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Utilization of Microalgae in Aquaculture System: Biological Wastewater Treatment

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Abstract

We recently developed an autotrophic biofloc technology (ABFT) system entailing simultaneous microalgae co-culturing with juvenile-farming-stage fish and shrimp in aquaculture and microalgae-based water treatment. The present study was conducted to confirm the potentialities of the ABFT system at the remaining stages (seedling to adult farming, Nile tilapia) for industrial-level implementation. In the results at the seedling stage, an excellent water-purification effect and significant water conservation (97% reduction) by microalgae were verified. Indeed, among the fish, there were not any significant differences, either in growth performance or in body composition, and the wastewater from this system was recycled by use for the growth of various plants. Further, the ABFT system was demonstrated to have a positive effect on production economics by simplifying the production process steps (simultaneous fish breeding and wastewater treatment) and providing for a natural hatching environment. In summary, the ABFT system can be integrated with existing systems on an industrial level as an effective and efficient means of achieving sustainable aquaculture.

Keywords:

Microalgae;
Biological Water Treatment;
Natural Production Environment;
Sustainable Aquaculture System.

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1- Introduction

Over the past few decades, water pollution has emerged as an important environmental issue alongside the longstanding water-scarcity problem; thus, concern for wastewater management has grown concomitantly. Contamination of large bodies of freshwater, principally by heavy metals, persistent organic pollutants (POP), eutrophication, sewage, and acidification, all due mainly to exponential human population increase and rapid industrialization, makes water unsuitable for human use [1-4]. One of the major problems encountered in efforts to reduce water pollution is nutrient enrichment, mainly by phosphorus and nitrogen. The biological processes conventionally utilized to remove such elements from wastewater entail anaerobic digestion that is followed by denitrification as well as nitrification of bacteria [5, 6]. Unfortunately, such methods require large energy inputs for mechanical aeration along with chemical/ physical technologies involving coagulation-precipitation and adsorption utilizing ion salts, which processes incur prohibitive operating costs and offer only mediocre nutrient-removal efficiencies [7, 8].

To overcome the drawbacks that are associated with conventionally employed treatment methods, alternative methodologies that use autotrophs, for example microalgae and macroalgae (which are excellent at heavy metals absorption and the reduction of both chemical oxygen demand [COD] and biochemical oxygen demand [BOD] while remaining resilient to highly concentrated organic wastewaters' toxic contaminants), have been extensively studied [1, 9-17]. Utilization of microalgae with their high capacities for uptake of organic/inorganic nutrients from wastewater

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(80–100%) has been reported in the fields of agriculture [18, 19], industry [2, 20-24], livestock [25] and aquaculture [26-29]. Recent research in the USA, Australia, Mexico, Italy, Belgium, Brazil, South Korea, China, and Taiwan has focused on the problem of efficient industrial-level microalgae cultivation and simultaneous wastewater treatment [30, 31].

Notwithstanding the many studies on microalgae-based water treatment that have already been published, applications involving microalgae/animal co-culturing have gone unreported. In our previous study exploring the aquaculture potentialities of autotrophic biofloc technology (ABFT) for microalgae (*Scenedesmus obliquus* and *Chlorella vulgaris*) and fish (Nile tilapia, *Oreochromis niloticus*) at the juvenile farming stage (60 – 125 g)[32], improved immune response by microalgae was demonstrated, with no statistically significant disparities in fish survival or growth or indeed of body composition. The results demonstrated, moreover, better cost effectiveness in the form of an 82%-reduced water exchange (46,000 L) as compared with the controls (50% water replacement daily), which established that *Scenedesmus obliquus* and *Chlorella vulgaris* can be employed effectively as sources of alternative amino acids for tilapia growth.

The present study, accordingly, set out to confirm the remaining possibilities from the seedling (after hatching, 0.02 g) to the adult farming stage (> 300 g) for industrial-level implementation. We focused on microalgae's effects on water-degradation efficiency and the growth performance of fish; wastewater utilization as an insertion experiment.

2- Materials and Methods

2-1- Strains and Growth Medium

Chlorella ellipsoidea (FBCC180008) and *Scenedesmus dimorphus* (FBCC110009), both acquired from the NNIBR (Nakdonggang National Institute of Biological Resources, Republic of Korea), were mixed in ten sets of cylindrical 10L plastic bottles for cultivation. SBG11 (10%-seawater-supplemented BG11) culture medium [33] with a constant 1%-CO_2 (v/v)-enriched air flow (250 mL/min) at 28°C under continuous illumination at a light intensity of 100 µmol photons $m^{-2}s^{-1}$ *(LI-189 Li-Cor, PAR quantumsensor, Lincoln, USA)* was used for strain maintenance. Preparatory to measurement of the microalgae's dry weight, the cells were filtered through filter paper (GF/C glass-fiber) and dried at a temperature of 60°C for 24 h, and subsequently, the filter paper's final weights were compared with the initial ones. Each measurement was performed in triplicate. Juvenile tilapia were purchased from a local fish farm (Changnyeong, Republic of Korea). The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Pukyong National University.

