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Effects of stocking density on the productivity and nutrient removal of *Agarophyton vermiculophyllum* in *Paralichthys olivaceus* biofloc effluent

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Abstract

Optimal stocking density of the marine red alga *Agarophyton vermiculophyllum* was determined to maximize the productivity and nitrogen removal in biofloc effluent. *Agarophyton vermiculophyllum* was cultured at 0.2, 2, 4 and 8 g L^{-1} (FW) stocking densities, 160 ± 10 μmol photons m^{-2} s^{-1} and 12:12 L:D photoperiod for 20 days. *Agarophyton vermiculophyllum* was cultured in effluent from a juvenile *Paralichthys* biofloc tank culture system and von Stosch–enriched (VSE) medium at 20 °C. The total nitrogen and phosphorus concentration of VSE medium was adjusted to the biofloc level, 1000 μmol L^{-1} and 33 μmol L^{-1}, respectively. Specific growth rate was significantly higher at 0.2, 2, 4 and 8 g L^{-1} in both media. However, the productivity was significantly higher at 8 than 0.2 g L^{-1} in both media. Tissue carbon contents were not significantly influenced by the medium at 8 g L^{-1} (34.9% in VSE and 34.0% in biofloc). However, tissue nitrogen content was significantly higher at VSE medium than at biofloc medium at 8 g L^{-1} (3.7% in VSE and 3.4% in biofloc). The carbon removal rate was highest at the highest stocking density, 1.98 mgC g^{-1} DW day^{-1} (VSE) and 1.89 mgC g^{-1} DW day^{-1} (biofloc), respectively. Also, the nitrogen removal rate was highest at the highest stocking density, 0.21 mgN g^{-1} DW day^{-1} (VSE) and 0.19 mgN g^{-1} DW day^{-1} (biofloc), respectively. The nutrient removal was not significantly influenced by medium at 4 and 8 g L^{-1}. These results show that *A. vermiculophyllum* can grow and have the potential to remove nutrients in the biofloc medium at high nitrogen concentrations.

Keywords *Agarophyton vermiculophyllum* · Rhodophyta · Biofloc · Nitrogen · Stocking density

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Introduction

The annual fish aquaculture production in Korea has increased about 13% in 2017 (86,387 t) compared with 2012 (76,308 t) (KOSIS 2018). The proportion of floating fish cages decreased to 63% in 2017 from 69% in 2012, whereas the proportion of land-based aquaculture has increased from 31 to 34% during the same time (KOSIS 2018). The floating fish cages and land-based aquaculture have faced many serious environmental issues. For example, fish escaping from floating and land-based systems causes environmental problems such as interbreeding of cultivated fish with wild ones (Naylor et al. 2005; Somarakis et al. 2013; Olaussen 2018), potentially becoming invasive species (Volpe et al. 2000; Naylor et al. 2005; Liao et al. 2010) and a source of infectious disease (Naylor et al. 2000; 2001; Krkošek et al. 2006). Apart from this, the floating fish cages accumulate nutrients at the bottom of the farms (Holmer 2010; Ruiz et al. 2010; Olaussen

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2018), whereas land-based farms could discharge nutrient-enriched effluents into local environment (Hanisak 1983; Tello et al. 2010; Hall et al. 2011).

One of the potential solutions to the aforementioned problems is recirculating aquaculture systems (RAS). Recently, some RAS has been replaced by biofloc technology (BFT) requiring zero or minimal water exchange (Schryver and Verstraete 2009). The BFT basically assimilates dissolved inorganic nitrogen (N) (e.g., ammonia, nitrite, and nitrate) into microbial proteins by bacteria in the presence of carbon (C) source (Avnimelech 1999; Azim et al. 2008). Because nitrogen removal rate by microorganisms is faster than nitrification rate, the carbon/nitrogen ratio of this system is controlled to increase microorganism growth rate, i.e., microorganism produces aggregation using carbon source and inorganic N (Hargreaves 2006). In addition, biofloc systems can minimize water exchange rates (0.5 to 1% day^{-1}) and the amount of aquatic animal food (Schryver and Verstraete 2009; Hargreaves 2013). Moreover, microbial proteins made by microorganism can be used as feed for aquatic animals (Avnimelech 2006). The BFT has the potential to reduce annual feed costs up to 15% per kg fresh weight (Schryver et al. 2008). Therefore, the biofloc technology can be an innovative environmentally friendly land-based aquaculture system (Crab et al. 2012; Luo et al. 2014). Previously, the BFT was principally applied to shrimp cultivation (Xu et al. 2012; Schveitzer et al. 2013; Furtado et al. 2014; Kim et al. 2014a; Krummenauer et al. 2014; Brito et al. 2016). The BFT still has challenges to maintain low ammonia- and nitrite-nitrogen levels and be successfully used on a commercial scale. The biofloc system require high oxygenation, i.e., sustainable aeration and sustainable supply of carbon source to keep the microorganism in optimal condition (for nitrification by microorganism) (Hargreaves 2013; Thong 2014). Nitrification resulted in high accumulation of nitrate, whereas high oxygenation disturbs denitrification for removal of high nitrate level (Thong 2014). Moreover, sustainable alkaline supplements are needed to maintain the appropriate pH level in BFT (Hargreaves 2013).

