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The Effect of Mixed Wastewaters on the Biomass Production and Biochemical Content of Microalgae

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Abstract: The effect of ammonia and iron concentration in Bold Basal Medium and mixed wastewater (including pretreated piggery wastewater and acid mine drainage) on biomass production and biochemical content (lipid and ß-carotene) of microalgae (*Uronema* sp. KGE 3) was investigated. Addition of iron to the Bold Basal Medium enhanced the growth, lipid, and ß-carotene of *Uronema* sp. KGE 3. The highest dry cell weight, lipid content, and lipid productivity of KGE 3 were 0.551 g L^{-1}, 46% and 0.249 g L^{-1} d^{-1}, respectively, at 15 mg L^{-1} of Fe. The highest ß-carotene was obtained at 30 mg L^{-1} of Fe. The biomass production of KGE 3 was ranged between 0.18 to 0.37 g L^{-1}. The microalgal growth was significantly improved by addition of acid mine drainage to pretreated piggery wastewater by membrane. The highest dry cell weight of 0.51 g L^{-1} was obtained at 1:9 of pretreated piggery wastewater by membrane and acid mine drainage for KGE 3. The removal efficiencies of total nitrogen and total phosphate was ranged from 20 to 100%. The highest lipid and ß-carotene content was found to be 1:9. Application of this system to wastewater treatment plant could provide cost effective technology for the microalgae-based industries and biofuel production field, and also provide the recycling way for pretreated piggery wastewater and acid mine drainage.

Keywords: microalgae; acid mine drainage; microalgae culture; lipid; ß-carotene

1. Introduction

Microalgae synthesize contain valuable biomass compounds, such as lipid, ß-carotene, astaxanthin, and lutein [1,2]. Biofuel has been obviously reported as a source of renewable energy for superseding the fossil fuel [3]. The biodiesel can be derived from lipids of microalgae, which is achievable since microalgae contains up to 50% of lipid with respect to dry cell weight [4]. Also, microalgae have advantages like high photosynthetic efficiency, high productivity yield, non-arable land use, and ability to capture and utilize CO_2 as a nutrient [5,6]. Carotenoids are commercially using as food coloring agent and feed cosmetic products, particularly ß-carotene acted as an antioxidant, anticancer and immune functions. The nutrient concentration is one of the vital sources for successful of cultivation microalgal, and efficient and improved synthesis of microalgae biomass for subsequent production of biodiesel and ß -carotene has also been reported [7]. Also, the concern has been increasing towards the biomass production from microbial to absorb heavy metal ions and bioaccumulation [8,9].

Acid mine drainage (AMD) is often net acidic and contains a high concentration of essential metals including iron, which can be used as micronutrients to improve the growth of microalgal with respect

to enhancing the lipid production [10,11]. Organic sources for microalgae growth was accounted as 80% of the total cost of the medium [12], hence it is necessary to use low-cost organic moieties or wastewaters to achieve maximum microalgal yield at commercial scale [13]. The development of livestock production has environmental problems. Manure characterization results show that it contains a high concentration of organic material like nitrogen and phosphorus. The manure treatment process tends to couple with anaerobic digestion, which reduces the treatment cost along with nutrient recovery for microalgae, and manure then becomes a resource [14]. The different type of wastewaters from municipal, piggery and food industries was used as resource for microalgae cultivation and its growth has been investigated [15,16].

The mixing of different wastewaters to acquire the ideal nutrients ratio for improved biomass production has not been well investigated. In addition to that, supplementation of manure wastewater (as source of TN and TP) with AMD (as source of Fe) has not yet been studied. Hence, the present investigation to explore the effect of mixed wastewater (including manure and AMD) on biomass production, biochemical content (lipid and ß-carotene) of microalgae (*Uronema* sp. KGE 3) was examined. The removal of total nitrogen (TN) and total phosphorus (TP) was monitored. The kinetic assessment of specific growth rate of microalgae was also evaluated. These results provide fundamental information to establish and cultivate the microalgae in Pipes inserted microalgae reactor (PIMR) system, which could be one of the alternate choices for a microalga-based biodiesel production strategy.

2. Materials and Methods

2.1. Preparation of Microalgal Suspension, Strain, and Culture Medium

Uronema sp. KGE 3 microalgae was derived from AMD at YD abandoned mine (Gangwon-do, South Korea). The resultant microalga was cultivated in 2.2 L column using 5% ($V_{inoculum}/V_{media}$) of 2.0 L of Bold's Basal Medium (BBM) [17]. The cultivated *Uronema* sp. KGE 3 microalgae was identified by rDNA D1-D2 sequence-based phylogenetic extraction analysis (Figure 1). The cultures were kept under a light irradiation of 130 µmol photon m^{-2} s^{-1} at 25 ± 1 °C for ten days with white fluorescent light illumination. Throughout the incubation, each column was constantly sparged with air through 0.2 µm sterilized filter at the flow rate value of 0.4 L min^{-1}, the suspension stirred, and concurrently supplied with atmospheric CO_2. The microalga biomass suspension in the medium, which adjusted to an absorbance of 0.01 at an optical density of 680 nm as measured by a spectrophotometer (Hach DR/2800, Loveland, Colorado, USA). Experiments were used stock microalgae to inoculum amount of three milliliters.

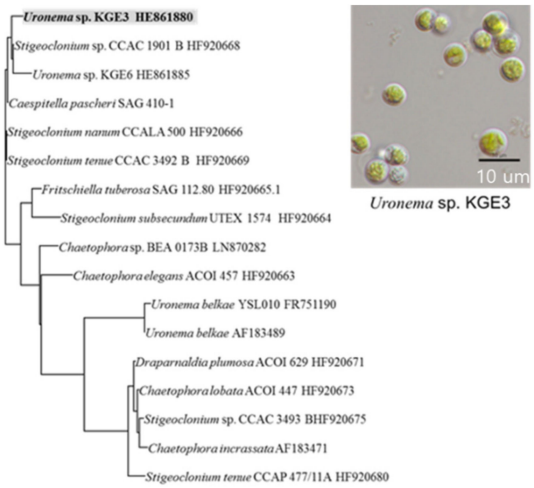

Figure 1. *Uronema* sp. KGE3 cell morphology and phylogenetic tree displays the connection between the NSU(Nuclear large subunit) rDNA D1-D2 sequences of the isolates *Uronema* sp. KGE3 and the maximum parallel sequences rescued from the NCBI nucleotide database.

