD_{Mn} was achieved by C-B25, with the highest removal efficiency and reduction rate of 44.93% and 6.446 mg/(L•d), respectively.

4 Discussion

4.1 Current Status of Tropical Mariculture Wastewater in Hainan

The present study determined that the main pollutants are SS, NH_4^+-N, and PO_4^{3-}-P. In general, SS values in 34 sites are high. High SS contents may be attributed to massive aquaculture feeds, antibiotics excretions of shrimp, and the algae baits in intensive shrimp mariculture mode.

A higher concentration of NH_4^+-N was observed in *Babylonia*, fishes, or shrimp cultivated sites for a long period. Under such circumstances, more bait residue, excrement, and plankton would be produced and accumulated, particularly at the late culturing period. Furthermore, when the drain outlet site is close to a port (i.e., Wenchang Port), the value of NH_4^+-N shows a high trend, which may result from the frequent shipping activities, including water cleaning ships bilges and fuel tanks, which produce large quantities of pollutants such as diesel fuels, oil, grease, food residues, solvents, cleaning agents, cigarette butts, paints, bacteria, and other solid particles (Bilgili et al., 2016).

Among 34 sites, three samplings showed a high level of PO_4^{3-}-P. One of them was a fish culturing, draining accumulated feed residue and fish excreta. While the others were close to a port, the high nutrients might also be attributed to the geographical locations, feedings, and culturing methods, in the same as high NH_4^+-N content, which was mainly affected by various pollution sources around the port.

The highest concentration of COD_{Mn} may be contributed by that outlet receives not only mariculture wastewater but also municipal sewage. Particularly, municipal wastewater is generally with a high concentration of organic pollutants (e.g., COD containing 95.33–126.33 mg/L and SS containing 80–130 mg/L) and ammonia nitrogen (8.7–40 mg/L) (Cheah et al., 2016).

4.2 Effects of Free-celled Microalgae on Nutrients Removal from AWW

In this study, *C. vulgaris*, *P. subcordiformis*, and *C. müelleri* all grew well under the conditions of this experiment. However, the biomass accumulation of microalgae is relatively smaller as compared with other studies in Table 3, and that may be related to the low concentration of nitrogen and phosphorus in AWW, and they are insufficient to support the cell growth of microalgae. A 107.86 mg/(L•d) *Chlorella sorokiniana* biomass was obtained after phototrophic cultured in aquaculture wastewater with NO_3^--N, NO_2^--N, NH_4^+-N, and PO_4^{3-}-P in concentrations of 40.67 mg/L, 5.52 mg/L, 5.32 mg/L, and 8.82 mg/L, respectively (Guldhe et al., 2017). Moreover, the biomass concentration of 3150 mg DCW/L was achieved when *Neochloris oleoabundans* was cultivated in artificial wastewater with sodium nitrate and phosphate concentrations of 140 mg/L and 47 mg/L, respectively (Wang & Lan, 2011). Interestingly, a previous study reported that *C. vulgaris* was cultivated in secondary treated domestic wastewater with a low NH_4^+-N concentration of 0.56 mg/L, but a higher biomass concentration of 1100 mg/L was reached, which may relate to the bubbling with sufficient air in a photobioreactor (Fernández-Linares et al., 2017).

In this study, PO_4^{3-}-P can be completely removed via the cultivation of such monoculture microalgae as

Table 3 Comparison of nutrients removal by different microalgae from the previous study

Microalgal species	Types of wastewater	Initial concentration (mg/L)			Removal efficiency (%)						Reference
		N_0	P_0	N_0/P_0	NH_4^+-N	NO_3^--N	NO_2^--N	T_N	PO_4^{3-}-P	T_P	
Chlorella	Freshwater aquaculture	17.94	6.75	2.66	97.71				49.73		Lananan et al. (2014)
Gracilaria birdiae		-	-	-	34	100			49.73		Marinho-Soriano et al., (2009)
Chlorella sorokiniana		51.31	8.82	5.82	98.21	75.76			100		Ansari et al. (2017)
Scenedesmus obliquus					88.7	77.7			100		
Ankistrodesmus falcatus					86.45	80.85			98.52		
C. vulgaris		6.81	0.42	16.21	46.2			86.1		82.7	Gao et al. (2016)
C. vulgaris	Sewage				53.8				49.6	49.6	Sydney et al. (2011)
C. vulgaris	Mariculture	11	1.5	7.33	100		99.86	98.43	100		In this study
P. subcordiformis							99.46	95.52	100		
C. müelleri							99.68	94.06	100		

