



Growth and nutrient removal of *Chlorella vulgaris* in ammonia-reduced raw and anaerobically-digested piggery wastewaters

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ABSTRACT

This study was aimed to investigate the possibility of using raw and anaerobically-digested piggery wastewater as culture media for a green microalga *Chlorella vulgaris* (*C. vulgaris*). Due to high concentration of ammonia and dark color, the microalga did not grow well in this wastewater. In order to solve this problem, air stripping and NaOCl-treatment were applied to reduce the concentration of NH₃-N and the color intensity from the wastewater. Algal growth was monitored in terms of specific growth rate, biomass productivity, and nutrient removal efficiency. As a result, *C. vulgaris* grew without any sign of inhibition in air-stripped and 10-folds diluted anaerobically-digested piggery wastewater with enhanced biomass productivity of 0.57 g/L-d and nutrient removal of 98.7-99.8% for NH₃-N and 41.0-62.5% for total phosphorus. However, NaOCl-treatment showed no significant effect on growth of *C. vulgaris*, although dark color was removed greatly. Interestingly, despite that the soluble organic concentration after air stripping was still high, the biomass productivity was 4.4 times higher than BG-11. Moreover, air stripping was identically effective for raw piggery wastewater as for anaerobic digestate. Therefore, it was concluded that air stripping was a very effective method for culturing microalgae and removing nutrients from raw and anaerobically-digested piggery wastewaters.

Keywords: Air stripping, Ammonia, Anaerobic digestate of piggery wastewater, Biomass productivity, *Chlorella vulgaris*, Nutrient removal

1. Introduction

Piggery wastewater is one of the most-produced wastewaters throughout the world [1-2]. In Korea, piggery wastewater accounted for approximately 53% of livestock excreta, and more than 32 million tons of piggery wastewater were produced in 2015 [3]. Piggery wastewater contains not only organic matters but also nitrogen (N) and phosphorus (P) that cause eutrophication when discharged into water bodies [1, 2, 4, 5]. Piggery wastewater is frequently subjected to anaerobic digestion using acid-forming and methane-producing microorganisms [4, 6-7], by which organic matters are degraded, resulting in the reduction of volatile suspended solids and chemical oxygen demand (COD). However, nitrogen and phosphorus are not removed during anaerobic digestion albeit that trace

amounts of both nutrients are absorbed by microbial cells for growth [4, 8, 9]. The nitrogen in anaerobic digestate of piggery excreta is mainly ammonia which is toxic to living organisms [9]. According to literatures [4, 10-11], anaerobically-digested piggery wastewater contains 1,196-3,141 mg/L of ammoniacal nitrogen and is known to be hardly treated by biological treatment [12].

Many studies have demonstrated that microalgae provide a great potential for removing inorganic nitrogen and phosphorus from wastewater [4, 13-16]. The main mechanism of nutrient removal by microalgae is assimilatory uptake into the cell [13, 17-18]. Microalgae provide other advantages in wastewater treatment, including no need for carbon source because of photosynthetic fixation of carbon dioxide, the possibility of utilizing algal biomass as a fertilizer, the discharge of oxygenated effluent into the water body, and conversion of algal biomass to biodiesel and hydrogen [4,



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12-13]. Thus, the utilization of wastewater as an inexpensive medium for microalgae cultivation could achieve the dual goals of wastewater treatment and biomass/bioenergy production. The most-widely-used microalgae for nutrient removal are species of *Chlorella* [13, 15, 19-22], *Scenedesmus* [4, 15, 22-27], and *Spirulina* [14]. In particular, *Chlorella vulgaris* (*C. vulgaris*) is known to show a high resistance to organic matters and stable growth in various wastewaters [2, 19]. Also, phosphorus, nitrogen and organic matters can be removed at the same time [2, 19, 21]. In terms of bioenergy production, *C. vulgaris* contains high concentration of palmitic acid and linolenic acid, which are suitable for biodiesel production [28]. Although, *Chlorella* sp. and *Scenedesmus* sp. are known to grow well in the presence of high concentrations of ammonia and nitrate [19], concentration of ammonia in anaerobic digestate of piggery wastewater is too high for those microalgae to grow. A high level of ammonia is known to retard the growth of microalgae by disturbing the photosynthesis and nitrate uptake [29-33].

Many studies employed 5-40 folds dilution as a pre-treatment strategy to decrease concentrations of inorganic nutrients (ammonia toxicity), turbidity, and color (light limitation) in raw and anaerobically-digested piggery wastewater prior to cultivation of green microalgae [1-2, 4-5, 34-35]. Also physicochemical pre-treatment technologies were adopted prior to cultivation of green microalgae by using filtration [4, 34, 36], coagulation [31, 36], air stripping [36], NaOCl treatment [34], and ozonation [24, 29].

Ammonia can specifically be removed from wastewater by biological, physical, chemical, and combinations of these methods. Available technologies include adsorption, chemical precipitation, reverse osmosis, ion exchange, air stripping, breakpoint chlorination and biological nitrification and denitrification [37-38]. Among these technologies, air stripping is known to be very efficient for removing ammonia. Bonmati and Flotats [39] reported that air stripping provided many advantages when used for pre-treating anaerobically-digested piggery wastewater, and ammonia removal efficiency was very dependent on the characteristics of piggery wastewater. Cheng *et al.* [40] also commented that air stripping might be an effective option for the piggery wastewater containing a high concentration of ammonia (854 mg/L). The breakpoint chlorination is a commonly applied and well-known method for removing the ammonia and color [24].

For removing strong color, it has been reported that wastewater can be treated with oxidizing agents (e.g., NaOCl, H₂O₂, O₃, etc.) [24, 34, 41-43]. Recently, NaOCl-treated domestic, dairy and piggery wastewaters were used for *Chlorella* cultivation [5, 44]. Other studies suggested that nutrients was separated from the wastewater containing colored substances by using coagulants, flocculants, and adsorbents and then added to the microalgae culture medium [30, 35, 41-42]. In previous studies on low-strength wastewater treatment using microalgal cells, concentrations of total nitrogen (TN) and total phosphorus (TP) were 25.4-53.2 mg/L and 1.2-12.2 mg/L, respectively [15, 23, 45-47]. A few studies have dealt with microalgae cultivation as a strategy for treating anaerobically-digested piggery wastewater [4, 36]. In case of high-strength wastewater containing various inhibitors that hinder the algal growth and nutrients removal, dilution method could be a solution overcoming this problem. However, information on effects of different pre-treatment methods for high-strength wastewater on the growth of microalgae

and nutrient removal is not sufficient.

In this study, ammonia concentration and intensity of color of the anaerobic digestate of piggery wastewater were reduced by air stripping and NaOCl treatment. *C. vulgaris* was incubated under 50 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ of continuous light illumination so that both mixotrophic and autotrophic growth were enabled. The cells of *C. vulgaris* were grown in these pre-treated digestates to determine growth kinetics. Finally, nitrogen and phosphorus removal by algal growth was analyzed. Based on these results, pre-treatment methods for the cultivation of *C. vulgaris* in anaerobically-digested piggery wastewater were suggested.