2.2. Wastewater Sampling and Analysis

Acid mine drainage (AMD) was collected from Yeong-dong mine at Gangneung, South Korea. The manure was collected from cattle farms at Jeollanam-do Province, South Korea. The manure was pretreated by anaerobic digestion and membrane bioreactor (MBR). Wastewater was immediately filtered using syringe filter (Polyvinylidene fluoride or polyvinylidene difluoride (PVDF), 0.2 μm) to remove the suspended solid particles [18]. Total nitrogen (TN), total phosphorus (TP), and ammonium was measured using Hach Kit (HACH, CO, USA), which are equivalent methods: Standard Methods 4500-N C and 4500-P B (5). Metal ions in AMD were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian 730-ES, USA). The Orion 5-Star pH/DO/ORP/Cond. Meter (Thermo Scientific, USA) was used to measure the solution pH. The major components of AMD were 237.8 mg L^{-1} of total iron, 187.3 mg L^{-1} of ferrous, 5.7 mg L^{-1} of manganese and 320.4 mg L^{-1} of sulfate, and solution pH was 3.2. The major components present in the pretreated piggery wastewater by membrane (PPWM) were 4645 mg L^{-1} of ammonium, 6230 mg L^{-1} of total nitrogen, and 365 mg L^{-1} of total phosphate, and solution pH was found to be 8.53.

2.3. Experimental Method

The experiments were conducted in two stages. In the first stage, BBM was supplemented with different concentration of iron and ammonium to optimize the concentration for further experimental. The second phase was the replacement of BBM with PM amended with AMD as a cheap source of iron. The mixed ratio of PPWM with AMD were 1:0, 1:1, 1:9, and 1:19. The biomass production, nutrients removal (including TN /TP), and biochemical composition (including lipids and ß-carotene) of the microalga in all tested condition were evaluated.

Growth rate was used to monitor the microalgae by absorbance (optical density (OD)) and gravity (dry cell weight (DCW)) method. OD was measured at 680 nm using a spectrophotometer (HS-3300; Humas, Daejeon, Korea). To measure the DCW, microalgae was harvested by centrifugation at 3000 rpm for 20 mins (1580R, Labogene, Korea). The harvested microalgal biomass was frozen at –70 °C by deep freezer (DFU-256CE, Operon, Korea). After freeze-dried the biomass, experiments sample were lyophilized by freezing dryer (FDB-550, Operon, Korea).

The DCW was used to calculate the microalgae growth by: biomass productivity (*P*), as demonstrated in Equation (1).

$$P = M_b - M_{bo}/T - T_0 \quad (1)$$

where M_b and M_{b0} are microalgae dry biomass at time T and initial time T_0. The specific growth rate (μ, day $^{-1}$) of the microalgae can be calculated using Equation (2) as follows [19].

$$\mu = \frac{\ln X_i - \ln X_0}{t_i - t_0} \quad (2)$$

2.4. Analysis of Lipid and ß-carotene

The dried biomass of freeze-dried microalgae was used to extract the total lipids contents by modified method reported by Bligh and Dyer [20]. After the extraction, chloroform-methanol layer contains the microalgae extracted lipids, which can be collected using a separate-funnel and followed by the rotary evaporator. The lipid content of microalgae was calculated by the following Equation (3):

$$\text{Lipid content (\%)} = (W_2 - W_3)/W_1 \times 100 \quad (3)$$

where W_1 is weight of dried cell biomass, W_2 is glass tube with extracted lipids W_3 is empty glass tube.

Various organics solvents including methanol, acetone, and chloroform were used to extract the ß-carotene from freeze-dried algal biomass. In this study, the extraction of ß-carotene from freeze-dried algal biomass using chloroform provides a quietly high amount of yield. Hence, 0.2 g of freeze-dried algal biomass was suspended in 5.0 mL of chloroform, then sonicated for 10 min under maximum

power in an ultrasound condition, and finally the suspension was kept at 4 °C for 24 h. The supernatant liquid in the suspension was harvested by centrifugation and stored until analysis was carried out. After 14 extractions, chloroform did not display any coloration while the pellet remained greenish in color. The ß-carotene was analyzed by LC-MS (e2695, Waters, USA) and analytical column with 5 mm C30-reversed phase material (250 mm L 4.6 mm ID) at 30 °C. Detail of pretreatment and analyze procedure followed the Gupta method [21].

3. Results and Discussion

3.1. The Influence of Iron and Ammonium on the Microalgal Growth

The impact of iron on the growth of lipid, ß-carotene, and *Uronema* sp. KGE 3 cultivated in BBM are appeared in Figure 2A–C, respectively. The Fe concentration used in this study was 5 mg L^{-1} (from BBM only), 10, 15, 25, and 35 mg L^{-1}. Fe acted as a vital element for the growth of higher plants and microorganisms including microalgae [22]. The growth and reproduction of microalgae also requires addition of Fe. In microalgae-based reactors, optimal iron dosage needs to be maintained for stable growth of microalgae biomass [23]. The impact of various concentration of Fe on the growth and lipid production of *Uronema* sp. KGE 3 was investigated to identify the required amount of Fe from AMD. Addition of iron (15-35 mg L^{-1}) to BBM enhanced the growth and biochemical content (including lipid and ß-carotene) of *Uronema* sp. KGE 3. The highest dry cell weight (Figure 2A), lipid content, and lipid productivity (Figure 2B) of KGE 3 were 0.551 g L^{-1}, 46%, and 0.249 g L^{-1} d^{-1}, respectively after the addition of 15 mg L^{-1} of Fe, and it was higher than control by 24% (Figure 2A). The highest ß-carotene was found at 35 mg L^{-1} of Fe (Figure 2C). The metals including Fe, Mn, Cu, Zn, and Co acted as micronutrients and possessed the ability to increase the growth of microalgae by certain concentration, while on the other hand, the higher concentrations of these metals can hinder the growth of microalgae [24,25]. The growth pattern of *Uronema* sp. KGE 3 cultivated in BBM under various concentration of ammonium is shown in Figure 3A–C. Ammonium concentration used in this study was 0 (BBM only) to 1,000 mg L^{-1}. The growth of KGE 3 was ranged between 0.18 to 0.37 g L^{-1} (Figure 3A). The growth pattern of KGE 3 under ammonium was below the control (Figure 3B), which revealed the toxicity of ammonium. The present result was reliable with previous report displaying that ammonium had strong effect on the microalgal growth [26]. The highest lipid and ß-carotene content was achieved at 1000 mg L^{-1} of ammonium (Figure 3C).