C. vulgaris, *P. subcordiformis*, and *C. müelleri*. Moreover, when *C. vulgaris* was continuously cultivated in aquaculture wastewater using a membrane photobioreactor, the PO_4^{3-}-P removal efficiency of 82.7% (Gao et al., 2016). Under autotrophic, heterotrophic, and mixotrophic conditions, almost total nitrogen and phosphorus removals (>99% for both total nitrogen and PO_4^{3-}-P) were achieved by microalgae *C. vulgaris* in centrate wastewater (Ge et al., 2018). Peng et al. (2020) also proved the reduction in dissolved inorganic nitrogen dissolved inorganic phosphorus in marine aquaculture wastewater after being treated by microalgae (i.e., *C. vulgaris*) in a biofilm membrane photobioreactor.

By comparison, *C. vulgaris* showed a relatively faster removal rate of NH_4^+-N than that of *P. subcordiformis,* followed by *C. müelleri*, and this was also confirmed in other studies. For example, monoculture microalgae of *Chlorella* sp. greatly removed NH_4^+-N from aquaculture wastewater of *L. calcarifer*, with an initial concentration of 7 mg/L (Lananan et al., 2014).

Microalgal cells remove nitrogen through direct uptake of inorganic nitrogen such as NH_4^+-N, NO_2^--N, NO_3^--N, from which NH_4^+-N is the easiest to assimilate. However, priority assimilation may apply to some species. For example, when both nitrate and ammonium are provided, only NH_4^+-N is utilized by microalgal cells (Fernández-Linares et al., 2017).

The results obtained in this study showed that three microalgae completely removed NO_2^--N at different removal rates. However, the removal efficiency is comparable to the previous research that *Chlorella sorokiniana* cultured in aquaculture wastewater removed NO_2^--N from 5.52 to 0.2 mg/L after the 7-day cultivation period (Guldhe et al., 2017). Additionally, in the current study, *C. vulgaris* presented the optimal bioremediator despite advantages such as shorter treatment time, higher removal efficiency, and vigorous cultures. In comparison to other nitrogen species, NO_2^--N must be converted into NH_4^+-N in two steps before being assimilated by microalgae. In the first step, NO_3^--N is reduced to NO_2^--N using nicotine-amide adenine dinucleotide phosphate (NADPH) as a reducing agent, and then NO_2^--N is converted to NH_4^+-N using nitrite reductase in a six-electron transfer reaction catalyzed by ferredoxin.

Nutrients removal from mariculture wastewater in this study is comparable to that achieved in previous studies; for instance, all T_N removal efficiencies of three species of *Dunaliella* sp., *Nannochloropsis* sp., and *Tetraselmis* sp. were reached higher than 90% in controlled simulated mariculture wastewater (Sacristán de Alva et al., 2018). The efficient T_N removal is partly attributed to the uptaking of NH_4^+-N and organic nitrogen by microalgal cells under the investigated condition (Cai, Ge, et al., 2013). It may also be related to the fact that abiotic removal processes, such as chemical precipitation and stripping, can decrease T_N (Salama et al., 2015).