2. Materials and Methods

2.1. Microalgal Strain and Culture Conditions

The microalgal strain used in this study was *C. vulgaris* KCTC AG10032 obtained from the Biological Resource Center of the Korea Research Institute of Bioscience and Biotechnology. *C. vulgaris* was maintained in the Blue-Green (BG-11) medium consisting of NaNO₃ (1.5 g/L), K₂HPO₄ (40 mg/L), MgSO₄·7H₂O (75 mg/L), CaCl₂·2H₂O (36 mg/L), Na₂CO₃ (20 mg/L) and FeCl₃·6H₂O (3.15 mg/L), citric acid (6 mg/L) and 1 mL/L of trace elements solution containing H₃BO₃ (2.86 mg/L), MnCl₂·4H₂O (1.81 mg/L), ZnSO₄·7H₂O (0.22 mg/L), Na₂MoO₄·2H₂O (0.39 mg/L), CuSO₄·5H₂O (0.08 mg/L) and Co(NO₃)₂·6H₂O (0.05 mg/L). The initial pH of the medium was adjusted to pH 6.8. Pre-cultured algal suspension ($2-3 \times 10^8$ cells/mL) was inoculated at 10% (v/v) ratio into 200 mL BG-11 medium contained in a 500 mL Erlenmeyer flask. Then the flask was incubated at 25°C and 50 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ of continuous cool-white fluorescent light illumination with rotation at 150 rpm. Although additional carbon dioxide or oxygen was not supplied, these gases contained in ambient air might be dissolved into the medium because flasks were closed with cotton plugs. Cells grown in this flask were inoculated into various media in Table S1.

2.2. Pre-treatment of Anaerobic Digestate of Piggery Wastewater

The raw piggery wastewater (PW) used in this study was obtained from storage tanks on a swine farm located in Yongin, Korea, which contained the mixture of feces, urine, and tap water that was used for cleaning pig pens. After arriving at the laboratory, the piggery wastewater was filtered through a 2 mm-mesh to remove the coarse particles and then stored at 4°C until use. This filtered wastewater has been anaerobically digested at the organic loading rate of 2.51 g COD/L·d in a 30 L reactor for longer than 3 y.

Air stripping of raw PW and anaerobic digestate (AD) was conducted in a cylindrical 1.0 L reactor (ID 90 mm × H 200 mm) with a working volume of 0.5 L. The pH of the wastewater was adjusted to pH 10.0 using 5 N NaOH and added into the stripping reactor kept at 37°C. Air was introduced directly into the liquid phase of the reactor via an aquatic air stone at the aeration rate of 1 L/min for 12 h. In order to compensate water loss during air stripping, distilled water was added up to the initial volume. Chlorination of wastewater was carried out with sodium hypochlorite (NaOCl) at the dosage of 1.2 g/L (Cl:NH₃-N mass ratio of 3.6:1) for 2 h, and then chlorination reaction was immediately stopped

by adding a stoichiometric amount of sodium sulfite (Na_2SO_3).

These pre-treated wastewaters were re-adjusted to pH 7.6 using 5 N HCl and used for growing microalgal cells under the same culture conditions as above. Before inoculation, all media in Table S1 except BG-11 were 10-folds diluted with distilled water to reduce the toxic influence of ammonia on algal growth as Park *et al.* [4] did.

2.3. Determination of Microalgal Growth

For monitoring microalgal growth, optical density was measured at 680 nm every day using a spectrophotometer (Genesys 20, Thermo spectronic, Waltham, Massachusetts, USA). Number of microalgal cells was also counted microscopically using a Neubauer hemocytometer (INCYTO C-Chip, Korea). For measuring dry cell weight of algal biomass, 10 mL of culture were filtered through a mixed cellulose ester membrane filter (0.2 μm , Advantec, Japan), rinsed with deionized water, dried in an oven at 105°C for 12 h, cooled in a silica gel desiccator and then weighed. The relationship between optical density (680 nm) and dry cell weight appeared linear as in Eq. (1).

$$\text{Dry cell weight (g/L)} = 0.42 \times \text{OD}_{680} - 0.13 \quad (R^2 = 0.98) \quad (1)$$

The specific growth rate (μ , d^{-1}) in an exponential phase of algal growth was calculated by using Eq. (2);

$$\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1) \quad (2)$$

where X_1 and X_2 were dry cell weights (g/L) at time t_1 and t_2 , respectively. The biomass productivity (P_{biomass} , g/L·d) was calculated according to Eq. (3);

$$P_{\text{biomass}} = (X_2 - X_1)/(t_2 - t_1) \quad (3)$$

The growth yield ($Y_{X/S}$, dimensionless) was calculated using Eq. (4);

$$Y_{X/S} = (X_2 - X_1)/(S_2 - S_1) \quad (4)$$

where X and S were the dry cell weight (g/L) and concentration of total dissolved nitrogen (TDN) (g/L), respectively.

2.4. Analytical Methods

COD was analyzed using the HACH colorimetric method 8,000 and the DR/2,500 spectrophotometer (HACH, Loveland, Colorado, USA). TN and TP were analyzed based on the persulfate digestion method using HACH type HR total nitrogen Test 'N Tube™ vials and HACH type HR total phosphorus Test 'N Tube™ vials, respectively. Concentrations of ammoniacal nitrogen ($\text{NH}_3\text{-N}$) and nitrate nitrogen ($\text{NO}_3\text{-N}$) were measured by using HACH type HR ammonia Test 'N Tube™ vials and HACH type HR nitrate Test 'N Tube™ vials, respectively. True and apparent colors were measured following the Platinum-Cobalt Standard Methods (120 color, 455 nm) [48]. Total solids (TS) were measured following the Standard Methods [49], and pH was measured using a pH meter (Orion star A211, Thermo spectronic, Waltham, Massachusetts, USA). For measuring soluble chemical oxygen demand (SCOD), TDN, and total dissolved phosphorous (TDP), samples were centri-

fuged at 1,800 g for 10 min, and the supernatants were filtered through 0.2 μm membranes (Advantec, Japan). Measurements were carried out in triplicate and expressed as means \pm standard deviations (SD). Results of nutrient removal were statistically analyzed by one-way analysis of variance (ANOVA) and then by Tukey's post hoc analysis with significance level at $p < 0.05$ using SPSS for Windows ver. 22.0 (IBM Corp., Armonk, New York, USA).