2-2- Water-degradation Experiment by Microalgae

The experiment was performed for 14 days. There were three treatment groups, as follows: 50% (50% water replacement daily); 0% (no water replacement); ABFT (no water replacement/including *Chlorella ellipsoidea* and *Scenedesmus dimorphus*). To 3 plastic tanks (150 L capacity), 100 L of freshwater was added. In each ABFT tank, the average microalgal concentration for the inoculum was 0.103 g L^{-1}. Illumination at a light intensity of 57 µmol photons $m^{-2}s^{-1}$ was measured on the surface of the water (16-h light, 8-h dark). Tanks were randomly assigned in triplicate, and each was stocked with 30 tilapia (15.10 ± 0.04 g/ total 453.00 ± 1.33 g). During the experiment, the fish were provided with a commercial diet consisting of 45% protein and 9% lipid (Jeil Feed Co., Hamman, Korea). They were fed 5 times per day (9:00, 12:00, 15:00, 18:00 and 21:00) at 8% of wet body weight. The oxygen level was maintained (> 5 mg L^{-1}) by continuous aeration from air stones positioned on the tank bottoms, and the water temperature was kept at a constant 26°C.

2-3- Water-quality Analysis

The supernatant was sent through a 0.2 µm syringe filter, and the total ammonia nitrogen (TAN), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were quantified by ion chromatography (883 Basic IC Plus, Metrohm, Switzerland). For analysis of the nitrate, nitrite, and phosphorus ions, an anion column Metrosep A Supp 5 was used, with the eluent, which consisted of 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$, supplied to the column at a flow rate of 0.7 ml/min. For additional quantification of TAN concentration, an cation column Metrosep C 4 with eluent containing 1.7 mmol/L HNO_3 and 0.7 mmol/L PDCA (2,6-Pyridinedicarboxylic acid) supplied at a flow rate of 0.9 ml/min was used.

2-4- Indoor 100 L ABFT (Fish-growth [Seedling Stage] Experiment)

The experiment was continued for 60 days. The treatments were "50%" (50% water replacement daily: control) and ABFT (no water replacement: experiment). In each ABFT tank, the average microalgal concentration for the inoculum was 0.037 g L^{-1}. Illumination at a light intensity of 34 µmol photons $m^{-2}s^{-1}$, as measured on the surface of the water (16-h light, 8-h dark), was provided. Tanks were randomly assigned in triplicate, each stocked with 100 fry tilapia (0.02 g average body weight), with water supplied every three days to compensate for evaporation loss. The fish were fed at a rate of 4 – 20% of wet body weight. The fish-rearing conditions were the same as those for the water-degradation experiment.

At the feeding trial's end, each tank's fish were measured for the survival rate, weight gain (WG), specific growth rate (SGR), and feed efficiency (FE). A proximate composition analysis of all of the fish from each tank as well as the microalgae was performed using the standard AOAC methods [34]. Prior to the analysis, samples were freeze-dried for a duration of 48 hours. Then, their moisture contents were measured by dry oven at 105°C, and their ash contents were determined by combustion at 550°C. The crude protein was calculated by the Kjedahl method, and the crude lipid was measured by soxhlet extraction based on the Soxhlet system 1046 (Tacator AB, Sweden) [35].

2-5- Amino Acid Analysis

Preparatorily to an amino acid (AA) analysis, microalgae and tilapia samples were freeze-dried. In total, 0.02g of each sample was subjected to hydrolyzation with 15 ml of 6 N HCl at a temperature of 110°C for 24 hours. Each hydrolyzed sample was evaporated in a 50 mL flask of distilled water and recovered in a sodium citrate buffer (0.2 N, pH 2.2). Following filtration (0.2 µm), each sample was examined with a S433 amino acid analyzer (Sykam, Gilching, Germany) using ninhydrin at 570 and 440 nm. For hydrolysis of methionine and cystine, performic acid was used instead of 6 N HCl.

2-6- Statistical Analysis

After confirming both the normality and the homogeneity of variance, all of the data were scrutinized by one-way ANOVA (SAS version 9.1; SAS Institute, Cary, NC, USA) in order to test for the treatment effects. In the cases where a significant treatment effect was evident, the means were compared using an LSD (Least Significant Difference) test. The treatment effects were considered at a level of significance of 5% ($P < 0.05$).