4.3 Effects of Microalgal-bacteria Symbiosis on Nutrients Removal in AWW

In this study, the symbiosis of *Bacillus* spp. and *C. vulgaris* promoted both of their growth in mariculture wastewater. Both cooperative and competitive interactions contributed to the outstanding performance despite the ups and downs during the treatment process, which was similar to the findings of Sun et al. (2020). Likewise, in a wastewater treatment study, Qi et al. (2018) reported the dominance of three bacteria, *Xiguobacterium aurantiacum*, *Stenotrophomonas acidaminiphila*, and *Chryseobacterium Scophthalmus species*, in fermentation wastewater treated by microalgae. They found more than 77.8% of NH4$^+$-N, 45.6% of total PO4^{3-}-P in all co-cultures. However, in TN contents, fluctuations occurred in the three pure cultures of bacteria, and the TN concentrations almost did not get reduced. In contrast, excellent removal efficiency was observed in three consortia groups, with TN nearly got completely removed by *Chlorella sorokiniana L3* and *C. scophthalmus*. Muradov et al. (2015) compared the concentrations of nutrients in 25% diluted swine wastewater before and after treatment with *C. protothecoides*, *T. suecica*, and their pellets with *A.fumigatus*. After 48 h of Af/Cp incubation in 25% diluted wastewater, NH_4^+-N and PO_4^{3-}-P concentrations reduced from 164.3 to 43.2 mg/L and from 38.7 to 17.2 mg/L with a corresponding elimination efficiency of 73.9% and 55.6%, respectively. This removal efficiency was higher than the efficiency of NH_4^+-N, and PO_4^{3-}-P removal was achieved separately by *C. protothecoides* (36% and 25%, respectively) and by *A. fumigatus* (46% and 20%, respectively). The microalgae-bacteria consortium showed higher purification efficiency, which was consistent with the current study (Table 2).

Bacteria can enhance the sewage purification effect based on a microalgae monoculture and promote the deposition of sugars and fats in the microalgae cells, thereby significantly improving the wastewater purification function of microalgae (Ramanan et al., 2016). Furthermore, integrating microalgae into activated sludge treatment facilitates the decline of various organic pollutants and inorganic nitrogen species (NH_3, NO_3^-, and NO_2^-) (Leong et al., 2020). Furthermore, up to a particular level, bacteria could be effective in the improvement of NH_4^+-N removal efficiency (Table 1). These findings are in agreement with the previous work, suggesting symbiosis of effective microorganisms (EM-1) and microalgae *Chlorella* sp. as an effective strategy to remove ammonia and phosphorus from aquaculture wastewater (Lananan et al., 2014). Likewise, the removal of PO_4^{3-}-P in microalgal-bacteria symbiosis was consistent with the previous study, which focused on treating L. calcarifer aquaculture wastewater using symbiotic bioremediation *Chlorella* sp. and EM (Lananan et al., 2014). The better cell growth of both microalgae and bacteria and the higher removal of PO_4^{3-}-P in the symbiosis could be explained as follows. First, microalgae can be a habitat for bacteria and protect them from adverse environmental conditions (Mandal et al., 2011). Second, microalgae (e.g., *Amphidinium carterae*) can release extracellular polymeric substances (EPS) to stimulate bacteria (*Bacillus pumilus*) growth (Mandal et al., 2011). Meanwhile, bacteria excreted growth-promoting factors to enhance microalgae generation (Subashchandrabose et al., 2011), and the EPS excreted by microalgae or bacteria surface adsorbed PO_4^{3-}-P from mariculture wastewater via the formation of hydrogen bonds (Li et al., 2013). Then, PO_4^{3-}-P was incorporated into organic compounds (e.g., DNA, RNA, lipids) through phosphorylation, involved in the generation of ATP from ADP (Cai, Park, et al., 2013) or were taken up and stored as intracellular polyphosphate (Schmidt et al., 2016). Moreover, higher pH resulting from microalgal growth would promote the precipitation of PO_4^{3-}-P with calcium and magnesium ions via hydroxyapatite formation (Li et al., 2013; Liu et al., 2017).

The COD_{Mn} removal efficiencies achieved by symbiotic bioremediation of microalgae and bacteria were slightly higher than monoculture *C. vulgaris*. This enhancement could be explained by the fact that

Table 4 Removal efficiency of nutrients in wastewater by different symbioses of microalgae and microbe