3. Results and Discussion

3.1. Characteristics of Anaerobically-digested Piggery Wastewater and Algal Growth

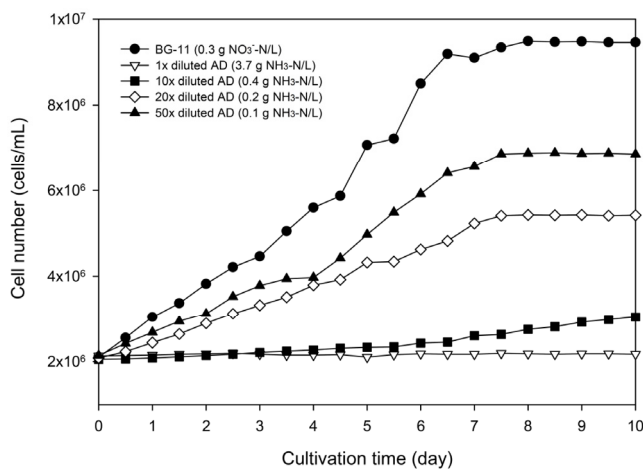
As shown in Table 1, strength of raw piggery wastewater was dramatically lowered after anaerobic digestion. Total COD (TCOD) and SCOD of raw PW decreased from 75.4 g/L and 24.4 g/L to 16.7 g/L (77.9% reduction) and 6.1 g/L (75.0% reduction), respectively, in AD. TN and TP were also reduced by anaerobic digestion from 5.9 g/L to 4.0 g/L (32.2% reduction) and from 470 to 300 mg/L (36.2% reduction), respectively. However, the $\text{NH}_3\text{-N}$, the main component of TN, decreased slightly from 3.95 g/L to 3.70 g/L. TS also decreased a little from 59.6 g/L to 53.4 g/L. Meanwhile color and pH obviously increased from 7,133 Pt-Co to 8,367 Pt-Co and from pH 7.6 to pH 8.5, respectively. Dark brown color of PW changed to black after anaerobic digestion probably due to formation of iron sulfide (FeS) and humic substances derived from incomplete breakdown of lignin in PW [41]. Characteristics of pre-treated anaerobic digestate (AD_C AD_S, and AD_M) will be discussed later.

C. vulgaris AG10032 growth as indicated by cell numbers in the BG-11 medium and 1-, 10-, 20- and 50-folds diluted anaerobic digestate of piggery wastewater (AD) are shown in Fig. 1. Although COD was greatly reduced by anaerobic digestion, *C. vulgaris* did not grow at all in the undiluted AD (1x diluted AD). This was thought to be due to high concentration of $\text{NH}_3\text{-N}$ in AD (3.7 g/L of $\text{NH}_3\text{-N}$). Tam and Wong [50] observed a complete inhibition of growth of *C. vulgaris* in a commercial Bristol medium containing 750 mg/L of $\text{NH}_3\text{-N}$. Park *et al.* [4] also observed that growth of *Scenedesmus* sp. was completely inhibited in anaerobic digestate of piggery wastewater containing 1 g/L of ammonium-nitrogen ($\text{NH}_4^+\text{-N}$). In case of 10-folds diluted AD containing 400 mg/L of $\text{NH}_3\text{-N}$, the alga did not grow until 5.5 d of cultivation and started to grow slowly after 6 d. For higher dilutions, the alga grew steadily in 20- and 50-folds diluted ADs (100-200 mg/L of $\text{NH}_3\text{-N}$) without noticeable lag phases. Similar results were also reported by Khanh *et al.* [29], Wang *et al.* [20] and Wang *et al.* [21] who observed algal growth in 20-folds diluted digestate (43-100 mg/L of $\text{NH}_3\text{-N}$). It was suspected that a high level of ammonia in AD delayed the growth of microalgal cells via the ammonia toxicity, high pH, and the uncoupling effect on electron transport in photosystems [29]. A simple way to overcome the toxic effect of ammonia was to dilute anaerobic digestate as shown by this and other studies.

In addition to high concentrations of $\text{NH}_3\text{-N}$, dark color of AD was also thought to attribute to the poor growth of *C. vulgaris*. As shown in Table 1, a high level of TS (53 g/L) and the strong black color (8,367 Pt-Co) of non-diluted AD obviously reduced light penetration into the culture medium and thereby hindered

Table 1. Characteristics of Various Wastewaters Used in This Study (mean \pm SD)

	PW	PW_S	AD	AD_S	AD_C	AD_M
pH	7.63	9.49	8.49	9.48	7.63	7.63
TS (g/L)	59.6 \pm 0.51	56.7 \pm 0.78	53.4 \pm 4.72	40.1 \pm 2.29	26.0 \pm 0.45	19.5 \pm 0.1
TCOD (g COD/L)	75.4 \pm 0.1	70.1 \pm 0.56	16.7 \pm 0.15	13.8 \pm 0.5	8.9 \pm 0.32	6.5 \pm 0.36
SCOD (g COD/L)	24.4 \pm 0.27	19.9 \pm 0.36	6.1 \pm 0.38	5.9 \pm 0.27	5.0 \pm 0.35	4.3 \pm 0.2
TN (g N/L)	5.9 \pm 0.3	2.2 \pm 0.1	4.0 \pm 0.12	2.0 \pm 0.15	3.8 \pm 0.06	1.4 \pm 0.2
Ammoniacal-N (g NH ₃ -N/L)	3.95 \pm 0.03	1.08 \pm 0.02	3.7 \pm 0.06	0.92 \pm 0.01	3.0 \pm 0.03	0.8 \pm 0.02
Nitrate-N (g NO ₃ -N/L)	0.04 \pm 0.01	0.04 \pm 0.00	0.12 \pm 0.01	0.12 \pm 0.00	0.48 \pm 0.02	0.49 \pm 0.02
TP (g P/L)	0.47 \pm 0.01	0.44 \pm 0.00	0.3 \pm 0.01	0.29 \pm 0.01	0.27 \pm 0.01	0.29 \pm 0.01
Color (Pt-Co)	7,133 \pm 153	4,067 \pm 231	8,367 \pm 208	7,767 \pm 306	5,583 \pm 95	4,400 \pm 458

**Fig. 1.** Cell numbers of *C. vulgaris* grown in BG-11 and diluted anaerobic digestates (AD) of piggery wastewater.

microalgal growth [42, 51-52]. According to Moheimani *et al.* [41], the dark color lowered cellular productivity by 20-30% when compared to a non-colored culture medium. These results together showed that successful algal growth in diluted anaerobic digestate was thought to be brought by not only reduced ammonia toxicity but also improved light transmittance.

3.2. Pre-treatment of Anaerobic Digestate for Ammonia and Color Removal

In many studies on cultivation of microalgae in animal-manure wastewater, ammonia toxicity and light limitation were avoided by diluting the wastewater with clean water [5, 13, 34, 41-42], and Fig. 1 also confirmed this observation. However, the dilution method for large-scale systems would be unsustainable because a large volume of water is demanded [41-42]. Therefore, it was necessary in this study to lower the ammonia concentration and color intensity for minimizing dilution with water. In this context, air stripping, chlorination, and a combination of these two methods were adopted as pre-treatment methods for AD.

As shown in Table 1 and Fig. 2, air stripping (AD_S) decreased concentrations of TN and NH₃-N of AD from 4,000 mg/L to 2,000 mg/L (50.0% removal) and from 3,700 mg/L to 920 mg/L (75.0%

removal), respectively. Concentrations of TS and TCOD were also lowered from 53.4 g/L to 40.1 g/L (24.8% removal) and from 16.7 to 13.8 g/L (17.0% removal), respectively, while intensity of color decreased slightly (from 8,367 Pt-Co to 7,767 Pt-Co). In case of NaOCl-treated AD (AD_C), removal efficiencies of TS, TCOD, and color were higher than those achieved by air stripping. Concentrations of TS and TCOD were reduced from 53.4 g/L to 26.0 g/L (51.1% removal) and from 16.7 to 8.9 g/L (46.4% removal), respectively. Color intensity decreased from 8,367 Pt-Co to 5,583 Pt-Co. Contrary to anticipation, chlorination did not decrease TN and NH₃-N much, *i.e.*, from 4,000 mg TN/L to 3,800 mg TN/L and from 3,700 mg NH₃-N/L to 3,000 mg NH₃-N/L. Interestingly, concentration of nitrate (NO₃-N) increased four folds (Table 1). It has been known that chlorination converts ammonia to nitrogen gas, but some of nitrogen still remains as chloramine, nitrate, and nitrogen trichloride (NCl₃) [53]. Pressley *et al.* [53] also reported that about 10% of NH₃-N was converted to nitrate at pH 8 or higher. As summarized in Fig. 2, air stripping efficiently removed ammoniacal nitrogen in AD (75.0%), but NaOCl did not (18.6%) remove ammonia as much as air stripping did. However, NaOCl-treatment was more efficient for removing color (33.3%) than air stripping (7.2%). These results suggested that NaOCl-treatment might be applicable only when light penetration is a limiting factor for microalgae cultivation.