The microalgal with high specific growth rate (μ) is important criteria to select the best microalgal species as it signifies a shorter doubling time [27]. The changes on the specific growth rate of *Uronema* sp. KGE 3 cultivated under the effect of iron and ammonium for 9 days in BBM is shown in Figure 4A,B, respectively. The highest specific growth rate of *Uronema* sp. KGE 3 was observed at 15 and 25 mg L^{-1} of Fe during the cultivation time (Figure 4A), while the trended specific growth rate under ammonium was not fixed, which might be due to the pernicious effect of ammonium (Figure 4B). The changes on specific growth rate was observed in this research because of the difference in specific cell biomass yield [28]. Yoshimura et al. reported that *Botryococcus braunii* possesses the specific growth rate and doubling time of 0.19–0.50 day^{-1} and 1.4–3.6 days, respectively [29]. Zhu et al. utilized artificial wastewater in photobioreactor for both cultivation and treatment of wastewater using *Chlorella zofingiensis*. The result shows that wastewater treatment was ranged from 0.208 to 0.260 day^{-1} with a doubling time of 2.67 to 3.34 days within 15 days of cultivation [30]. The difference in values of specific growth rate and doubling time for *Chlorella zofingiensis* might be owing to variances in cultivation period, culture medium, and cultivation conditions [31].

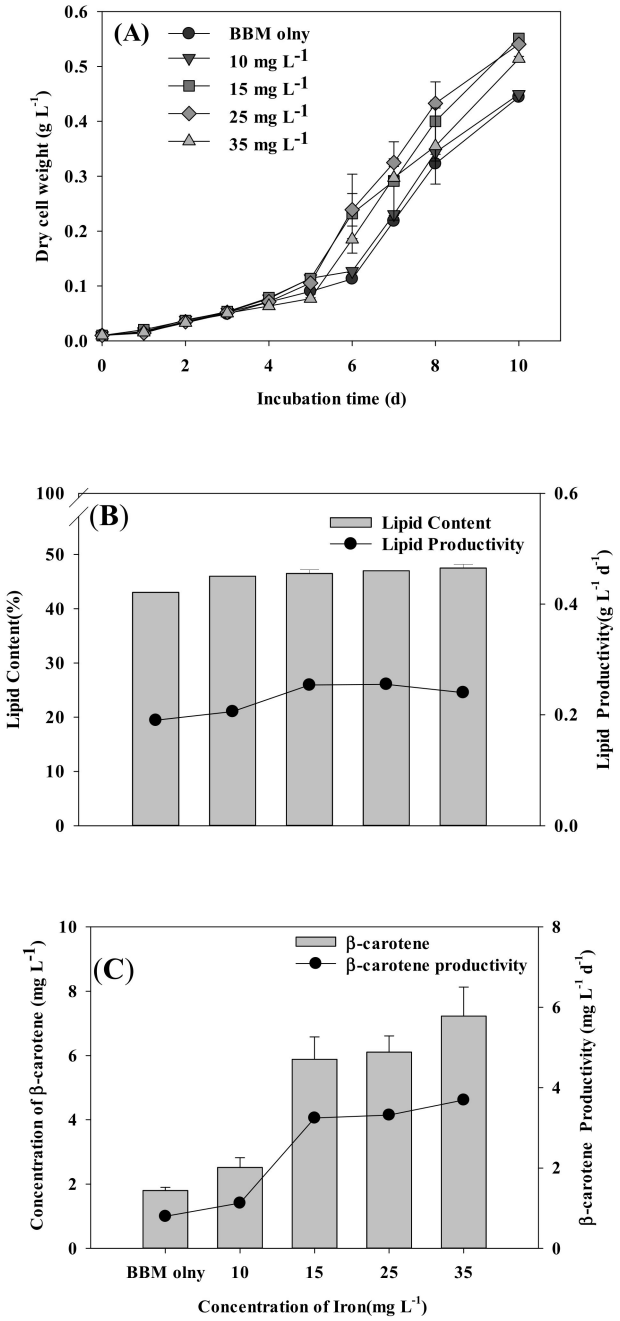

Figure 2. The effect of iron on the growth (**A**), lipid (**B**) and ß-carotene (**C**) of *Uronema* sp. KGE 3 cultivated in Bold's Basal Medium (BBM).