	Microbe	Wastewater	Pollutants removal effects (%)			
			TN	TP	COD	Reference
Mixed algae *Chlorella* sp. dominant	*Proteobacteria, Bacteroidetes*	Swine lagoon	-	93	88	Ye et al. (2016)
Chlorella sorokiniana L3	*Stenotrophomonas acidaminiphila*	Simulated fermentation wastewater	80.3	46.7	63.7	Qi et al. (2018)
Chlorella vulgaris	*Aspergillus* sp.	Molasses wastewater	67.09	88.39	70.68	Li et al. (2019)
Chlorella sp.	*Exiguobacterium*	Piggery wastewater	78.3	87.2	86.3	Wang, Jiao, et al. (2020), Wang, Lei, et al. (2020), Wang, Wang, et al. (2020))
Chlorella vulgaris	*Bacillus licheniformis*	Synthetic wastewater	88.9	80.2	86.5	Ji et al. (2018)
Chlorella sp.	*Proteobacteria*	Piggery wastewater	72	100	-	García et al. (2017)
Chlorella vulgaris	*Ganoderma lucidum*	Swine wastewater	74	84	79	Guo et al. (2017)
Chlorella protothecoides	*Aspergillus fumigatus*	10% anaerobically digested swine manure wastewater	93	87	-	Muradov et al. (2015)
Chlorella vulgaris	*Rhodosporidium sphaerocarpum*	Shrimp culture wastewater	86.2 (NH_4^+) 100 (NO_2^-) 100 (NO_3^-)	100 (PO_4^{3-})	100	Luo et al. (2021)
Chlorella sp.	*Effective Microorganisms, EM-1*	Aquaculture wastewater	99.82 ± 1.09 (NH_4^+)	93.07 ± 7.22 (PO_4^{3-})	-	Lananan et al. (2014)
Coelastrella	*Rhodobacteraceae*	Aquaculture wastewater	84.9 (NH_4^+) 50.0 (NO_2^-) 70.8 (NO_3^-)	84.2 (PO_4^{3-})	64.8	Fan et al. (2020)
Chlorella vulgaris	*Mucor indicus*	Synthetic fish wastewater	> 99 (NH_4^+, NO_2^-, NO_3^-)	> 99 (PO_4^{3-})	-	Barnharst et al. (2018)
Chlorella vulgaris	*Mucor indicus*	Synthetic shrimp wastewater	> 99 (NH_4^+, NO_2^-, NO_3^-)	> 99 (PO_4^{3-})	-	Barnharst et al. (2018)
Chlorella sp.	*Pseudomonasputida Flavobacterium*	Artificial wastewater	99.2 (NH_3-N)	83.9	87.3	Wang et al. (2016)
Chlorella pyrenoidosa Chick	*Photosynthetic bacteria*	Aquaculture wastewater	84.5 (NH_4^+)	76 (PO_4^{3-})	-	Liu et al. (2017)
Chlorella vulgaris	*Bacillus* spp.	Synthetic aquaculture wastewater	96.57 (NH_4^+)	89.89 98.62 (PO_4^{3-})	39.09	Ma et al. (2019)

Bacillus spp. not only mineralizes organic carbon but also decomposes some organic matter into forms that are easier to be absorbed by microalgae (Zhang et al., 2012). Besides, CO_2 produced in the metabolism of bacteria can be further utilized as a carbon source by microalgae (Muñoz and Guieysse, 2006). The low removal efficiencies achieved in the current study may be attributed to less nitrogen and phosphorus, which limited cell generation of *C. vulgaris* and *Bacillus* spp., and therefore inhibited the decomposition and absorption of organic matters since high phosphorus concentration (e.g., higher than 6 mg/L) could lead to the explosive growth of microalgae (Ahmad et al., 2011). In addition, previous studies also suggest that micro algae-microbe symbiosis also showed effective performance in the treatment of TN, TP, and OD, as summarized in Table 4.

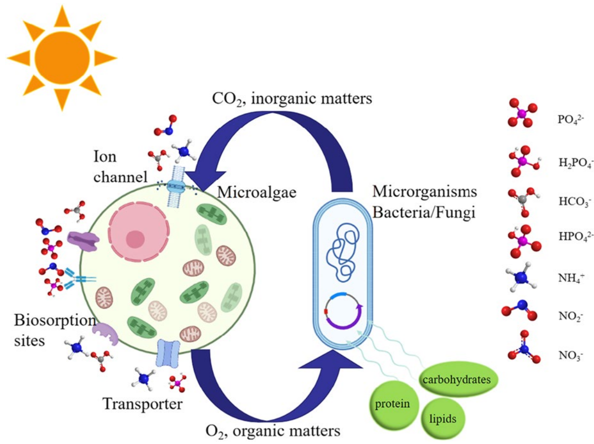

Fig. 7 The mutual interactions between bacteria and microalgae during the process of pollutants removal