Although the toxic ammonia and nitrite concentrations are low in the biofloc system, the nitrate concentration is extremely high, reaching > 2000 μmol L^{-1} in Korean BFT (Table 1). Nitrate (NO_3^-) is relatively safe but can accelerate increasing disease organisms. High concentration of NO_3^- can also affect immune responses and hematological and biochemical parameters (Tucker 1998). Some marine fish species can survive up to 4862 μmol L^{-1} NO_3–N. However, high concentration of nitrate (e.g., 1621 μmol L^{-1}) negatively influenced growth and survival of common clownfish larvae and juveniles (Frakes and Hoff 1982). High nitrate concentration is also detrimental when the Pacific white shrimp (*Litopenaeus vannamei*) were exposed to > 4800 μmol L^{-1} NO_3–N (Furtado et al. 2014). Therefore, it is important to maintain low NO_3–N concentration in the BFT systems (e.g., < 800 μmol L^{-1}; Tucker 1998).

The red alga *Agarophyton/Gracilaria* is one of the most cultivated genera of seaweeds in the world. Cultivation of *Agarophyton/Gracilaria* exceeds 4.0 million tonnes with nearly an economic value of US$ 1.7 billion (Kim et al. 2014b, 2017; FAO 2018). This alga is used as feed for animals (Qi et al. 2010; Johnson et al. 2014) and also is used as main source of food-grade agar, which is accounted for approximately 66% of the world agar production (Pereira and Yarish 2008; Rocha et al. 2019). Additionally, *Agarophyton/Gracilaria* has gained much attention in the field of environmentally friendly aquaculture because of its high nutrient removal capacity (Yang et al. 2016; Zhou et al. 2006; Marinho–Soriano et al. 2009; Huo et al. 2012; Kim and Yarish 2014; Kim et al. 2014b, 2017).

Agarophyton vermiculophyllum (formerly known as *Gracilaria vermiculophylla*) is native to Korea (Kim et al. 2010; Gorman et al. 2017) and an invasive species in North America and Europe (Freshwater et al. 2006; Hammann et al. 2008; Weinberger et al. 2008; Kim et al. 2016). This alga also provides ecosystem services by providing shelter and feed to other organisms, and nutrient bioextraction (Wallentinus and Nyberg 2007; Thomsen et al. 2010; Byers et al. 2012; Kim et al. 2014b; Rose et al. 2015). Additionally, this species is highly tolerant to a wide variety of environmental parameters. For example, *A. vermiculophyllum* can grow in a wide range of salinities (5 to 50 psu) (Wu et al. 2018) and temperatures (5 to 34 °C) (Nejrup et al. 2013; Gorman et al. 2017). Additionally, recent research on the cultivation of *A. vermiculophyllum* in aquaculture effluent showed that *A. vermiculophyllum* has a high potential for nutrient bioextraction (Nejrup et al. 2013; Barceló–Villalobos et al. 2017; Gorman et al. 2017).

There have been a few studies on seaweed cultivation in the BFT systems. For example, *Litopenaeus vannamei* was co-cultivated with *Gracilaria* sp. (Sánchez-Romero et al. 2013; Brito et al. 2014a; 2018) and *Ulva* sp. (Brito et al. 2014b) in the BFT. However, none of these studies were conducted to see the nutrient removal rate of macroalgae, independent from the biofloc system. In this study, we cultivated *A. vermiculophyllum* in an independent land-based culture system using biofloc effluent. The goals of this study were to determine optimal stocking density for efficient nutrient removal by *A. vermiculophyllum* in biofloc effluent and to determine the nitrate removal capacity in biofloc effluent.

Table 1 Water quality of biofloc technology systems in previous studies

Cultivation species	Dissolved Inorganic nitrogen			Cultivation environments		References
	TA–N (μmol L^{-1})	NO_2–N (μmol L^{-1})	NO_3–N (μmol L^{-1})	Salinity (psu)	Temperature (°C)	
Litopenaeus vannamei	2.6–4.6	8.3–8.7	52.5–74.9	35.8–36.7	26.0–28.1	Brito et al. 2016
Litopenaeus vannamei	0.0–127.4	0.0–672.5	0.0–1231.8	26.0–28.0	27.2–29.1	Furtado et al. 2014
Litopenaeus vannamei	5.5–66.5	8.7–57.2	2833.1–4398.7	32.2–33.8	27.5–29.2	Kim et al. 2014a
Litopenaeus vannamei	0.0–61.5	30.8–882.3	0.0–375.7	34.3–36.5	27.3–30.9	Krummenauer et al. 2014
Litopenaeus vannamei	0.0–221.6	0.0–109.2	1134.5–3241.5	32.0–34.6	28.6–30.5	Schveitzer et al. 2013
Litopenaeus vannamei	0.0–26.6	0.2–19.9	0.3–64.5	31.2–33.2	23.6–27.1	Xu et al. 2012
Paralichthys olivaceus	17.7–68.1	6.6–39.3	810.4–2431.1	30.3–35.0	20.2–24.2	Kim et al. 2018
Penaeus monodon	3.3–7.8	0.4–14.0	8.1–2.4	13.10–15.40	28.8–32.50	Shyne Anand et al. 2017