3- Results and Discussion

3-1- Effect of Microalgae on Nutrient Removal (Water-degradation Experiment)

The values of the measured water-quality parameters (nitrogenous compounds) are plotted in Figure 1. Excessive nitrogenous compounds (total ammonia nitrogen (TAN), nitrite (NO_2^-) and nitrate (NO_3^-)) accumulated from high fish densities and high feed loads in aquaculture systems need to be carefully controlled, because in aquacultures, they are responsible for reduced animal growth and decreased survival [36, 37]. TAN (Total ammonia nitrogen) represents the sum of un-ionized (NH_3) and ionized (NH_4^+) ammonia. Elevated levels of ammonia (NH_3) in the body have a large number of deleterious effects [38]; acute ammonia (NH_3) toxicity, for example, affects the central nervous system of vertebrates, leading quickly to convulsions and death [39]. Ammonia (NH_3)'s toxicity is due to its lipid solubility and lack of charge, which enable its ready diffusion across the membranes of gills; ionized ammonia (NH_4^+), meanwhile, is in a larger hydrated form, with charged entities that do not readily pass through the gill membranes' hydrophobic micropores [40]. The toxicity of forms ammonia (NH_3) chemically increases with water pH (ammonia/ammonium ratio increases at pH 7 and above), which is one of the reasons for the importance of pH control in aquaculture. As for NO_2^- (nitrite), it arises in fresh waters as a natural result of the nitrification of ammonia and the denitrification of nitrate, and its concentration tends to be enhanced by ammoniacal discharges' partial oxidation. Its effect is manifested by haemoglobin's conversion to methaemoglobin, which is not capable of oxygen transport; as such, NO_2^- is toxic to vertebrate fish [41-43]. The toxicity of NO_3^- (nitrate) to aquatic animals, similarly to that of NO_2^-, is due to the conversion of oxygen-carrying pigments (i.e., hemoglobin, hemocyanin) into the non-oxygen-carrying form (i.e., methemoglobin) [44-46].