In case of the combined treatment of air stripping and NaOCl-treatment (AD_M), concentrations of TN, NH₃-N, TS, TCOD, and color intensity were reduced to 1,400 mg/L (65.4% removal), 800 mg/L (78.4% removal), 19.5 g/L (63.3% removal), 6.5 g/L (61.0% removal), and 4,400 Pt-Co (47.5% removal), respectively. Meanwhile, the concentration of nitrate increased to 490 mg/L. Concentration of TP did not decrease in all cases (AD_S, AD_C, and AD_M). By the combined treatment of air stripping and NaOCl treatment, the removal efficiencies of TS, TCOD and color in AD_M were improved by 12.2%, 14.6% and 14.2% compared to AD_C. TN removal efficiency was also improved by 15.8% compared to AD_S. However, in the case of ammoniacal nitrogen removal efficiency, AD_M was only 3% higher than AD_S. Thus it was summarized that the combined treatment of air stripping and NaOCl treatment was somewhat effective for removing TS, TCOD, color and TN, but the removal efficiency of ammoniacal nitrogen was not significantly improved.

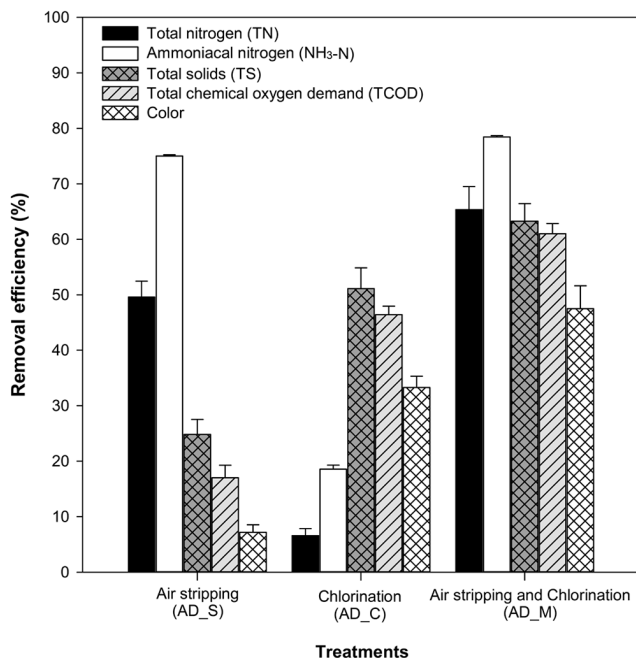


Fig. 2. Changes of characteristics of anaerobic digestate by air stripping, chlorination, and combined treatments (The error bars represent the standard deviation of three replicates).

3.3. Assessment of Microalgal Growth

C. vulgaris was grown in five different culture media for 14 d and growth parameters were estimated and summarized in Table 2. *C. vulgaris* showed slow or nearly no growth in the 10-folds diluted AD and AD_C as also shown in Fig. 1. Specific growth rates (μ) of cultures in the 10-folds diluted AD and AD_C were only 0.026 and 0.004 d⁻¹, respectively. Doubling times of *C. vulgaris* in AD and AD_C were longer than 26 d. By contrast, *C. vulgaris* grew fast in 10-folds diluted AD_S and AD_M media, in which ammonia concentration was greatly reduced by air stripping. Concentrations of TDN and NH₃-N in AD_S and AD_M were 102.7-103.3 mg TDN/L and 79.7-92.3 mg NH₃-N/L, respectively. Specific growth rates of *C. vulgaris* in AD_S and AD_M were 0.195

and 0.112 d⁻¹, respectively. Thus doubling time in AD_S and AD_M were 3.56 and 6.19 d, respectively. In particular, specific growth rate and doubling time of *C. vulgaris* grown in AD_S were equal or comparable to those grown in the BG-11 medium (0.195 d⁻¹ and 3.55 d). Surprisingly, even 10-folds diluted and air stripped PW (0.1x PW_S) supported growth of *C. vulgaris* (will be discussed further later). Meanwhile, *C. vulgaris* did not grow at all in the undiluted four different anaerobic digestates (AD, AD_S, AD_C, AD_M) (data not shown).

The maximum biomass concentration (X_{max}) and biomass productivity ($P_{biomass}$) of *C. vulgaris* grown in the BG-11 medium were 0.35 g/L and 0.05 g/L-d, respectively. Those obtained in 10-folds diluted AD_S were the highest, reaching 1.51 g/L and 0.22 g/L-d. Although *C. vulgaris* survived in 10-fold diluted AD, the biomass concentration and biomass productivity of *C. vulgaris* were markedly lower than those in the ammonia-stripped media (AD_S and AD_M). Again culture in AD_C showed the lowest biomass concentration (0.02 g/L) and biomass productivity (0.003 g/L-d) because ammonia concentration was still high (Table 1). The biomass productivity in AD_S was a little lower than that of Zhu *et al.* [5], who achieved 0.296 g/L-d when they cultivated *Chlorella zofingiensis* in the autoclaved 1.8-folds-diluted piggery wastewater (1,900 mg/L of COD, 80 mg/L of TN, and 85 mg/L of TP). A similar $P_{biomass}$ was also reported by Cheng *et al.* [40], who achieved 0.22 g/L-d for *Desmodesmus* sp. CHX1 cultivated in air-stripped and autoclaved piggery wastewater (1,800 mg/L of COD, 100 mg/L of TN, and 7.5 mg/L TP). Therefore, it was suspected that ammonia removal was essential not only for enhancing biomass productivity of *C. vulgaris* in anaerobically-digested piggery wastewater but also for cultivating other microalgal species in other ammonia-rich wastewaters. The growth yield coefficients (Y) ranged from 1.59 to 24.22 mg/mg (Table 2). High yields were found in AD_S (24.22 mg/mg) and AD_M (13.22 mg/mg). Ji *et al.* [1] reported that growth yield of *Scenedesmus obliquus* was 6.7-7.1 mg/mg in diluted-piggery wastewater containing 280-961 mg/L of NH₃-N. In case of 10-fold diluted AD and AD_C, very low growth yields were obtained at 1.59 and 3.49 mg/mg, respectively. This retarded algal growth in AD and AD_C was attributed to the high concentration of ammonia (> 300 mg/L of NH₃-N). This observation confirmed that the ammonia concentration was a critical parameter rather than color intensity

Table 2. Growth Parameters of *C. vulgaris* Grown in Five Different Culture Media

Medium ^a	Specific growth rate (μ , d ⁻¹) ^b	Doubling time (t_d , d) ^b	Maximum biomass concentration (X_{max} , g/L) ^c	Biomass productivity ($P_{biomass}$, g/L-d) ^b	Growth yield (Y , mg/mg) ^d
0.1 × PW _S	0.204	3.40	3.98	0.57	37.67
BG-11	0.195	3.55	0.35	0.05	3.03
0.1 × AD	0.026	26.36	0.32	0.03	1.59
0.1 × AD _C	0.004	189	0.02	0.003	3.49
0.1 × AD _S	0.195	3.56	1.52	0.22	24.22
0.1 × AD _M	0.112	6.19	0.93	0.07	13.22

^a Media were 10-fold diluted (0.1x) except BG-11.