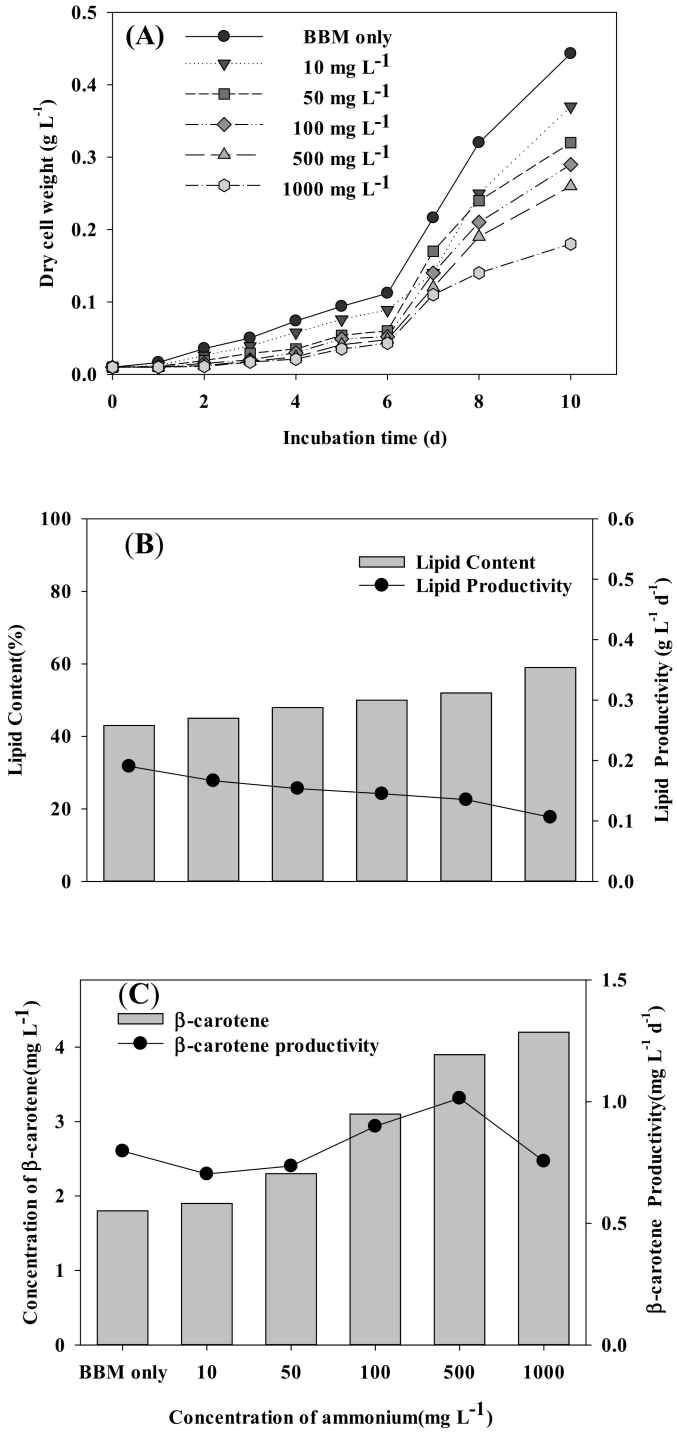

Figure 3. The effect of ammonium the growth (**A**), lipid (**B**) and ß-carotene (**C**) of *Uronema* sp. KGE 3 cultivated in Bold's Basal Medium (BBM).

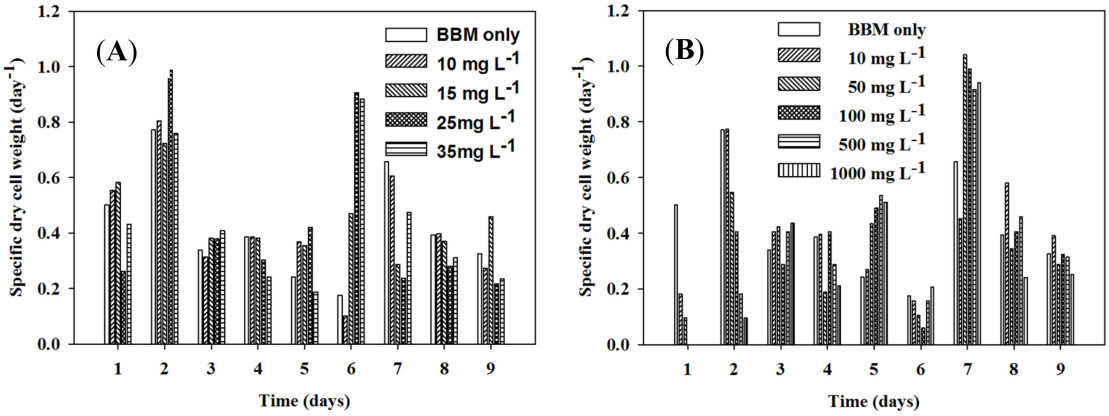

Figure 4. Changes on the specific growth rate of *Uronema* sp. KGE 3 under the effect of iron (**A**) and ammonium (**B**) cultivated for 9 days in Bold's Basal Medium (BBM).

3.2. Effect of Pretreated Piggery Wastewater by Membrane (PPWM) and Acid Mine Drainage (AMD) on the Microalgal Growth

The growth of *Uronema* sp. KGE 3 in various ratio of PPWM present of AMD is presented in Figure 5. The physico-chemical characteristics of experimental variations are presented in Table 1. The suitable selection of wastewater, robust microalgal species, and pre-treatment techniques are the primary factors for microalgae-based biofuel production, as well as the development of technology with the combination of wastewater treatment and microalgae cultivation for biomass and lipid production [32,33]. The dry cell weight of microalgae ranged between 0.19 to 0.51 g L^{-1} in all the tested conditions. The microalgal growth was significantly improved by addition of AMD to PPWM. The highest dry cell weight (0.51) was obtained at 1:9 of PPWM and AMD for *Uronema* sp. KGE 3 (Figure 5). Pervious studies have reported that the addition of AMDs to the microalgal culture enhanced the microalgal growth due to AMDs provided an optimal C:N:P ratio and suitable initial pH [18]. Microalgae can grow copiously when provide the adequate amount of nutrients and appropriate conditions. The algal growth is also directly influenced by the temperature, nutrients (TN, TP), light intensity, iron concentration, and the initial pH level [34,35]. Addition of AMD in this study provided suitable pH, which resulted in a higher growth rate than without addition of AMD (Figure 5A). The initial pH level of microalgal culturing media (including Bold's basal medium) was 6.8 [36].