Materials and methods

Algal material and culture

A strain of *Agarophyton vermiculophyllum* (GV-KR-ST1) was used in this experiment. This strain was originally collected from Byunsan, Jeonbook, Korea. This strain was cultured at Marine Ecology and Green Aquaculture Laboratory, Incheon National University, Korea, for more than 2 years. Prior to the experiment, *A. vermiculophyllum* was acclimated to biofloc and von Stosch–enriched (VSE) medium for 5 days. *Agarophyton vermiculophyllum* was cultivated in VSE medium (Ott 1965) and juvenile *Paralichthys olivaceus* biofloc effluent collected from the National Institute of Fisheries Science (Taean, Chungnam, South Korea).

In this biofloc system, 500 fish (*Paralichthys olivaceus*) with an average weight of 296.4 ± 31.7 g were cultivated in a 12-m^3 tank. Glucose was supplied to the system as a carbon source. The fish were fed at 2% of total body weight using extruded pellets 4 times per day. The C:N ratio in the biofloc effluent was maintained below 10 (Kim et al. 2018).

The nitrogen concentration in biofloc was different each time when the fresh effluent was replaced every 5 days, and the concentration was 1000 to 3000 μmol L^{-1}. The phosphorous concentration in biofloc was rather consistent 30–40 μmol L^{-1}. The total nitrogen and phosphorus concentrations in VSE medium was adjusted to the biofloc level, 1000 μmol L^{-1} and 33 μmol L^{-1}, respectively (Table 2). To prepare VSE medium, VSE stock solution was added to ambient seawater which was pumped from the Taean coastal water. Biofloc effluents and the seawater were filtered using a series of cartridge filters (Filter Tech, BDM-250, Daejeon, South Korea; pore size, 10, 5, 1, and 0.1 μm). The salinity of both media was maintained at 35 psu.

Experimental design

Agarophyton vermiculophyllum was cultured in 2.5-L transparent acrylic cylinders (diameter = 13 cm, height = 25 cm) at 20 °C, 35-psu salinity and 12:12 L:D photoperiod for 20 days. Light was supplied by white light–emitting diode lamps (Crystal lighting, CL-1200, Seosan, South Korea). The LED lamps were located at above and below the cylinders, and photosynthetically active radiation (PAR) was measured at the water surface using a light meter (MQ-200 Quantum Separate Sensor with Handheld Meter, USA). PAR was 160 ± 10 μmol photons m^{-2} s^{-1}.

Agarophyton with four different stocking densities, 0.2, 2, 4, and 8 g L^{-1}, respectively, was cultured in two different media (VSE and biofloc). These stocking densities were selected based on a previous study on *Chondrus* and *Palmaria* (Kim et al. 2013). A randomized block design was applied to avoid positional effects in each block. VSE and biofloc medium without algae were cultured to observe the changes of nutrient concentrations due to factors other than algae in the medium under the same conditions. Aeration was provided. The culture medium was changed every 5 days.

Calculations of growth parameters

Fresh weight (FW) of algae in each culture was measured every 5 days over 20 days. *Agarophyton* was cleaned with distilled water prior to weight measurement. Each condition was restored to the initial stocking density. The specific growth rate (SGR) was determined by the following formula:

$$\mathrm{SGR}\left(\%\mathrm{day}^{-1}\right) = \frac{(\ln S_2 - \ln S_1)}{(T_2 - T_1)} \times 100$$

where S_1 and S_2 are the initial and final FW at T_1 and T_2, respectively.

Table 2 Nitrogen and phosphorus concentrations and C:N and N:P ratio in VSE and the media used in the present study

	VSE (Ott 1965)	VSE (present study)	Biofloc (present study)
Nitrate (μM)	500	1000	1000~3000
Phosphorus (μM)	30	30	33
C:N ratio	NM	NM	Below 1:10
N:P ratio	1:17	1:33	1:30~1:91

NM, not measured

Tissue carbon and nitrogen analysis

Agarophyton vermiculophyllum was collected from each condition every 5 days over 20 days to measure tissue carbon and nitrogen content. Samples were oven-dried at 60 °C to constant weight, then ground to powder using MM400 ball mill (Retsch, Germany). Tissue carbon and nitrogen contents were analyzed by using a CHN analyzer (Series II, CHNS/O 2400 Analyzer; Perkin Elmer Inc., USA). Carbon and nitrogen removal rate was calculated using the following equations:

$$\text{C (or N) removal } \left(\text{mg C (or N) L}^{-1}\text{day}^{-1}\right) = \frac{(B_t - B_0) \times \text{tissue C (or N)}}{t} \times \frac{\text{DW}}{\text{FW}}$$

where B_0 and B_t are biomass (in gram) at day 0 and day t. Tissue C (or N) is tissue C (or N) (percent DW) on day t.