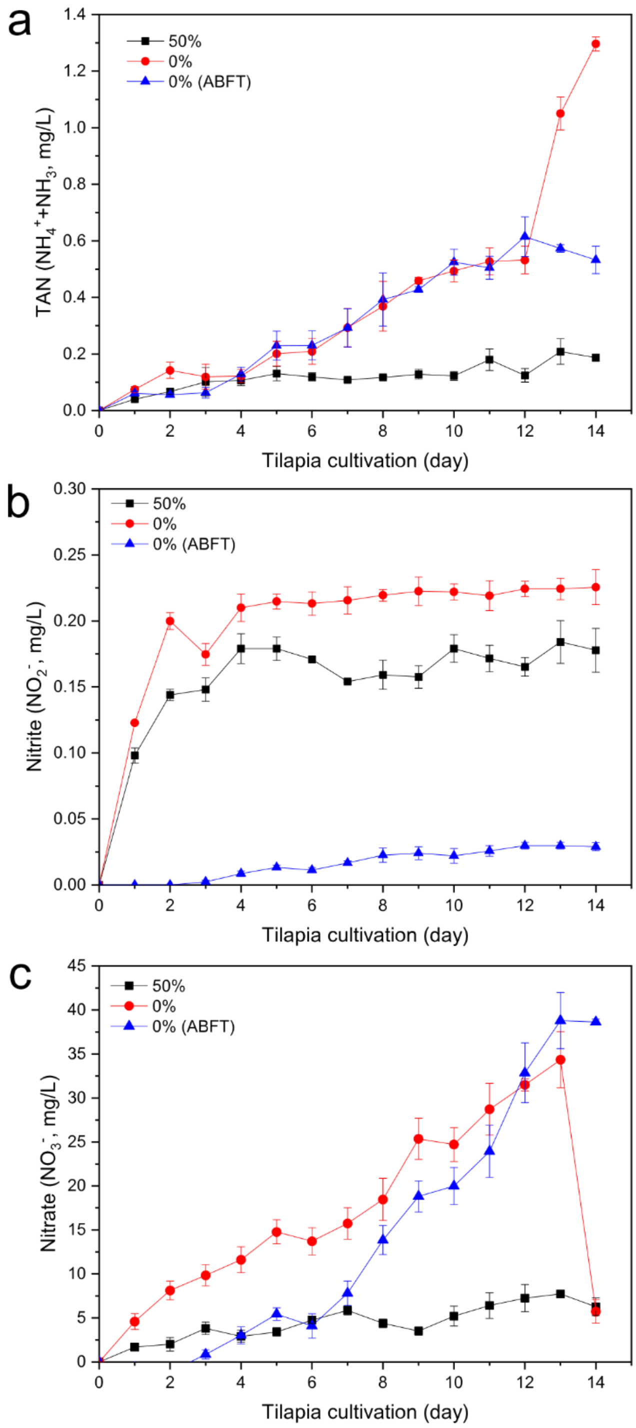

Figure 1. Nutrient-removal efficacy: (a) TAN (NH_4^+ and NH_3), (b) Nitrite (NO_2^-) and (c) Nitrate (NO_3^-). Groups: 50% (closed squares; water replacement daily); 0% (closed circles; no water replacement); ABFT (closed triangles; no water replacement; including *Chlorella ellipsoidea* and *Scenedesmus dimorphus*).

TAN (Figure 1(a), NH_4^+ and NH_3) of 0.04-0.21 mg/L (50%), 0.08-1.30 mg/L (0%), and 0.06-0.62 mg/L (0%, ABFT), nitrite (Figure 1(b), NO_2^-) of 0.10-0.18 mg/L (50%), 0.12-0.23 mg/L (0%), and 0.00-0.03 mg/L (0 %, ABFT), and nitrate (Figure 1(c), NO_3^-) of 1.70-7.74 mg/L (50%), 4.59-34.35 mg/L (0%), and 0.86-38.79 mg/L (0 %, ABFT), were detected respectively, which showed a similar result (viable concentration) in a previous study [47-51]. The conclusions that can be drawn are as follows. **(i)** Similarly to the relevant previous studies [52-57] (water treatment by microalgae), the present values of NO_2^- and NO_3^- showed lower levels at 0% ABFT than at 0%, suggesting that the water quality in the fish-culture tank was improved by microalgae. It is considered that microalgae selected the NO_2^- (exponential stage) and NO_3^- (lag stage) for growth (Figure 1(b) and (c)). **(ii)** The concentrations of all of the factors (TAN, NO_2^- and NO_3^-) were considered to be accumulated due to the gradual increase of the continuous feed supply and of fish secretion

(Figures. 1(a)-(c)). **(iii)** Mortality started on day 13 in the 0% tanks, and all of the fish died within two days, whereas in the ABFT tanks, all of the fish survived (Figure 2(a)). **(iv)** Although no mortality occurred in the ABFT tank (Figure 2(b)), its concentration of NO_3^- (Figure 1(c)) would be expected to increase continuously until mortality occurs, especially considering the result of a previous study using larger fish (> 85 g) [32] than did the present study (< 16 g). **(v)** The higher pH in 0% ABFT group compared to 0% (Figure 2 (a)) is considered to be due to microalgae as in a previous study [58-61], which has been a common conclusion that photosynthesis causes an increase in pH with increases of cultivation time. The values of pH, from the microbiological point of view, can decrease by waste in the form of excess carbon dioxide (CO_2) released into the water from the microbial nitrification process [62]. Low pH values in the 0% group compared to 50% are considered to be due to microorganisms by abiogenesis in the course of the aquaculture process (fish secretion or influx or external inflow). In summary, water quality was improved by microalgae (ABFT tank), which consequently had a positive impact on the fish survival rate.