^b Specific growth rate, doubling time, and biomass productivity were calculated in terms of dry cell weight at 7 d cultivation.

^c Maximum biomass productions were measured over 14 d of cultivation.

^d Growth yields were calculated based on TDN uptake for 14 d of cultivation.

for determining growth of *C. vulgaris*.

Markou *et al.* [43] treated olive-oil mill wastewater with sodium hypochlorite (NaOCl) for phenol and color removal, by which biomass productivity of *Arthrospira* was enhanced. However, Zhu *et al.* [34] reported that cultivation of *C. zofingiensis* in NaOCl-treated piggery wastewater did not enhance nutrient removal, algal growth and biodiesel production. In this study, *C. vulgaris* grown in NaOCl-treated anaerobic digestate (AD_C) also showed the lowest specific growth rate and biomass productivity. One possible reason for the poor growth of *C. vulgaris* might be high levels of inorganic nitrogen species including ammonia (300 mg/L of NH₃-N) and nitrate (48 mg/L of NO₃-N). Another reason could be found in inhibitory effects of chlorinated disinfection by-products (DBPs). Liang *et al.* [54] suspected that formation of DBPs from chlorination of organic pollutants might be responsible for the growth inhibition of *Chlorella pyrenoidosa*. Yang *et al.* [55] also observed the toxicological effect of halogenated DBPs in the chlorinated wastewater effluent against growth of marine alga *Tetraselmis marina*.

Biomass productivities of 10-fold diluted PW_S, AD_S and AD_M, all of which were air stripped, were 11.4 times, 4.4 times and 1.4 times higher than that of BG-11, respectively. On the other hand, biomass productivity decreased in 10-fold diluted AD_C possibly due to toxicity of chlorinated disinfection by-products. Therefore, it was decided to apply air stripping for pre-treating AD rather than NaOCl-treatment.

3.4. Effect of SCOD Concentration on Growth of *C. Vulgaris*

Since *C. vulgaris* grew very slowly even in 10-folds diluted AD (Fig. 1 and Table 2), it was not expected at all at first that *C. vulgaris* might grow in raw piggery wastewater. However air stripping appeared very effective to remove ammonia and enable *C. vulgaris* to grow in high-strength wastewater. Thus, 10-fold diluted raw piggery wastewater was air-stripped under the same conditions as in AD_S and AD_M to examine if *C. vulgaris* grew. SCOD and NH₃-N concentrations in this 10-fold diluted ammonia-stripped piggery wastewater (0.1x PW_S) were 2,000 mg/L and 108 mg/L (refer to Table 1), respectively, which was higher than 0.1x AD_S and 0.1x AD_M (400-600 mg/L of SCOD and 80-92 mg/L of NH₃-N). On the other hand, color levels of 10-fold diluted PW_S (400 Pt-Co) was lower than 0.1x AD_S and 0.1x AD_M (440-780 Pt-Co). Surprisingly, cultivation of *C. vulgaris* in 10-fold diluted PW_S for 14 d resulted in high nutrient removal efficiencies of 99.8% for NH₃-N, 76.9% for TDN, 62.5% for TDP, and 81.9% for SCOD (data not shown), high specific growth rate (0.20 d⁻¹), high biomass productivity (0.57 g/L-d), and high growth yield (37.67 mg/mg) (Table 2). Again as summarized in Table 2, the biomass productivities (P_{biomass}) of the 10-fold diluted ammonia-stripped wastewaters (0.1x PW_S, 0.1x AD_S, and 0.1x AD_M) were much higher than that of BG-11.

Moreover, it was found that P_{biomass} was linearly correlated with the initial SCOD concentration ($R^2 = 0.97$). As shown in Fig. 3, initial SCOD concentrations of BG-11, 0.1x AD_M, 0.1x AD_S, and 0.1x PW_S were 0.3 g/L, 0.43 g/L, 0.59 g/L and 1.99 g/L, respectively, and P_{biomass} were 0.05 g/L-d, 0.07 g/L-d, 0.22 g/L-d, 0.57 g/L-d, respectively. The growth yield (Y) was also well correlated

with the log of initial SCOD concentration ($R^2 = 0.95$). Accordingly, Y was obviously well correlated with P_{biomass} ($R^2 = 0.97$, data not shown). Li *et al.* [19] reported that when *Chlorella* sp. was grown in autoclaved (initial concentration of 2,390 mg COD/L) and raw (initial concentration of 2,304 mg COD/L) wastewaters, the removal efficiency of COD were 90.3% and 90.8%, respectively. Li *et al.* [19] also reported that, in the presence of high COD in the wastewater, the microalgal growth was vigorous without a lag phase. These results suggested that a high concentration of COD might enhance the growth of *C. vulgaris* and correspondingly nutrients removal if ammonia concentration was appropriately adjusted. Previous studies have reported that some *Chlorella* sp. are mixotrophic so that CO₂ and organic carbon could be used simultaneously [56-57]. Especially, Martinez *et al.* [56] found that the *C. pyrenoidosa* completely consumed the organic substrates when cultured under a weak light condition (~1,500 Lux). Lalucat *et al.* [57] also reported that *Chlorella* sp. grew heterotrophically by utilizing various organic compounds.

The P_{biomass} in 10-fold diluted PW_S, 0.57 g/L-d, was higher than that 0.222 g/L-d of a previous report of Cheng *et al.* [40] that was obtained from cultivation of *Desmodesmus* sp. CHX1 in ammonia-stripped piggery wastewater. Furthermore, when they supplemented CO₂ in the same media, P_{biomass} increased to 0.869 g/L-d. Cheng *et al.* [40] and Kim *et al.* [36] reported that microalgal biomass concentration, productivity, and specific growth rate increased in the presence of CO₂. These results indicated that CO₂ supplementation could enhance microalgal growth and biomass productivity in ammonia-stripped raw and anaerobically-digestated piggery wastewater. The biomass productivities of 0.1x AD_S and 0.1x AD_M, which contained high concentrations of organic matters were 4.4 and 1.4 times, respectively, higher than that of a mineral medium, BG-11.