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics 25 (IBM, USA). Repeated measures two-way ANOVA ($\alpha = 0.05$) was used to determine the effects of medium and stocking density on SGR, productivity, tissue C and N, C:N, and C and N removal. Tukey's honestly significant difference (HSD) analysis was used as post hoc test to make pairwise comparisons of treatment means. A regression was used to determine relationships between stocking density and SGR, productivity, or carbon and nitrogen removal.

Results

Specific growth rate and productivity

Specific growth rate was significantly influenced by medium, stocking density ($p < 0.001$) and by the interaction of these two factors ($p = 0.001$) (Table 3). Stocking density significantly influenced the specific growth rate in both media. The stocking density of 0.2 g L^{-1} showed the highest specific growth rate in comparison with other stocking densities in both media. At the same stocking density, growth rate was higher in VSE medium (17.4% day^{-1}) than that in biofloc (12.7% day^{-1}). The lowest growth rate was observed at 8 g L^{-1} stocking density in both media (3.2% day^{-1}). The growth rate at 2 g L^{-1} stocking density (9.4% day^{-1}) was significantly higher than that at 4 and 8 g L^{-1} stocking densities in VSE. In case of biofloc, there was no significant difference at different stocking densities except 0.2 g L^{-1} (Fig. 1a).

Productivity also was significantly influenced by medium ($p = 0.025$), by stocking density ($p < 0.001$), and by the interaction of these two factors ($p = 0.008$) (Table 3). In contrast to the specific growth rate, productivity at 0.2 g L^{-1} stocking density was the lowest in comparison with other stocking densities at both media (Fig. 1b). Productivity at 0.2 g L^{-1} stocking density was 0.04 to 0.05 g FW L^{-1} day^{-1}, and no significant differences were observed in both media. The lowest productivity was observed at 4 and 8 g L^{-1} stocking densities (0.28 g FW L^{-1} day^{-1}), and no significant differences were observed in between media. Productivity in VSE medium (0.24 g FW L^{-1} day^{-1}) was higher than that in biofloc (0.14 g FW L^{-1} day^{-1}) at 2 g L^{-1} stocking density (Fig. 1b).

Tissue carbon and nitrogen and C:N ratio

Tissue carbon content was significantly influenced by medium ($p = 0.017$), whereas stocking density ($p = 0.166$) and the interaction of medium and stocking density ($p = 0.22$) did not affect tissue carbon content (Table 3). Tissue carbon content was ranged from 34.5 to 35.1% DW in VSE medium, while it was 33.0 to 34.7% DW in biofloc (Fig. 2a).

Tissue nitrogen content was also significantly influenced by medium ($p < 0.001$), whereas stocking density ($p = 0.847$) and the interaction of medium and stocking density ($p = 0.506$) did not affect tissue nitrogen content (Table 3). The tissue nitrogen content at 8 g L^{-1} (3.7% DW) in VSE medium was significantly higher than that at 4 (3.4% DW) and 8 g L^{-1} (3.4% DW) in biofloc medium (Fig. 2b). In other stocking densities, tissue nitrogen contents were not significantly influenced by medium. Tissue nitrogen content was ranged from 3.6 to 3.7% DW in VSE medium, while it was 3.4 to 3.5% DW in biofloc medium (Fig. 2b). The C:N ratio was ranged from 9.4 to 9.9 in VSE medium and 10.1 to 10.2 in biofloc medium (Fig. 2c). No significant differences were observed in different stocking densities.

Carbon and nitrogen removal

Carbon removal was significantly influenced by stocking density ($p < 0.001$), whereas medium ($p = 0.053$) and interaction ($p = 0.075$) of these two factors did not affect carbon removal (Table 3). The pattern of carbon removal in both media exhibited a similar trend, with the lowest carbon removal at lowest stocking density and highest removal at higher stocking densities (4 to 8 g L^{-1}) (Fig. 3a). The highest carbon removal was recorded in VSE (from 1.60 to 1.98 mg C g^{-1} DW day^{-1}) and in biofloc (1.67 to 1.89 mg C g^{-1} DW day^{-1}) at 4 and 8 g L^{-1} stocking densities (Fig. 3a). The lowest carbon removal was observed at 0.2 g L^{-1} stocking density, 0.40 mg C g^{-1} DW day^{-1} in VSE medium and 0.26 mg C g^{-1} DW day^{-1} in biofloc medium (Fig. 3a).

Nitrogen removal was significantly influenced by medium ($p = 0.004$) and stocking density ($p < 0.001$), whereas the interaction ($p = 0.072$) of these two factors did not affect nitrogen removal (Table 3). Like carbon removal, both media exhibited a similar pattern with lowest nitrogen removal at lowest stocking density and highest removal at higher stocking densities (Fig. 3b). Highest nitrogen removal in VSE was observed at 2 to 8 g L^{-1} stocking densities, from 0.17 to 0.21 mg N g^{-1} DW day^{-1}. In the case of biofloc, the highest nitrogen removal was observed at 4 and 8 g L^{-1} stocking densities, from 0.16 to 0.19 mg N g^{-1} DW day^{-1}. The lowest nitrogen removal was recorded at 0.2 g L^{-1} stocking density, 0.04 mg N g^{-1} DW day^{-1} in VSE and 0.03 mg N g^{-1} DW day^{-1} in biofloc (Fig. 3b).