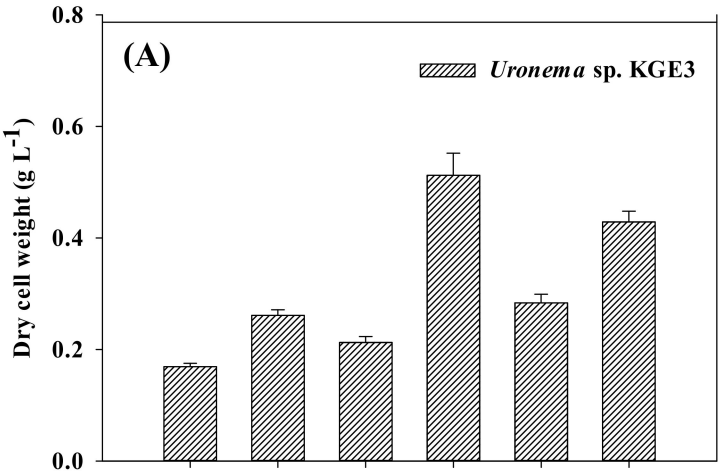

Figure 5. *Cont.*

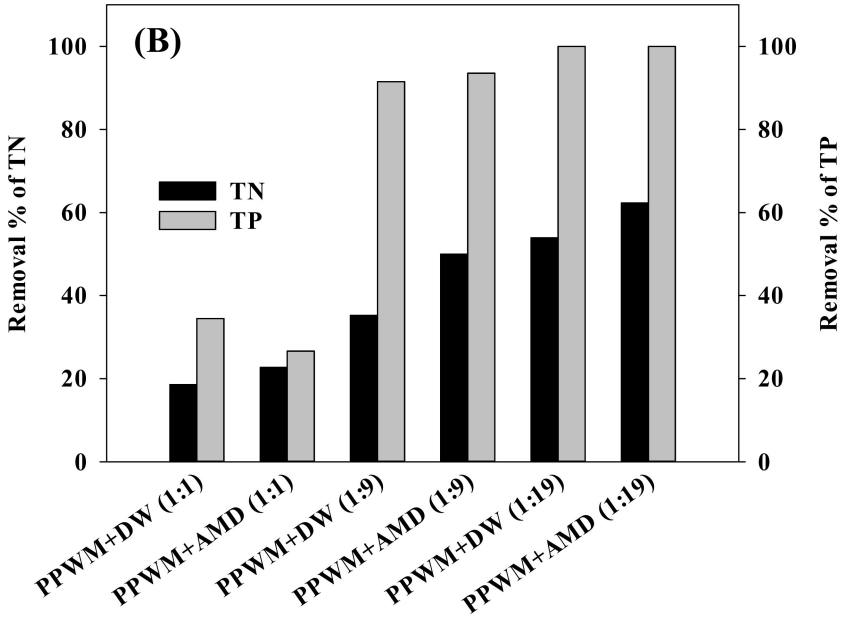

Figure 5. The effect of various mixing ratios between pretreated piggery wastewater by membrane (PPWM) and acid mine drainage (AMD) on the growth (**A**) and nutrients removal (**B**) of *Uronema* sp. KGE 3.

Table 1. Experimental condition for studying the effect of mixture from Pretreated manure and acid mine drainage.

Experimental Condition / Parameters	Pretreated Manure + Distilled Water (v/v)			Pretreated Manure + Acid Mine Drainage (v/v)		
	1/1	1/9	1/19	1/1	1/9	1/19
Total nitrogen (mg L^{-1})	743	173	76	762	168	69
Ammonium (mg L^{-1})	726	162	79	732	148	69
Nitrate (mg L^{-1})	13	3	1	15	2	1
Total phosphorous (mg L^{-1})	29	5.9	2.9	30	6.2	3.1
Manganese (mg L^{-1})	-	-	-	2.9	0.6	0.3
Sulfate (mg L^{-1})	-	-	-	160.2	32	16
Iron (mg L^{-1})	4.6	0.9	0.5	39.6	69.8	73.7

3.3. Effect of Microalgal Growth on Nutrient Removal

The microalgae growth can be enhanced by utilization of trace elements and nutrients present in the wastewater and subsequently the nutrient concentration in the wastewater reduced, which supports the advanced of wastewater recycling process and biomass production. Aliquots of wastewater samples were collected after 10 days of cultivation period to calculate the TN and TP removal from various dilutions of PPWM with AMD by *Uronema* sp. KGE 3, in order to assess its efficiency of nutrient removal. The removal efficiency was ranged from 18.6 to 62.3% for TN, while TP was ranged from 26.7 to 100% (Figure 5B). Nitrogen is one of the main macro elements for microalgae growth, which ranges from 1.0 to 10.0% of total dry biomass and is also an indicator for determining lipid content in microalgae biomass [37]. The TP was not fully used as nutrient by microalgae. Only part of the TP was utilized as a nutrient for growth and metabolism, including energy transfer and the biosynthesis of DNA, while the remaining TP was precipitated and assimilated into biomass through intracellular polyphosphate. Schreiber et al., reported that most of the consumed P accumulated in their body [38].

3.4. The Production of Lipid and ß-carotene after Cultivation in Pretreated Piggery Wastewater by Membrane (PPWM) Supplemented with Acid Mine Drainage (AMD)

After cultivation of *Uronema* sp. KGE 3 in PPWM supplemented with AMD, lipid and ß-carotene content was evaluated (Figure 6). The lipid content was ranged between 36 to 52% (Figure 6A), while ß-carotene content was ranged between 0.5 to 5.9% (Figure 6B). The highest lipid and ß-carotene content was found at 1:9 (PPWM: AMD ratio) medium, when *Uronema* sp. KGE 3 cultivated in BBM showed an optimal growth and obtained a higher cell growth than 1:9 (PPWM: AMD ratio) medium. Even though, the value of DCW in BBM (1.04 g L^{-1}) was higher than PPWM: AMD medium (0.91 g L^{-1}), 1:9 (PPWM: AMD ratio) medium was more suitable media for productivity of lipid and ß-carotene. The result indicated that iron in AMD could promote the biomass productivity (Figure 7). The addition of PPWM (as a source of nutrients) and AMD to the system increases the microalgal biomass from 0.19 to 0.51 g L^{-1} and also increases the lipid/carotenoids productivity from 0.166 g L^{-1} d^{-1} and 1.01 mg $g^{-1}d^{-1}$ to 0.251 g L^{-1} d^{-1} and 3.01 mg $g^{-1}d^{-1}$ respectively, along with increasing nutrient removal efficiencies from 18.6 to 62.3%.