In the presence of light and organic matters, *C. vulgaris* is known to convert to mixotrophic growth consuming both organic matters and CO₂ [56-60]. According to Li *et al.* [60] and Liang *et al.* [61], the C/N ratio affected the mixotrophic growth and lipid content of *C. vulgaris*. Other researchers also reported that the optimal C/N ratio was 5-15 for cultivating *C. vulgaris* mixotrophically [57-58]. Based on well-known microalgal cell composition (C_{1.06}H_{1.81}O_{0.45}N_{0.16}P_{0.01}) [62], the C/N molar ratio is 6.6 (mass ratio of 8.9), and sufficient carbon and nitrogen are required for vigorous growth. In this study, the initial C/N ratios were different for different wastewaters and the pre-treatment methods. As shown in Fig. 4, the initial C/N ratios (*i.e.*, SCOD/TDN ratios) of 0.1x PW_S, 0.1x AD, 0.1x AD_S, 0.1x AD_C and 0.1x AD_M were 14.53, 1.79, 5.73, 1.56 and 4.17, respectively. C/N ratios of both 0.1x PW_S and 0.1x AD_S were in the optimal C/N ratio range and showed higher specific growth rates (0.204 and 0.195 d⁻¹, respectively) than the other pre-treatments (0.004-0.112 d⁻¹) (Table 2). Although the chlorination method was commonly used, AD_M with a high C/N ratio showed a specific growth rate about 28 times higher than that of AD_C. On the other hand, poor growth were observed in 0.1x AD and 0.1x AD_C with low C/N ratios. The characteristics of PW_S and AD_S (also 0.1x PW_S and 0.1x AD_S) were not significantly different except for COD, color and TP (Table 1 and Fig. 4). However, specific growth rate, maximum biomass concen-

tration, biomass productivity and growth yield of *C. vulgaris* grown in 0.1x PW_S were 1.05, 2.62, 2.59 and 1.56 times higher than those obtained in 0.1x AD_S, respectively. These results suggested that mixotrophic growth of *C. vulgaris* was dependent on not only C/N ratios but also refractory organic matters. According to Zeng *et al.* [63], a high proportion of refractory organic matters were found after anaerobic digestion of piggery wastewater. Thus, when microalgae were subjected to mixotrophic growth, 0.1x PW_S containing high levels of easily-biodegradable organic seemed to support stronger growth than AD_S.

Thus, it was clear that mixotrophic microalgal cells could be cultured in a medium containing a high concentration of organic matters if ammoniacal nitrogen concentration is reduced to an uninhibitory level and suitable C/N ratio.

To summarize, *C. vulgaris* used in the present study grew without any sign of inhibition in the 10-folds diluted air-stripped PW (PW_S) containing initial concentrations of 2,000 mg SCOD/L, 220 mg TN/L (137 mg TDN/L), 100 mg NH₃-N/L, 40 mg TP/L (15 mg TDP/L) (Tables 1 and 2). These results were similar to Zhu *et al.* [5], who also investigated the growth of *Chlorella* sp. under the controlled conditions. They reported that the diluted piggery wastewater containing 1,900 mg/L COD, 80 mg/L TN and 85 mg/L TP provided optimal conditions for *C. zofingiensis* cultivation. Chen *et al.* [30] also reported that the optimal growth rate of *Chlorella* sp. was obtained in an anaerobic digestate containing 200 mg/L TN and 2.5 mg/L TDP. Although high strength-wastewater contained organic matters, the biomass productivity of 0.1x PW_S was 2.5 times higher than Cheng *et al.* [40] and the results of 10-fold diluted AD_S was similar to those of a previous study [40]. Thus it was reasonable to conclude that air stripping was an effective method for culturing microalgae in the raw and anaerobically-digested piggery wastewaters.

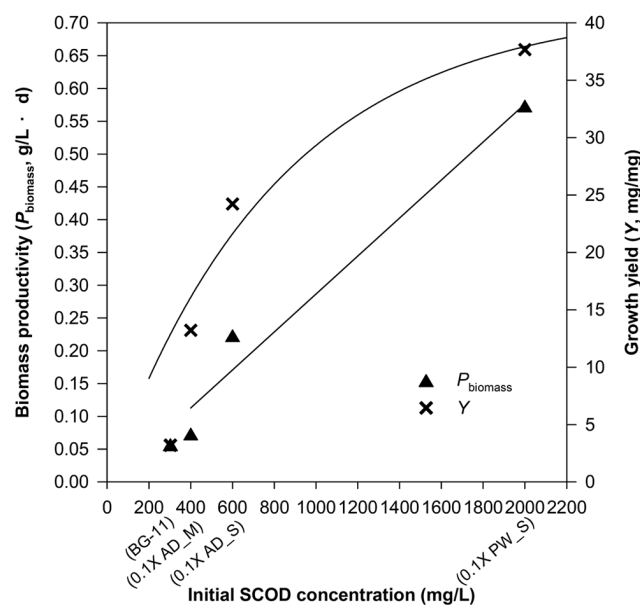


Fig. 3. Effects of initial SCOD concentration on biomass productivity ($P_{biomass}$) and growth yield (Y) of *C. vulgaris* grown in BG-11 medium and 10-folds diluted AD_M, AD_S, and PW_S.

3.5. Nutrient Removal by *C. vulgaris*

Fig. 4 shows NH₃-N, TDN, TDP, and SCOD removal by *C. vulgaris* grown for 14 d in 10-folds diluted PW_S, AD, AD_C, AD_S, and AD_M. Nutrient uptake of *C. vulgaris* appeared clearly different according to pre-treatment methods (air stripping and NaOCl-treatment). The culture in 0.1x AD_C showed the lowest nutrient removal efficiency ($p < 0.05$), because *C. vulgaris* did not grow well in this medium (Table 2). The culture in air-stripped media (0.1x PW_S, 0.1x AD_S and 0.1x AD_M) showed the high NH₃-N removal efficiencies of 98.7-99.8% ($p < 0.05$). The concentrations of NH₃-N decreased from 108.0 ± 2.0 to 0.2 ± 0.2 mg/L in 0.1x PW_S, from 92.3 ± 0.6 to 1.2 ± 0.4 mg/L in 0.1x AD_S and from 79.7 ± 1.5 to 0.6 ± 0.1 mg/L in 0.1x AD_M. The removal efficiency of TDN was also high in 0.1x PW_S and 0.1x AD_M ($p < 0.05$). As shown in Fig. 4(b), concentrations of TDN decreased from 137.3 ± 1.5 to 31.7 ± 4.9 mg/L in 0.1x PW_S (76.9% uptake) and from 102.7 ± 2.1 to 32.3 ± 0.6 mg/L in 0.1x AD_M (68.5% uptake). Similar results were reported by Wang *et al.* [35], who found that *C. pyrenoidosa* cultivated in 10-, 15-, 20-, and 40-folds diluted piggery wastewater (34.7-138.8 mg/L of initial NH₃-N concentration) showed removal efficiencies of 91.2-95.1% for NH₃-N, 54.7-74.6% for TN, and 31.0-77.7% for TP. Kumar *et al.* [64] also reported that the maximum NH₃-N reduction by *C. vulgaris* was 61.8% in 50-folds diluted anaerobic digestate of piggery wastewater containing 20 mg/L of initial NH₃-N. *C. vulgaris* grown in 0.1x PW_S and 0.1x AD_S removed 62.5% and 58.9% of TDP, respectively. From these final concentrations, it seems to be possible to satisfy the effluent limitation guidelines for livestock excreta in Korea (60 mg/L of TN and 8 mg/L of TP) [65] by combining air stripping and algal cultivation. The removal efficiencies of SCOD in 0.1x PW_S, 0.1x AD, 0.1x AD_C, 0.1x AD_S and 0.1x AD_M were 81.9% (residual concentration (RC): 360 mg/L), 58.3% (RC: 255 mg/L), 47.0% (RC: 265 mg/L), 59.2% (RC: 240 mg/L) and 53.6% (RC: 199 mg/L) (Fig. 4(d)). Thus it was obvious that microalgal culture removed not only nitrogen and phosphorus but also dissolved organic matters at the same time.