Linear regression analysis

Linear regression analysis was used to determine relationships between stocking density and growth rate, productivity, or carbon and nitrogen removal. Specific growth rate revealed a significant negative relationship with stocking density in both media (Fig. 4a), while positive relationships were found for productivity and carbon and nitrogen removal (Fig. 4b–d).

Discussion

Specific growth rates were higher at a lower stocking density while productivity and nutrient removal were highest at higher stocking densities. The average specific growth rate at the lowest density (0.2 g L^{-1}) was significantly higher than other densities. However, the optimal stocking density in terms of productivity was observed at higher stocking densities, i.e., 2 to 8 g L^{-1} at VSE medium and 4 to 8 g L^{-1} in biofloc. Kim and Yarish (2014) also reported a similar growth and productivity pattern in *Gracilaria tikvahiae*, higher growth rate at a low stocking density (0.5 g L^{-1}) and higher productivity at higher

Table 3 Results of analysis of variance examining the effects of medium (VSE and Biofloc) and different stocking densities (0.2, 2, 4, and 8 g L^{-1}) on specific growth rate (SGR), productivity, tissue C and N contents, C:N ratio, and C and N removal. Significant differences are shown in italics with *p* values

Source		Medium	Stocking density	Medium × stocking density	Error
SGR	df	1	3	3	86
	F	12.947	123.187	4.765	
	Sig.	*0.001*	*< 0.001*	*0.004*	
Productivity	df	1	3	3	86
	F	5.24	71.584	4.152	
	Sig.	*0.025*	*< 0.001*	*0.008*	
Tissue carbon	df	1	3	3	87
	F	5.89	1.733	1.502	
	Sig.	*0.017*	0.166	0.22	
Tissue nitrogen	df	1	3	3	87
	F	21.719	0.27	0.785	
	Sig.	*< 0.001*	0.847	0.506	
C:N	df	1	3	3	87
	F	15.262	0.242	0.207	
	Sig.	*< 0.001*	0.866	0.891	
Carbon removal	df	1	3	3	84
	F	3.852	49.417	2.386	
	Sig.	0.053	*< 0.001*	0.075	
Nitrogen removal	df	1	3	3	84
	F	8.873	54.063	2.413	
	Sig.	*0.004*	*< 0.001*	0.072	

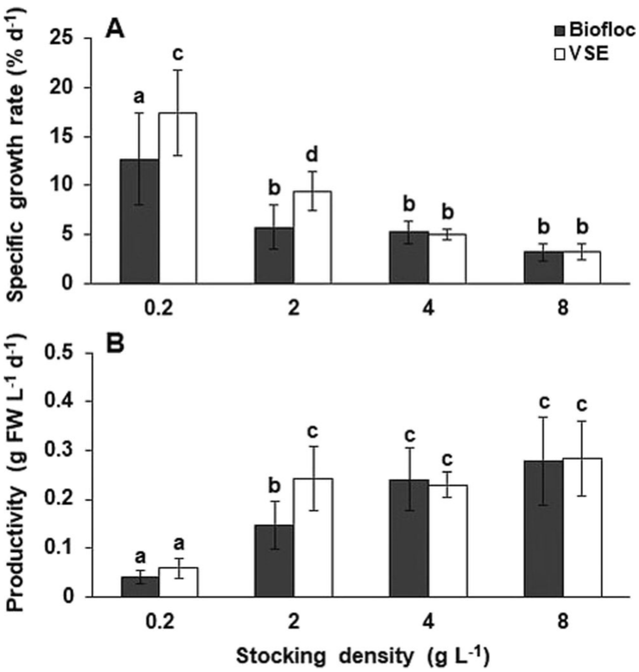

Fig. 1 Specific growth rate (**a**) and productivity (**b**) of *Agarophyton vermiculophyllum*. Each coordinate is the overall mean (SD) of three replicates measured every 5 days for 3 weeks. The means sharing the same letter within each medium–stocking density combination are not significantly different ($p > 0.05$)

stocking density (10 g L^{-1}). In another study, *G. parvispora* was cultivated at different stocking densities (2, 4, and 8 g L^{-1}) and the higher relative growth rate was recorded at 2 g L^{-1}, whereas higher production (kg cage^{-1}) was recorded at the highest stocking density, 8 g L^{-1} (Nagler et al. 2003). These results suggest that higher stocking density may cause a decrease in photosynthesis and nutrient uptake through self-shading, therefore a lower growth rate (Demetropoulos and Langdon 2004).