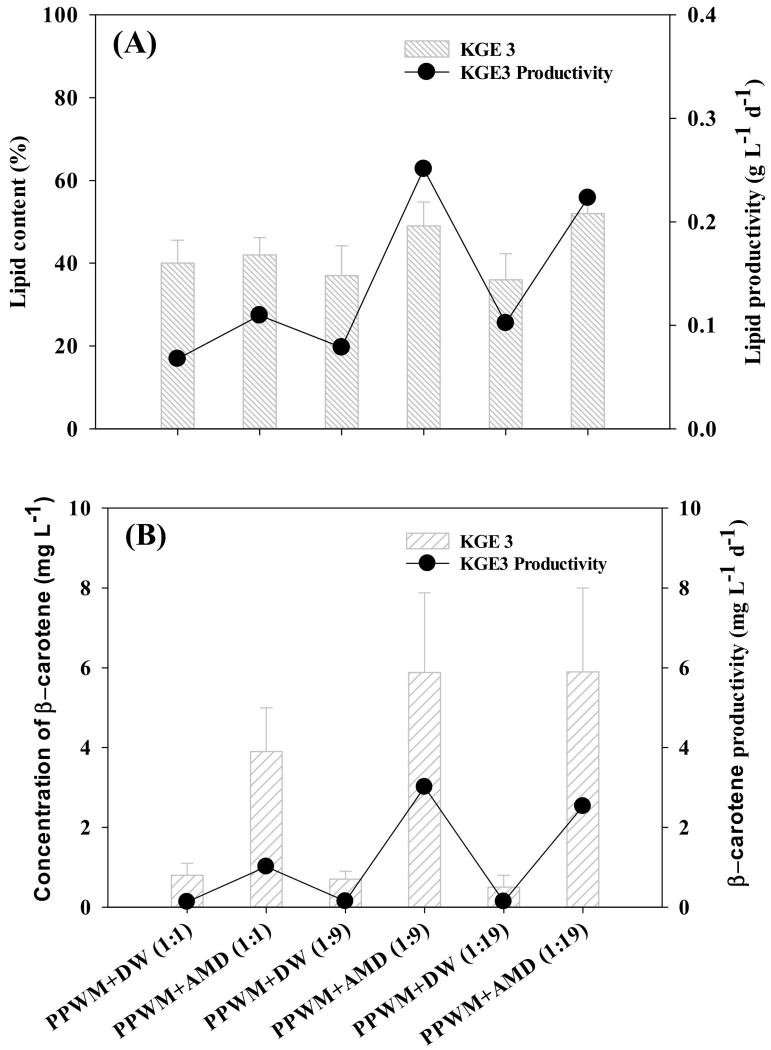

Figure 6. Effect of various mixing ratios between pretreated piggery wastewater by membrane (PPWM) and acid mine drainage (AMD) on the lipid production (**A**) and ß-carotene (**B**) of *Uronema* sp. KGE 3.

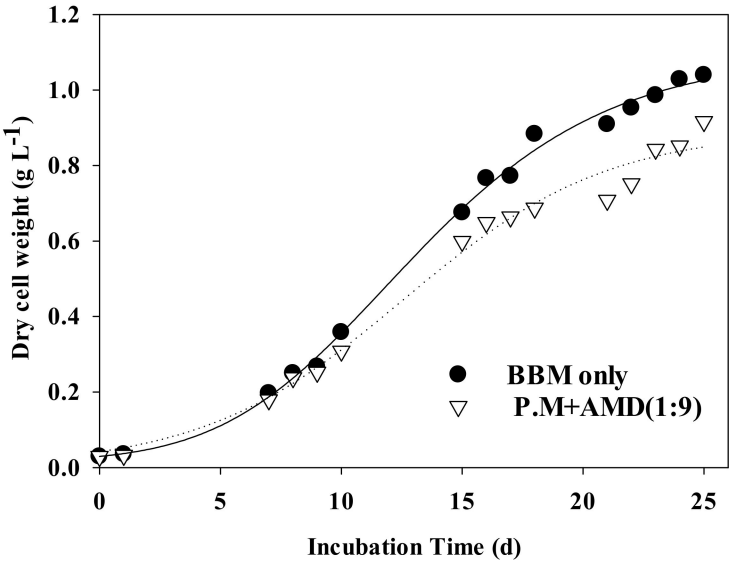

Figure 7. Time courses of biomass concentration for *Uronema* sp. KGE 3 cultivated in Bold's Basal Medium (BBM). with pretreated piggery wastewater by membrane (PPWM) + acid mine drainage (AMD) (1:9) medium during Pipes inserted microalgae reactor (PIMR) operation for 25 days.

3.5. Comparison of Other Researches for Lipid and ß-carotene Production

Iron may affect the productivity of lipid content, while ammonia affects both lipid and ß-carotene contents. Table 2 lists the microalgae production capacities of lipid and β-carotene. Kim et al. (2007) [39] reported that fermented swine urine was used as source of ammonia. It is worth noticing that while increasing the amount of ammonia, the ß-carotene production was also increased. In this study, ß-carotene production is well related to the concentration of PPWM, because PPWM acts as an ammonia source which correspondingly increases the production of ß-carotene. Feng et al. (2011) [40] also added ammonium in artificial wastewater to enhance the yield of ß-carotene. Singh et al. (2015) [41] reported that lipid productivity was 3–5 times higher than the conventional media.

Table 2. Comparison of other researches for lipid and ß-carotene production.

Strain	Cultivation Condition	Lipid (g/L d)	β-carotene (mg/g d)	Reference
Uronema sp. KGE 3[a]	Iron	0.249	3.69	This study
Uronema sp. KGE 3[a]	Ammonia	0.166	1.01	This study
Scenedesmus spp. -Mixed culture[b]	Fermented swine urine	-	0.05	Kim et al. (2007) [39]
C. vulgaris FACHB1068[c]	Artificial wastewater	0.147	-	Feng et al. (2011) [40]
Ankistrodesmus falcatus KJ671624	Iron	0.074	-	Singh et al. (2015) [41]
Uronema sp. KGE 3[a]	AMDS+PPWM	0.251	3.01	This study

[a] This study; [b] Kim et al. (2007); [c] Feng et al. (2011); [d] Singh et al. (2015).