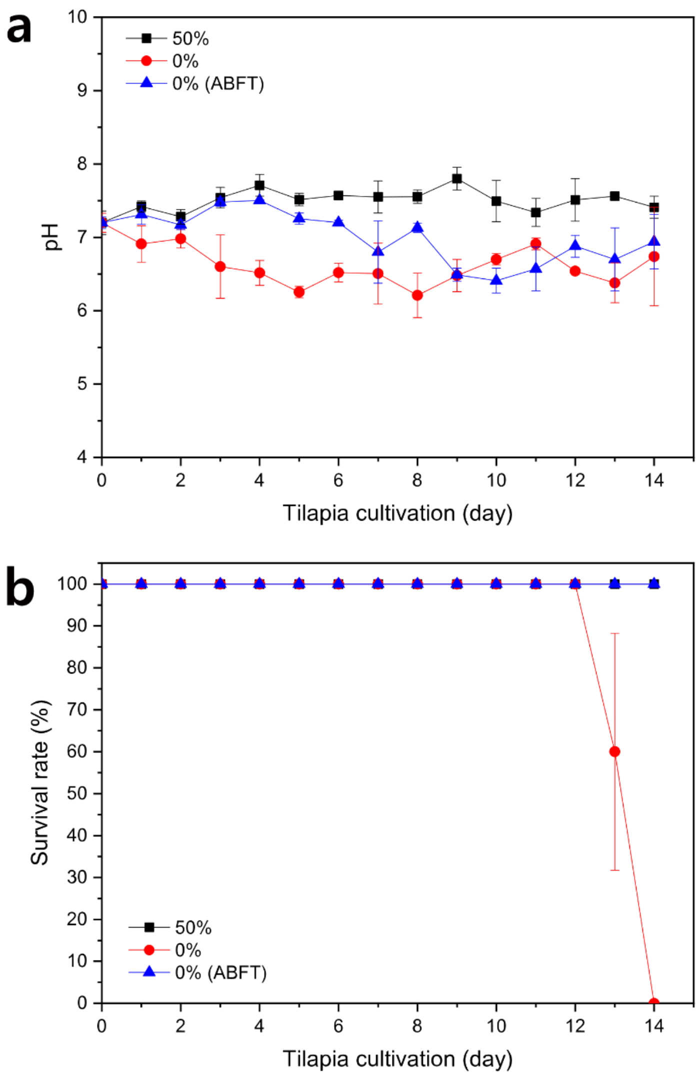

Figure 2. (a) pH-change pattern during fish cultivation and (b) Tilapia survival rates. Groups: 50% (closed squares; water replacement daily); 0% (closed circles; no water replacement); ABFT (closed triangles; no water replacement; including *Chlorella ellipsoidea* and *Scenedesmus dimorphus*).

3-2- Evaluations of ABFT System (Fish-growth [Seedling Stage] Experiment)

Similarly to previous research (juvenile stage, >85g) [32], there were no significant differences in growth performance (Table 1) or body composition (Table 2) or whole-body amino acid composition (Table 3) among the fish for 60 days, and the ABFT groups, notably, showed a 97% reduction of water usage (from 3,050 L to 100 L) relative to the control (50%) group. This reconfirmed the excellent water-purification effect and significant water conservation by microalgae, with no deleterious effect on the growth of the targeted aquaculture fish. Considering that microalgae, which have traditionally been used as primary food sources (especially protein sources), are mass-produced on the industrial level and utilized for their established biological functions [63, 64], it is not surprising that they are considered to be one of the best nutrient sources in aquaculture, many studies already having demonstrated their suitability as an animal food source or feed additive in various fields [18, 65-69].

Table 1. Growth performance of tilapia (as cultured in 50% and ABFT groups) at end of 60-day experiment [a]

	CON	ABFT
Initial mean weight (g $fish^{-1}$)	0.024 ± 0.001	0.024 ± 0.002
Initial number (fish $tank^{-1}$)	100	100
Final mean weight (g $fish^{-1}$)	12.37 ± 0.33	12.81 ± 0.63
WG (%) [b]	51855 ± 3522	53216 ± 2570
FE (%) [c]	129.64 ± 8.81	133.04 ± 6.43
SGR (%/day) [d]	10.42 ± 0.11	10.46 ± 0.08
Survival rate (%) [e]	98.00 ± 2.00	97.67 ± 1.53

[a] Values are means from triplicate groups of fish where the values in each row with different superscripts are not significantly different ($P > 0.05$)

[b] Weight gain (WG, %) = (final weight - initial weight) × 100 / initial weight

[c] Feed efficiency ratio (FE, %) = (wet weight gain / dry feed intake) × 100

[d] Specific growth rate (SGR, %/day) = (log_e final weight - log_e initial weight) × 100 / days

[e] Survival rate (%) = (initial number of fish - dead fish) × 100 / initial number of fish

Table 2. Whole-body proximate compositions of tilapia (as cultured in 50% and ABFT groups) at end of 60-day experiment (%) [a]

	Tilapia [b]		Microalgae [c]
	CON	ABFT	
Moisture	75.47 ± 0.58	76.33 ± 0.48	2.31
Protein	16.06 ± 0.41	15.66 ± 0.43	57.73
Lipid	4.13 ± 0.12 [b]	4.54 ± 0.16 [a]	7.69
Ash	3.88 ± 0.19	3.69 ± 0.03	5.46

[a] Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[b] Wet matter basis

[c] Dry matter basis (*Chorella ellipsoidea* and *Scenedesmus dimorphus*)

Table 3. Amino acid compositions of tilapia (as cultured in 50% and ABFT groups) at end of 60-day experiment (%) [a]