In addition to the stocking density, the water transparency affects the growth. Though the PAR of this study was maintained at 160 ± 10 μmol photons m^{-2} s^{-1} at the water surface, biofloc medium is opaque compared with VSE. Therefore, *A. vermiculophyllum* cultured in biofloc might not receive the same amount of PAR as VSE. The growth rate of *A. vermiculophyllum* at 20 °C was higher at 150 to 250 μmol photons m^{-2} s^{-1} than at 100 μmol photons m^{-2} s^{-1} (Nejrup et al. 2013). Also, *Gelidiella acerosa* showed significantly higher photosynthetic activity at 500 μmol photons m^{-2} s^{-1} than 100 μmol photons m^{-2} s^{-1} (Fujimoto et al. 2014). Nitrate uptake also depends on PAR because of the utilization of photophosphorylation ATP in active transport (Falkowski and Stone 1975). Therefore, an additional study is required to determine the optimum PAR for *A. vermiculophyllum* in a biofloc medium. If light was limited at the biofloc condition in the present study, the growth rate and nitrogen uptake of *A. vermiculophyllum* will probably increase if higher PAR is provided.

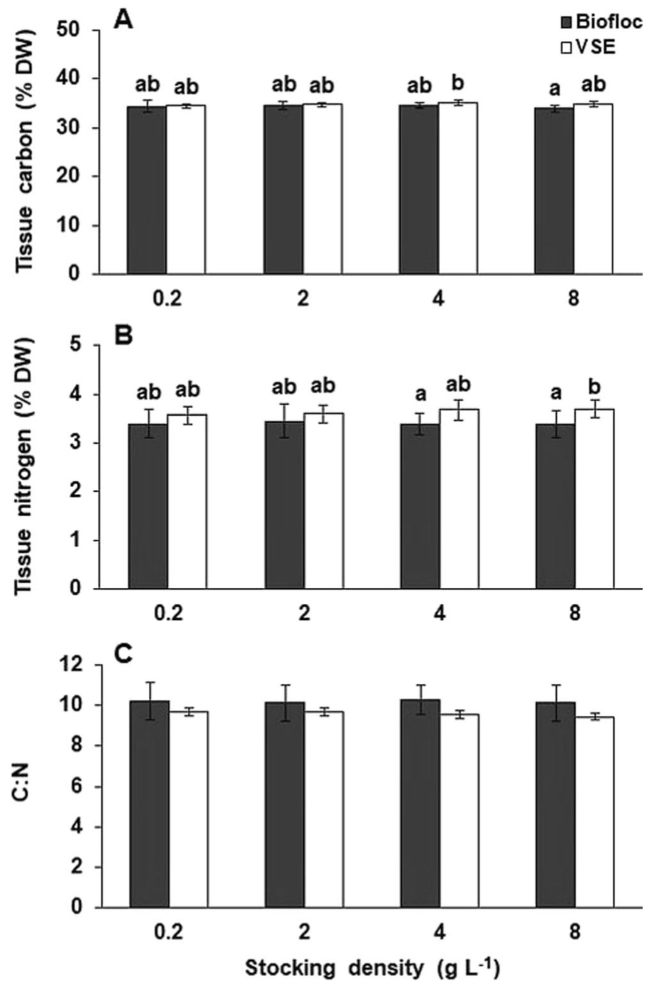

Fig. 2 Tissue carbon (**a**) and nitrogen (**b**) contents, and C:N ratio (C) in *Agarophyton vermiculophyllum* cultivated at different stocking densities (0.2, 2, 4, and 8 g L^{-1}) and medium (VSE and biofloc). Each coordinate is the overall mean (SD) of three replicates measured every 5 days for 3 weeks. The means sharing the same letter within each medium–stocking density combination are not significantly different ($p > 0.05$)

Tissue nitrogen content was not significantly affected by stocking density at both media. However, nitrogen removal rate was significantly higher at higher stocking densities (VSE, 2, 4, and 8 g L^{-1}; Biofloc, 4 and 8 g L^{-1}) than lower stocking densities (VSE, 0.2 g L^{-1}; Biofloc, 0.2 and 2 g L^{-1}). In this study, a positive relationship between N removal rate and productivity was observed in both media. The average tissue nitrogen content was 3.6 to 3.7% DW at VSE and 3.4 to 3.5% DW at biofloc. This value is similar to the tissue nitrogen content of *Agarophyton/Gracilaria* cultivated at VSE in other studies (2 to 4% DW) (Gorman et al. 2017; Kim et al. 2014b).

Abreu et al. (2011) cultivated *A. vermiculophyllum* in diluted fish effluent (nitrate, 117.0 μmol L^{-1}; total ammonium, 121.9 μmol L^{-1}; orthophosphate, 19.0 μmol L^{-1}) and reported that N content in *A. vermiculophyllum* was approximately 6% DW. This value was higher than the initial N value (5% DW).