4. Conclusions

The condition of PPWM: AMD = 1:9 and PPWM: AMD = 1:19 leads to the production of microalga feedstock and is suitable for obtaining highly efficient lipid and ß-carotene. Utilization of *Uronema* sp. KGE 3 as microalgae for the generation of biofuel material and recycling of wastewater could be a cost-effective and environmentally sustainable strategy. The optimal iron concentration of 15 mg L^{-1}

has potential application for microalgae cultivation because of distribution of biomass productivity increase. The change in iron and ammonium concentration can induce the lipid and ß-carotene accumulation. A pilot-scale study using PIMR proved that PPWM 1: AMD 9 can be used as effective cultivation media instead of synthetic BBM. This culture approach and appropriate microalgae strains will provide the cost-effective technology to microalgae-based industries and biofuel production fields and a recyclable way for PPWM and AMD. Application of this approach to pig wastewater treatment system could be an alternative strategy to reduce contaminant concentration such as TN and TP, and to increase facility for bioenergy production.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sigamani, S.; Ramamurthy, D.; Natarajan, H. A review on potential biotechnological applications of microalgae. *J. Appl. Pharm. Sci.* **2016**, *6*, 179–184. [CrossRef]
2. Del Campo, J.A.; Garcia-Gonzalez, M.; Guerrero, M.G. Outdoor cultivation of microalgae for carotenoid production: Current state and perspectives. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 1163–1174. [CrossRef] [PubMed]
3. Crutzen, P.J.; Mosier, A.R.; Smith, K.A.; Winiwarter, W. N2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. *Atmos. Chem. Phys.* **2008**, *8*, 389–395. [CrossRef]
4. Xiong, W.; Li, X.; Xiang, J.; Wu, Q. High-density fermentation of microalga Chlorella protothecoides in bioreactor for micobio-diesel production. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 29–36. [CrossRef]
5. Wang, B.; Li, Y.Q.; Wu, N.; Lan, C.Q. CO2 bio-mitigation using microalgae. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 707–718. [CrossRef]
6. Biller, P.; Ross, A.B. Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. *Bioresour. Technol.* **2011**, *102*, 215–225. [CrossRef] [PubMed]
7. Wu, Z.; Duangmanee, P.; Juntawong, N.; Ma, C. The effects of light, temperature, and nutrition on growth and pigment accumulation of three Dunalliella salina strains isolated from saline soil. *Jundishapur J. Microbiol.* **2016**, *9*, e26732. [CrossRef]
8. Benmalek, Y.; Halouane, A.; Hacene, H.; Fardeau, M.L. Resistance to heavy metals and bioaccumulation of lead and zinc by Chryseobacterium solincola strain 1YB–R12T isolated from soil. *Int. J. Environ. Eng.* **2014**, *6*, 68–77. [CrossRef]
9. Harguinteguy, C.A.; Cirelli, A.F.; Pignata, M.L. Heavy metal accumulation in leaves of aquatic plant Stuckenia filiformis and its relationship with sediment and water in the Suquia river (Argentina). *Microchem. J.* **2014**, *114*, 111–118. [CrossRef]
10. Das, B.K.; Roy, A.; Koschorreck, M.; Mandal, S.M.; Wendt-Potthoff, K.; Bhattacharya, J. Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. *Water Res.* **2009**, *43*, 883–894. [CrossRef]
11. Park, Y.T.; Lee, H.; Yun, H.S.; Song, K.G.; Yeom, S.H.; Choi, J. Removal of metal from acid mine drainage using a hybrid system including a pipes inserted microalgae reactor. *Bioresour. Technol.* **2013**, *150*, 242–248. [CrossRef] [PubMed]
12. Bhatnagar, A.; Chinnasamy, S.; Singh, M.; Das, K.C. Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. *Appl. Energ.* **2011**, *88*, 3425–3431. [CrossRef]
13. Liang, Y.; Sarkany, N.; Cui, Y. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol. Lett.* **2009**, *31*, 1043–1049. [CrossRef] [PubMed]