Closely examining the experimental data of Table 2 and Fig. 4, it was suspected that microalgal growth and concomitant nutrient removal might be affected by initial concentration of nitrogen or N/P ratio in the wastewater. To investigate the effect of initial nitrogen concentration on the removal efficiency of nitrogen and phosphorus, the concentrations of TN and TP in various wastewaters and removal efficiencies were surveyed and summarized in Table 3. Microalgae were used to treat municipal wastewater [15, 23, 66], industrial wastewater [67-68], piggery wastewater [5, 33, 69], anaerobic digestate [69-70] and synthetic wastewater [13, 71-72]. Commonly-used microalgae were *C. vulgaris* [13, 73], *C. emersonii* [22], *C. pyrenoidosa* [67], *Chlorella* sp. [70], *Scenedesmus obliquus* [15, 74], *Scenedesmus* sp. [25], etc. In some studies, naturally-borne microalgae [75] or more than two species of microalgae [76-77] were used for wastewater treatments. The initial concentrations of nitrogen and phosphorus ranged from 2.5 to 410 mg/L and from 2.1 to 199 mg/L, respectively. For these initial concentrations of nutrients, the removal efficiencies of nitrogen and phosphorus were 2.5-100% and 6.0-100%, respectively. Different illumination intensity, initial concentration of nutrients and molar ratio of N/P seemed to result in different removal efficiencies.

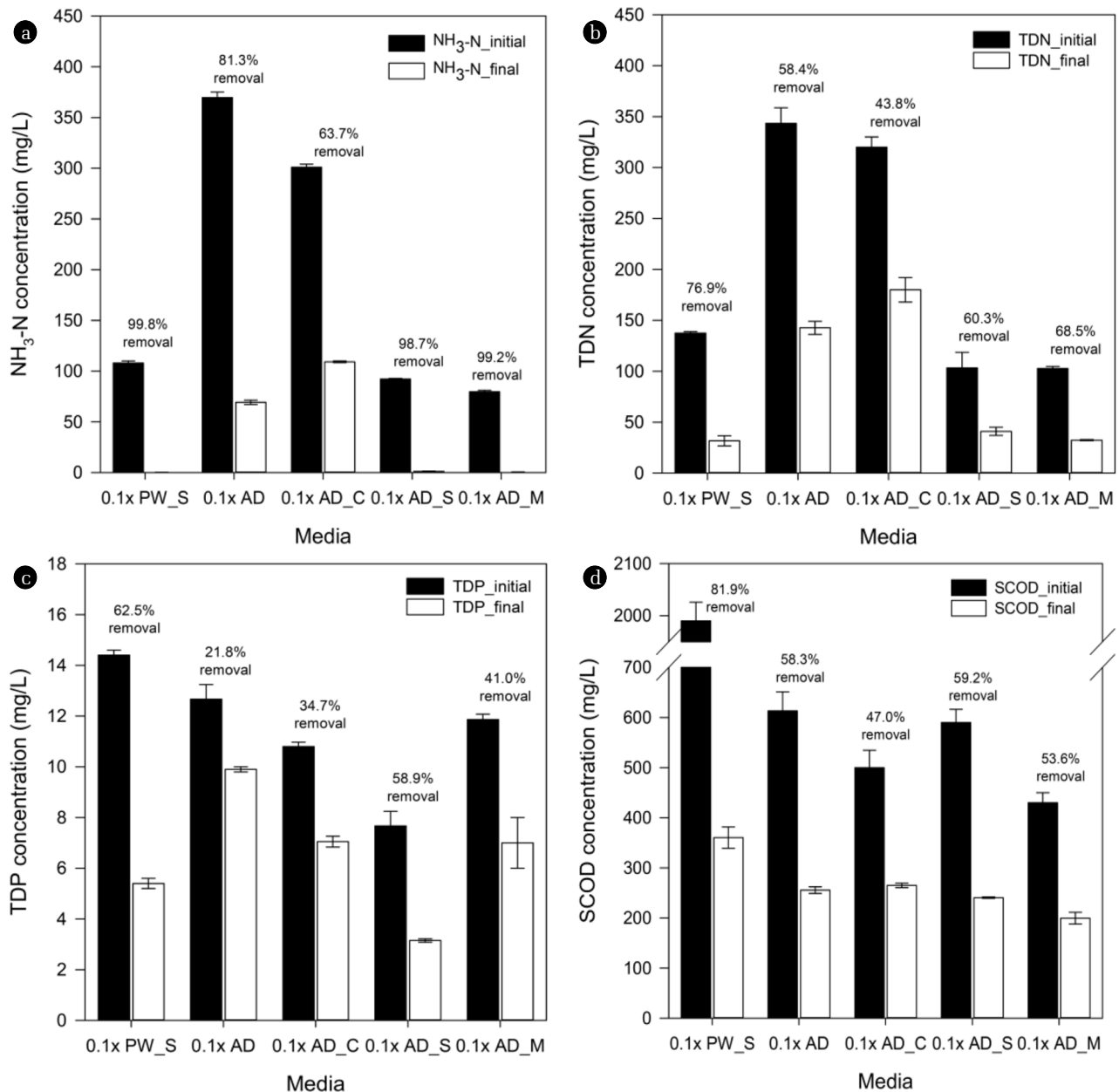


Fig. 4. The removal of (a) NH₃-N, (b) TDN, (c) TDP, and (d) SCOD by *C. vulgaris* grown for 14 d in 10-folds diluted (0.1x) wastewaters. The error bars represent the standard deviation of three replicates.

Fig. 5 that was drawn using data of Table S3 and Fig. 4 shows correlation between the removal efficiencies of TN and TP, and the initial concentration of TN. Regardless of wastewater characteristics, light intensity, temperature and microalgal species, the removal efficiencies of TN and TP appeared to decrease when the initial concentration of TN was higher than 100 mg/L. When the initial concentration of TN is was lower 100 mg/L, the removal efficiency seemed to be determined by various factors of the illumination intensity, the growth of microalgae species, the consumption rate of nitrogen and the presence of inhibitors in wastewater. One of the reasons why the high initial TN concentration lowered re-

moval of TN and TP might be the negative effect of free ammonia (NH₃) on microbial metabolism. It is known that free ammonia easily diffuses into cells of microorganisms because of lipophilicity [78] and then converted to ammonium ions in the cell for satisfying the equilibrium constant [79]. For forming ammonium ions from ammonia inside the cells, hydrogen ions are pumped from outside, causing cation imbalance in the cells. Therefore, microorganisms undergo potassium reversal, in which potassium ions move out of cells [80-81]. In this biochemical process, the cellular energy is continuously consumed for maintaining an ionic equilibrium in the cell, which exerts a negative effect on the microorganisms