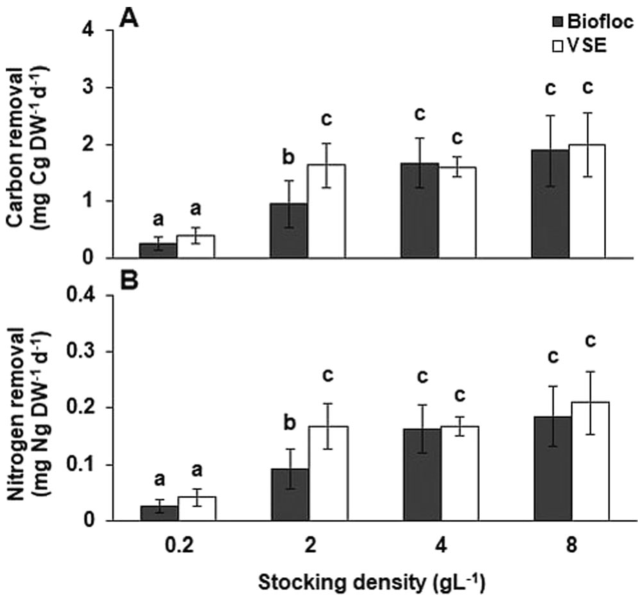

Fig. 3 Carbon (**a**) and nitrogen removal (**b**) of *Agarophyton vermiculophyllum* cultivated at different stocking densities (0.2, 2, 4, and 8 g L^{-1}) and medium (VSE and biofloc). Each coordinate is the overall mean (SD) of three replicates measured every 5 days for 3 weeks. The means with different letters indicate a significant difference ($p < 0.05$) at each medium and stocking density regime combination

It is expected that *A. vermiculophyllum* cultivated at nitrogen-enriched medium has a higher tissue N content than at lower N concentration medium. Although this study used higher N concentration media, the tissue N contents in the present study was lower than those reported in Abreu et al. (2011). This result is probably due to P limitation in both media.

In the present study, *A. vermiculophyllum* efficiently removed nitrate in biofloc effluent at 4 and 8 g L^{-1} stocking densities. It is calculated that, in a 100-t hypothetical biofloc system with 3000 μmol L^{-1} of nitrate, approximately 200 kg of *A. vermiculophyllum* can remove 5% of nitrate within 1 week. This suggests that *A. vermiculophyllum* can be integrated into a BFT system. In this recirculation biofloc system, nitrate in biofloc effluent can be removed by *Agarophyton*, and the seawater with lower nitrate can be returned to the fish cultivation tanks.

This study was the first attempt to measure the nutrient removal rate and growth rate of *A. vermiculophyllum* in a biofloc system. Recently, seaweeds have been integrated with whiteleg shrimp BFT systems (Brito et al. 2014a, b; 2018). For example, *Ulva* reduced inorganic nitrogen (TA–N by 25.9%, NO_2–N by 72.8%) and phosphate (PO_4^{3-}–P by 24.6%) in the BFT systems as compared with control (BFT system only) (Brito et al. 2014b). Also, when *Crassiphycus birdiae* (previously known as *Gracilaria birdiae*) was cultivated in a BFT system, this alga reduced 19–34% of DIN and 19–38% of NO_3–N in comparison with the control (BFT system only) (Brito et al. 2018). The growth rate of shrimps was higher in the shrimp- and seaweed-BFT system than in the shrimp-only system (Brito et al. 2014b, 2018). The findings from these studies along with the present study suggest that seaweed can be integrated with shrimp or flounder BFT systems and can improve the water quality in the systems.

Biofloc effluent contains extremely high concentrations of nitrogen. The nitrogen concentration in biofloc was different each time when the fresh effluent was replaced, but the