14. Ledda, C.; Schievano, A.; Scaglia, B.; Rossoni, M.; Fernandez, F.G.A.; Adani, F. Integration of microalgae production with anaerobic digestion of dairy cattle manure: An overall mass and energy balance of the process. *J. Clean. Prod.* **2016**, *112*, 103–112. [CrossRef]
15. Su, Y.Y.; Mennerich, A.; Urban, B. The long-term effects of wall attached microalgal biofilm on algae-based wastewater treatment. *Bioresour. Technol.* **2016**, *218*, 1249–1252. [CrossRef] [PubMed]
16. Matamoros, V.; Uggetti, E.; Garcia, J.; Bayona, J.M. Assessment of the mechanisms involved in the removal of emerging contaminants by microalgae from wastewater: A laboratory scale study. *J. Hazard. Mater.* **2016**, *301*, 197–205. [CrossRef] [PubMed]
17. Bischoff, H.W.; Bold, H.C. Phycological studies IV. Some soil algae from enchanted rock and related algal species. *Univ. Tex. Publ.* **1963**, *6318*, 1–95.
18. Ji, M.K.; Kabra, A.N.; Salama, E.S.; Roh, H.S.; Kim, J.R.; Lee, D.S.; Jeon, B.H. Effect of mine wastewater on nutrient removal and lipid production by a green microalga Micratinium reisseri from concentrated municipal wastewater. *Bioresour. Technol.* **2014**, *157*, 84–90. [CrossRef]
19. Wang, M.; Kuo-Dahab, W.C.; Dolan, S.; Park, C. Kinetics of nutrient removal and expression of extracellular polymeric substances of the microalgae, *Chlorella* sp. and *Micractinium* sp., in wastewater treatment. *Bioresour. Technol.* **2014**, *154*, 131–137. [CrossRef]
20. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **1959**, *37*, 911–917. [CrossRef]
21. Gupta, P.; Sreelakshmi, Y.; Sharma, R. A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography. *Plant Methods* **2015**, *11*. [CrossRef] [PubMed]
22. Wang, C.; Wang, X.; Wang, P.F.; Chen, B.; Hou, J.; Qian, J.; Yang, Y.Y. Effects of iron on growth, antioxidant enzyme activity, bound extracellular polymeric substances and microcystin production of Microcystis aeruginosa FACHB-905. *Ecotoxicol. Environ. Saf.* **2016**, *132*, 231–239. [CrossRef] [PubMed]
23. Zhao, Z.M.; Song, X.S.; Wang, W.; Xiao, Y.P.; Gong, Z.J.; Wang, Y.H.; Zhao, Y.F.; Chen, Y.; Mei, M.Y. Influences of iron and calcium carbonate on wastewater treatment performances of algae based reactors. *Bioresour. Technol.* **2016**, *216*, 1–11. [CrossRef] [PubMed]
24. Schowanek, D.; McAvoy, D.; Versteeg, D.; Hanstveit, A. Effects of nutrient trace metal speciation on algal growth in the presence of the chelator [S, S]-EDDS. *Aquat. Toxicol.* **1996**, *36*, 253–275. [CrossRef]
25. Miazek, K.; Iwanek, W.; Remacle, C.; Richel, A.; Goffin, D. Effect of metals, metalloids and metallic nanoparticles on microalgae growth and industrial product biosynthesis: A review. *Int. J. Mol. Sci.* **2015**, *16*, 23929–23969. [CrossRef] [PubMed]
26. Tan, X.B.; Zhang, Y.L.; Yang, L.B.; Chu, H.Q.; Guo, J. Outdoor cultures of Chlorella pyrenoidosa in the effluent of anaerobically digested activated sludge: The effects of pH and free ammonia. *Bioresour. Technol.* **2016**, *200*, 606–615. [CrossRef]
27. Vidyashankar, S.; VenuGopal, K.S.; Swarnalatha, G.V.; Kavitha, M.D.; Chauhan, V.S.; Ravi, R.; Bansal, A.K.; Singh, R.; Pande, A.; Ravishankar, G.A.; et al. Characterization of fatty acids and hydrocarbons of Chlorophycean microalgae towards their use as biofuel source. *Biomass Bioenerg.* **2015**, *77*, 75–91. [CrossRef]
28. Abou-Shanab, R.A.I.; El-Dalatony, M.M.; EL-Sheekh, M.M.; Ji, M.K.; Salama, E.S.; Kabra, A.N.; Jeon, B.H. Cultivation of a new microalga, Micractinium reisseri, in municipal wastewater for nutrient removal, biomass, lipid, and fatty acid production. *Biotechnol. Bioproc. E* **2014**, *19*, 510–518. [CrossRef]
29. Yoshimura, T.; Okada, S.; Honda, M. Culture of the hydrocarbon producing microalga Botryococcus braunii strain Showa: Optimal CO2, salinity, temperature, and irradiance conditions. *Bioresour. Technol.* **2013**, *133*, 232–239. [CrossRef]
30. Zhu, L.D.; Hiltunen, E.; Shu, Q.; Zhou, W.Z.; Li, Z.H.; Wang, Z.M. Biodiesel production from algae cultivated in winter with artificial wastewater through pH regulation by acetic acid. *Appl. Energ.* **2014**, *128*, 103–110. [CrossRef]
31. Yang, I.S.; Salama, E.S.; Kim, J.O.; Govindwar, S.P.; Kurade, M.B.; Lee, M.; Roh, H.S.; Jeon, B.H. Cultivation and harvesting of microalgae in photobioreactor for biodiesel production and simultaneous nutrient removal. *Energ. Convers. Manag.* **2016**, *117*, 54–62. [CrossRef]
32. Papadimitriou, C.A.; Papatheodouiou, A.; Takavakoglou, V.; Zdragas, A.; Samaras, P.; Sakellaropoulos, G.P.; Lazaridou, M.; Zalidis, G. Investigation of protozoa as indicators of wastewater treatment efficiency in constructed wetlands. *Desalination* **2010**, *250*, 378–382. [CrossRef]

33. Ji, M.K.; Yun, H.S.; Park, Y.T.; Kabra, A.N.; Oh, I.H.; Choi, J. Mixotrophic cultivation of a microalga Scenedesmus obliquus in municipal wastewater supplemented with food wastewater and flue gas CO2 for biomass production. *J. Environ. Manag.* **2015**, *159*, 115–120. [CrossRef] [PubMed]
34. Wang, L.; Li, Y.C.; Chen, P.; Min, M.; Chen, Y.F.; Zhu, J.; Ruan, R.R. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. *Bioresour. Technol.* **2010**, *101*, 2623–2628. [CrossRef] [PubMed]
35. Abou-Shanab, R.A.I.; Hwang, J.H.; Cho, Y.; Min, B.; Jeon, B.H. Characterization of microalgal species isolated from fresh water bodies as a potential source for biodiesel production. *Appl. Energ.* **2011**, *88*, 3300–3306. [CrossRef]
36. Laura, G.P. *Algae: Anatomy, Biochemistry, and Biotechnology*; CRC Press: Boca Raton, FL, USA, 2006.
37. Chisti, Y. Biodiesel from microalgae beats bioethanol. *Trends Biotechnol.* **2008**, *26*, 126–131. [CrossRef] [PubMed]
38. Schreiber, C.; Schiedung, H.; Harrison, L.; Briese, C.; Ackermann, B.; Kant, J.; Schrey, S.D.; Hofmann, D.; Singh, D.; Ebenhöh, O.; et al. Evaluating potential of green alga Chlorella vulgaris to accumulate phosphorus and to fertilize nutrient-poor soil substrates for crop plants. *J. Appl. Phycol.* **2018**, *30*, 2827–2836. [CrossRef]
39. Kim, M.K.; Park, J.W.; Park, C.S.; Kim, S.J.; Jeune, K.H.; Chang, M.U.; Acreman, J. Enhanced production of *Scenedesmus* spp. (green microalgae) using a new medium containing fermented swine wastewater. *Bioresour. Technol.* **2007**, *98*, 2220–2228. [CrossRef]
40. Feng, Y.; Li, C.; Zhang, D. Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium. *Bioresour. Technol.* **2011**, *102*, 101–105. [CrossRef]
41. Singh, P.; Guldhe, A.; Kumari, S.; Rawat, I.; Bux, F. Investigation of combined effect of nitrogen, phosphorus and iron on lipid productivity of microalgae *Ankistrodesmus falcatus* KJ671624 using response surface methodology. *Biochem. Eng. J.* **2015**, *94*, 22–29. [CrossRef]

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