	Tilapia [b]	
	50 %	ABFT
Essential amino acids (EAA)		
Arginine	1.02 ± 0.01 [a]	0.95 ± 0.02 [b]
Threonine	0.62 ± 0.03	0.61 ± 0.01
Valine	0.71 ± 0.04	0.68 ± 0.01
Isoleucine	0.62 ± 0.02	0.59 ± 0.01
Leucine	1.05 ± 0.04	1.01 ± 0.01
Methionine	0.40 ± 0.02	0.39 ± 0.01
Lysine	1.17 ± 0.03	1.12 ± 0.01
Phenylalanine	0.59 ± 0.02	0.56 ± 0.01
Histidine	0.51 ± 0.01	0.49 ± 0.01
Non-essential amino acids (NEAA)		
Serine	0.58 ± 0.03	0.56 ± 0.01
Glutamic acid	2.09 ± 0.07	2.01 ± 0.02
Proline	0.91 ± 0.04	0.86 ± 0.01
Glycine	1.24 ± 0.11	1.10 ± 0.03
Alanine	0.98 ± 0.01 [a]	0.91 ± 0.01 [b]
Tyrosine	0.33 ± 0.01	0.32 ± 0.01
Aspartic acid	1.52 ± 0.06	1.48 ± 0.01
Cystine	0.33 ± 0.02	0.36 ± 0.04

[a] Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[b] Wet weight basis

The discovery of dead fish in the course of the experimentation (survival rate, Table 1) was considered to have been due to cannibalism rather than water quality or stocking density. Studies on this phenomenon have already been reported for other tilapia species [68-70]. The rationale for the utilization of aquaculture sludge as a fertilizer for direct land application [45] or for compost production [67] is that its concentrations of toxic and other health-threatening constituents are low in relation to those evident in domestic- and industry-origin sludge, even though it is treated with domestic and industrial wastes (along with other livestock waste) in centralized facilities entailing the same primary, secondary and tertiary treatment steps [71]. Recent studies have shown that plant growth and germination were improved when microalgae biomass was added to fertilizer or media for plant cultivation [72-74]. Additionally, aquaculture wastewater is itself used directly for plant growth [75-77], since it contains dissolved rich nutrients (feed residue, gill excretion, feces and urine) that are composed of both soluble and solid organic compounds solubilizable to the ionic form in water and assimilable by plants [78]. Therefore, it is expected that the ABFT system's microalgal-biomass-containing sludge can be indirectly utilized as natural fertilizer, and that its wastewater can be directly used for plant cultivation (Figure 3).