4.4 Mechanisms of Microalgae-bacteria Symbiosis System for Nutrients Removal

Biodegradation is one of the major approaches in the microalgae-bacteria symbiosis system during wastewater remediation. Once microalgae and bacteria are co-cultivated in the wastewater treatment system, they would compensate each other with carbon dioxide or oxygen, and also some extracellular chemicals excreted by both of them may trigger or strict their growth which would further influence the efficiency of pollutants removal. Mutual interactions between bacteria and microalgae would occur via biodegradation, bioadsorption, and bioaccumulation during the pollutant removal process, as shown in Fig. 7. In the microalgae-bacteria symbiotic system, microalgae can capture light and nutrients from their surroundings and grow photoautotrophically, heterotrophically, and mixotrophically, facilitating biomass accumulation (polyphosphate, carboxylic acid, and polysaccharides) and decontamination efficiency (Hussain et al., 2021). On the other hand, Bacteria can assimilate organic matter and produce carbon dioxide and other inorganic substances as heterotrophic organisms. Bacterial respiration uses the oxygen produced by microalgal cells during photosynthesis. As a result, organic carbon such as carbohydrates, proteins, polysaccharides, and growth factors that produced to ensure the entire life process (Liu et al., 2017; Wang, Lei, et al., 2020). Simultaneously, inorganic substances produced by bacteria serve as raw materials such as NH_4^+-N, NO_2^--N, and PO_4^{3-}-P for microalgae photosynthesis with the help of enzymes and photosynthetic pigments and are eventually converted into amino acids, lipids, and proteins via nitrification, denitrification, substrate-level phosphorylation, oxidative phosphorylation, or photophosphorylation which can be regarded as a synergistic stage (Chu et al., 2021; Leng et al., 2021; Rahimi et al., 2020; Wang et al., 2014).

Unlike biodegradation, in bioabsorption and bioaccumulation, contaminants (e.g., heavy metals, antibiotics, organochlorines, and pesticides) are accumulated on the surface and or inside living organisms. Therefore, these two processes have become more popular because they are environmentally friendly, effective, and cheap. In addition, EPS secreted by microalgae and bacteria in the system can provide adsorption sites (like covalent bonding, electrostatic neutralization, and molecular interactions) (Kanamarlapudi et al., 2018; Wang et al., 2021). Composed of polysaccharides and proteins with carboxyl, phosphoric, amine, and hydroxyl groups, EPS is supposed to be regarded as a gap for mitigating the toxicity load and protecting the cell membrane (Wang et al., 2021).

5 Conclusions

The field investigation showed that the main culturing method is industrial factory and most of the wastewater is directly discharged into the surrounding sea in Hainan. The results of indoor experiments with monoculture microalgae indicated that nutrient removal efficiency of *C. vulgaris* was higher, followed by *P. subcordiformis* and *C. müelleri*, respectively.

Furthermore, the symbiotic bioremediation of microalgae *C. vulgaris* and bacteria *Bacillus* spp. with C-B5 treatment was the optimal treatment strategy for treating tropical mariculture wastewater.

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Author Contribution Ruiyang Ma contributed to the formal analysis, experiment investigation and original draft. Data curation was performed by Linhai Pu and Huiting Jia. Manuscript review and editing were prepared by Ruiyang Ma, Shiyu Xie, Linhai Pu, Huiting Jia, Licheng Peng and Tariq Mehmood. This study was supervised by Licheng Peng and Chengjun Ge. All authors read and approved the final manuscript.

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Data Availability All the original data was integrated in the file named as Supplementary Information.

Declarations

Ethics Approval This article does not contain any studies with animals performed by any of the authors.

Consent to Participate All the people involved in this work gave their consent to participate.

Consent for publication All the authors have given their consent for the publication of the study.

Conflict of Interest The authors declare no competing interests.

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