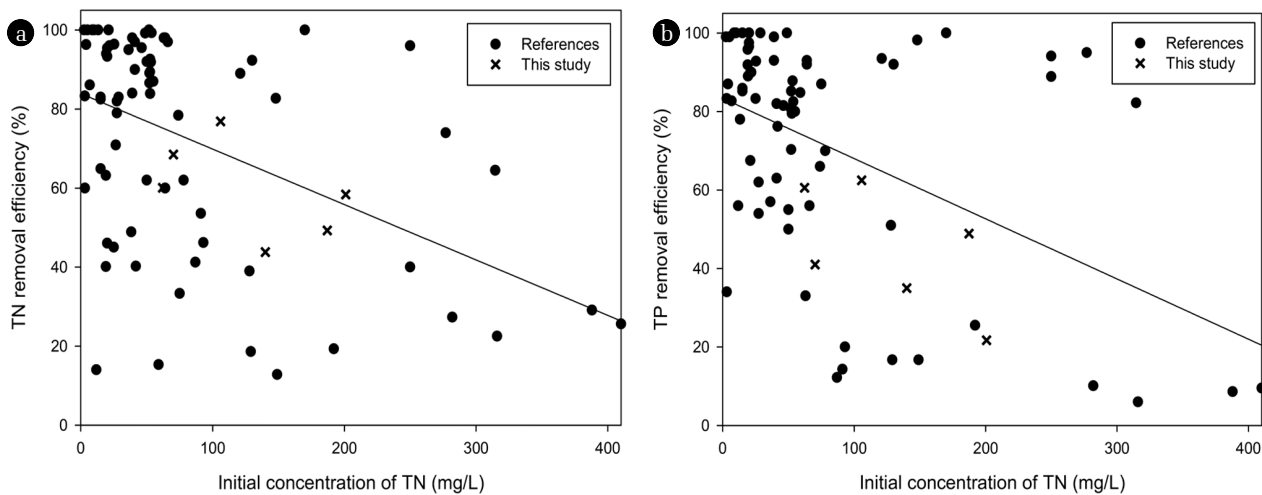


Fig. 5. TN and TP removal efficiencies by microalgal cultivation in various wastewaters (data were taken from Table S3 and Fig. 4).

Table 3. The TDN and TDP Removal by *C. vulgaris* in Five Different Media^a

Media	TDN removed		TDP removed		Consumed TDN/TDP ratio
	Conc. (mg/L)	Removal ratio (%)	Conc. (mg/L)	Removal ratio (%)	
0.1 × PW_S	105.7 ± 4.0	76.9	9.0 ± 0.4	62.5	11.74
0.1 × AD	200.7 ± 9.0	58.4	2.8 ± 0.6	21.8	71.68
0.1 × AD_C	140.0 ± 2.0	43.8	3.8 ± 0	35.0	36.84
0.1 × AD_S	62.3 ± 11.4	60.1	4.9 ± 0.1	60.6	12.71
0.1 × AD_M	70.3 ± 1.5	68.5	4.9 ± 0.8	41.0	14.35

^aMedia were 10-fold diluted, and data were acquired from Fig. 4

on growth [78]. It is also known that accumulated intracellular ammonium ions inhibit the microbial growth because protein synthesis is hindered [82].

The N/P ratio may also affect microalgal growth. Microalgal cells ($C_{1.06}H_{1.81}O_{0.45}N_{0.16}P_{0.01}$) undergoing phototrophic growth need nitrogen and phosphorus at the N/P molar ratio of 16:1 (mass ratio of 7.2:1) [62]. For cultivating freshwater microalgae (*i.e.*, *Chlorella* sp. and *Scenedesmus* sp.), Wang *et al.* [35] and Li *et al.* [19] reported that the optimal N/P ratio was 5-10. Table 3 (data extracted from Fig. 4) shows the removed TDN and TDP concentrations and their TDN/TDP ratios obtained for 14-d of *C. vulgaris* cultivation. The mass ratios of consumed TDN to TDP in 0.1x AD and 0.1x AD_C were 71.68 and 36.84, respectively, which were much higher than the mass ratio of 7.2:1 obtained by Redfield [62]. Chen *et al.* [31] observed similar ratios from freshwater algal cultivation in anaerobically-digested manure (72.2-76.9 of consumed N/P ratio). However, it should be noted that *C. vulgaris* did not grow well in AD and AD_C (Table 2). These results indicated that AD and AD_C contained excessive nitrogen (phosphorus limitation) and their N/P ratios were much higher than optimal ratio for this strain. Wang *et al.* [83] found that the consumed N/P ratios by *Oedogonium* sp. grown in digested piggery wastewater varied from 4.4 to 16.0. Wang *et al.* [21] also reported that *Chlorella* sp. showed consumed N/P ratios of 7.5-9.5 when grown in a growth media supplemented with digested dairy wastewater. Taking a close look at the air-strip-

ped three different media, it was noticed that PW_S, AD_S and AD_M supported active growth of *C. vulgaris* (Table 2) and removal of TDN and TDP (Fig. 4) at close-to-optimal ratios of 11.7-14.4 (Table 3). In short, compared to the untreated and chlorinated anaerobic digestate (AD and AD_C), ammonia-reduced (PW_S, AD_S, and AD_M) wastewaters provided favourable conditions for the growth of *C. vulgaris*. Aslan and Kapdan [13] suggested that the optimized N/P ratios enhanced the nutrient removal capability of *C. vulgaris* at high N and P concentrations.

The high concentrations of ammonia exert negative effects on microalgal growth and nutrient removal via two mechanisms. First, as discussed above, ammonia diffuses into the cell, and then cell continuously consumes cellular energy to maintain intracellular equilibria. Second, the inadequate molar ratio of N/P may also lower cell growth and nutrient removal. The results in the present study and previous studies [13] showed that the nutrients removal was enhanced when the N/P ratio was adjusted to the optimal ratio. Therefore, if ammonia is removed through air stripping, N/P ratio need to be re-adjusted.

4. Conclusions

The raw and anaerobically-digested piggery wastewaters containing nutrients and trace elements are promising microalgal culture

media. Due to high ammoniacal nitrogen and various inhibitors, however, growth rate and removal efficiency of nutrients by microalgae in these wastewaters are known to be inhibited. Thus previous many studies have reduced the concentrations of nutrients and inhibitors by highly diluting the wastewater with water. In this study, air stripping, NaOCl treatment, and the combined treatment of both methods were applied to grow microalgae in minimally-diluted raw and anaerobically-digested PWs. When raw and anaerobically-digested piggery wastewaters were air-stripped and 10-folds diluted, microalgal cultivation in these media reduced ammoniacal nitrogen concentration from 2,000-3,700 mg/L to 0.2-1.2 mg/L and, as a result, biomass productivity (0.22-0.57 g/L·d), removal efficiency of ammoniacal nitrogen (98.7-99.8%), TDP (41.0-62.5%) and TDN (60.3-76.9%) were 7.33-19, 1.21-1.23, 2.70-2.87 and 1.03-1.32 times higher than non-stripped anaerobically-digested PWs. Furthermore, despite the high concentration of SCOD, the biomass productivity was 1.4-4.4 times higher than BG-11 that did not contain any organic substances. However, NaOCl treatment did not remove ammonia effectively, and microalgal cells did not grow well. Therefore, it was concluded that efficient biomass production and nutrients removal could be accomplished by air-stripping high-strength wastewater.

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