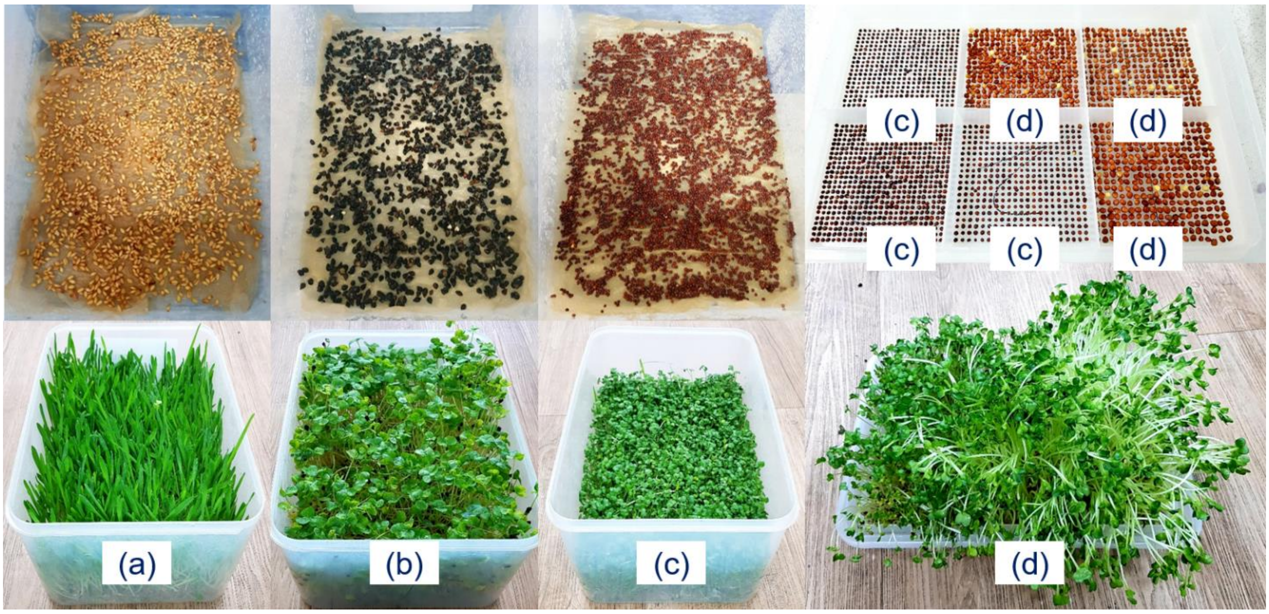

Figure 3. Efficacy of wastewater from ABFT tank including microalgae (*Chlorella ellipsoidea and Scenedesmus dimorphus*) in enhancing growth of leafy vegetables. (a) Barley (*Hordeum vulgare*), (b) Buckwheat (*Fagopyrum esculentum*), (c) Pak choi (*Brassica rapa*) and (d) Radish sprouts (*Raphanus sativus*). Three independents were performed. Each seed was purchased from an agriculture company (Chanamu Co., Republic of Korea) and the experiment was performed for 14 days in accordance with the guidelines.

Another advantage of the ABFT system is that it can have a positive effect on production economics by simplifying the production process steps so that fish breeding and wastewater treatment are performed simultaneously, rather than independently as in existing onshore systems such as biofloc technology (BFT) [79], recirculating aquaculture systems (RAS) [80], and aquaponics [73]. The ABFT system can also help to solve the problem of global climate change by atmospheric carbon dioxide emissions, because autotrophs such as microalgae convert carbon dioxide to carbohydrates through photosynthesis. The disadvantage of the ABFT system is the fact that fish finally all die with (> 85g, 12 days), as in our previous study [32], due to the formation rates of water pollutants such as nitrogenous compounds generated by feed or fish feces being higher than the treatment rate with other factors affecting fish mortality. However, under controlled conditions, water can be saved without incurring fish mortality if the sludge in the tank is removed in advance (Figure 4(a) and (b)), which can also provide for a natural hatching environment (Figure 4(c) and (d) [81], and Figure 5).