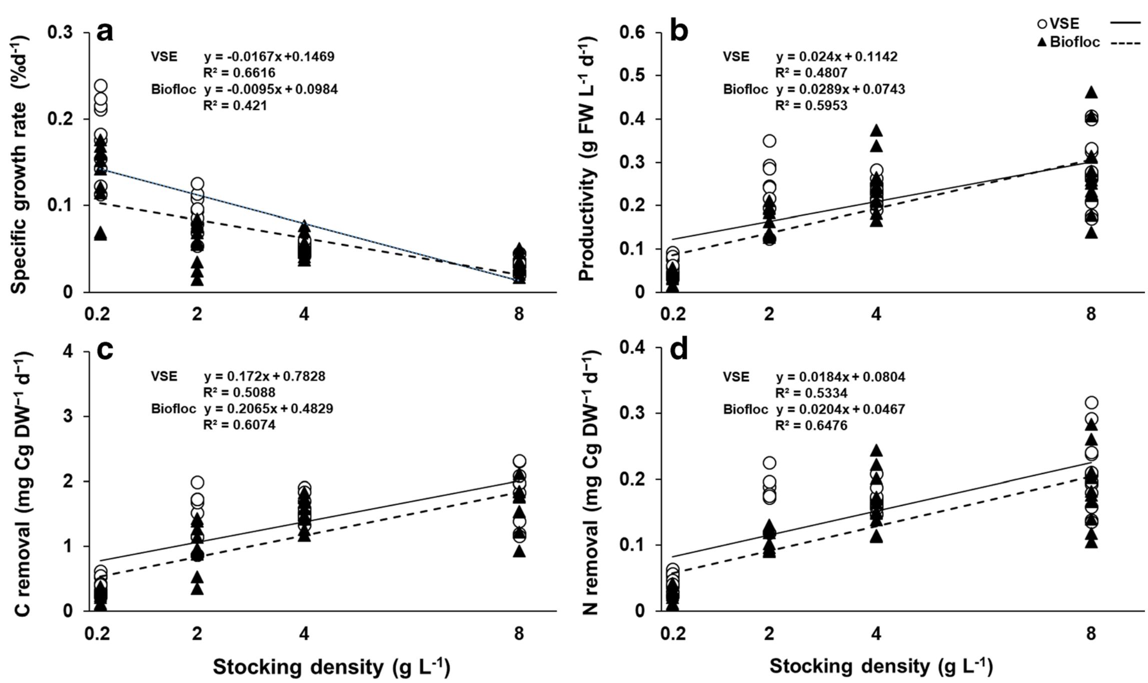

Fig. 4 Linear regression graphs to determine relationships between stocking density and SGR (**a**), productivity (**b**), or carbon (**c**) and nitrogen removal (**d**)

concentration was 1000 to 3000 μmol L^{-1}. The phosphorous concentration in biofloc was rather consistent 30–40 μmol L^{-1}. Therefore, the nitrate and phosphorus concentrations of VSE were adjusted to 1000 and 33 μmol L^{-1}, respectively. The nitrate variations between the biofloc media during the culture period probably did not affect the result in this study. The limiting nutrient in both media should be phosphorus, and the phosphorus in both media conditions was similar throughout the experiment. VSE and biofloc used in this study had N:P ratio of 30:1 and ~ 91:1, respectively. The optimal N:P ratio for *Agarophyton/Gracilaria* was demonstrated at 10:1 (Navarro-Angulo and Robledo 1999). Similarly, Abreu et al. (2011) reported that *A. vermiculophyllum* cultivated at N:P ratio of 13:1 showed the highest tissue N content (7.8% DW). Lapointe (1987) also reported that growth rate and photosynthesis of *G. tikvahiae* were higher at the condition of low N–high P (9:1) and high N–high P (18:1) than at condition of high N–low P (35:1) and low N–low P (18:1). Although the N:P ratio of high N–high P and low N–low P was the same, 18:1, the actual concentration of P in these media were different (i.e., high N–high P, 40 μmol L^{-1} of P; low N–low P, 20 μmol L^{-1} of P). Therefore, *Agarophyton/Gracilaria* requires higher *P* values, and P can be a limiting factor for the growth of *Agarophyton/Gracilaria*. The same phenomenon may have also occurred in the present study.

In biofloc, bacteria tend to congregate and make floc about 0.1 mm to several millimeters in diameter (Avnimelech 2009). The biofloc medium used in the present study was filtered through cartridge filters down to 0.5 μm pore size. Therefore, the role of microbes in biofloc in *Agarophyton* cultivation remains unknown. Generally, macroalgae are known to provide microbial habitats in marine ecosystem and to secrete a variety of organic substances necessary for bacterial growth and microbial biofilms formation (Steinberg et al. 2002; Staufenberger et al. 2008; Singh et al. 2013). It has also been demonstrated that nitrogen-fixing bacteria inhabiting the macroalgal surface induced the formation of morphogenesis and macroalgal growth (Chisholm et al. 1996; Matsuo et al. 2005). There might be some positive interactions between *A. vermiculophyllum* and microorganisms in biofloc, enhancing nutrient removal capacity. For example, Singh et al. (2011) reported that microorganisms induced regeneration of new branches of *A. vermiculophyllum* and, therefore, possibly increasing growth and nutrient removal efficiency. Cultivating *Agarophyton/Gracilaria* together with shrimps in a biofloc system was recently conducted, and this system enhanced the growth of shrimp and reduced cyanobacteria density (Brito et al. 2014a, b).

The required temperature for the growth of *Paralichthys olivaceus* and *Litopenaeus vannamei* in BFT are 20 to 24 °C and 27 to 29 °C, respectively (Kim et al. 2014a; 2018). These two species are commonly cultivated in BFT systems in Korea. Recent studies indicate that *A. vermiculophyllum* grew well in these temperature ranges (Kim et al. 2016; Gorman et al. 2017; Park et al. 2018), suggesting that *A. vermiculophyllum* could be a good species to be integrated in BFT systems in Korea.

The findings from this study suggest that *A. vermiculophyllum* can grow well and has the potential to remove nutrients in the medium of high opacity and high nitrogen concentrations (i.e., 3000 μmol L^{-1}). The optimal stocking density for efficient nitrogen removal by *A. vermiculophyllum* in biofloc effluent was recorded at 4 and 8 g L^{-1}. However, because a biofloc medium is opaque, and because the biofloc medium used in this experiment was filtered, additional experiments with high PAR and unfiltered biofloc effluent will be needed to determine an optimal light condition and bacteria effect on the growth and nutrient bioextraction capacity of *A. vermiculophyllum*.

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