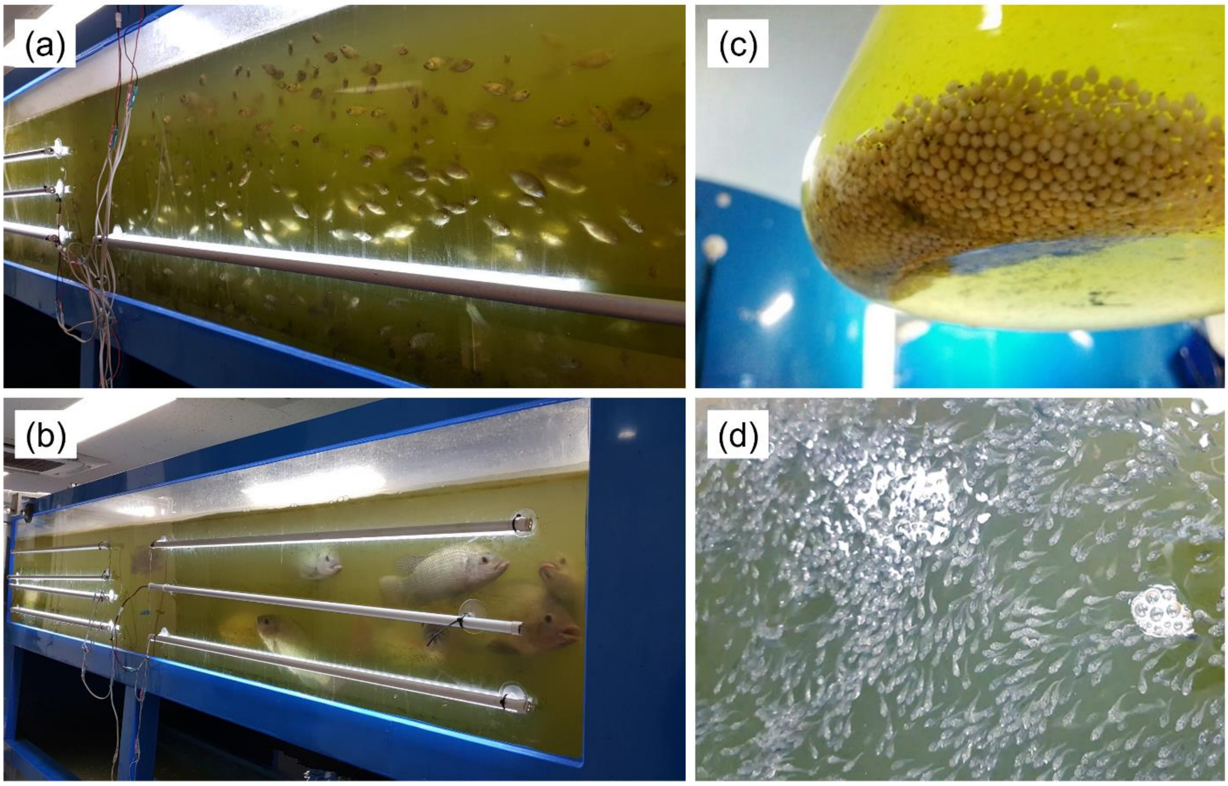

Figure 4. In ABFT tanks, average concentration of initial microalgal cells for inoculum every 10 days was less than 0.2 g L^{-1}. (a) Growth from hatching to juvenile stage (> 600 fish, < 10 g) in ABFT system for 4 weeks with no water replacement at 2,000 L water capacity. The fish were fed 3 times per day (9:00, 13:00 and 17:00) at 3-5% of wet body weight. (b) Growth to adult stage (> 130 fish, 350 - 550 g) in ABFT system for 6 weeks with 5% water replacement every 3 days at 2,000 L water capacity. The fish were fed 3 times per day (9:00, 13:00 and 17:00) at 1-3% of wet body weight. (c) Eggs and (d) fry produced from ABFT system at 2,000 L water capacity.

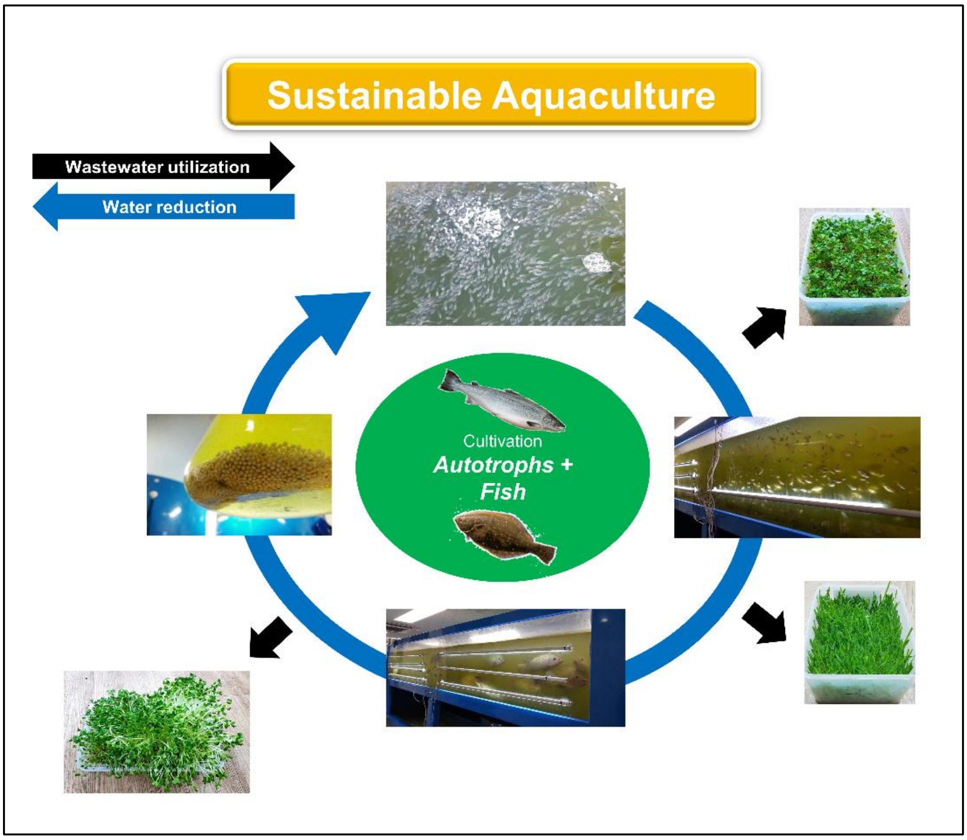

Figure 5. The autotrophic biofloc technology (ABFT) as sustainable aquaculture system.

4- Conclusion

The objective of the present study was to evaluate the potentialities of the ABFT system at the remaining stages (seedling to adult farming). Among the fish tested, there were no significant differences either in growth performance or in body composition, and the ABFT groups showed a 97% reduction of water usage relative to the control group at the seedling stage, and the wastewater from this system was recycled by use for the growth of various plants. In addition, the ABFT system also provided for a natural hatching environment. In short, the ABFT system demonstrated that it can be implemented — as integrated with existing systems — on an industrial scale. Indeed, it has considerable potential as an economical aquaculture system for sustainable aquaculture, especially in developing countries' rural areas.

5- Funding and Acknowledgments

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6- Conflict of Interest

The authors declare no conflict of interest.

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