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CO_2-Inorganic Carbon Auto-Buffering System for Efficient Ammonium Reclamation Coupled with Valuable Biomass Production in a Euryhaline Microalga *Tetraselmis subcordiformis*

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Abstract: The performance of microalgae-based wastewater treatment processes for ammonium-N (NH_4^+-N) removal depends on the maintenance of a favorable pH that is critical for minimizing nitrogen escape in the form of free ammonia (NH_3) and preventing high-NH_3 or extreme-pH stress. This study developed a CO_2-inorganic carbon (CO_2-IC) buffering system that automatically stabilized pH with the supply of a carbon source for efficient photosynthetic reclamation of NH_4^+-N by a euryhaline microalga *Tetraselmis subcordiformis*. The soluble ($NaHCO_3$) and insoluble ($CaCO_3$ and $MgCO_3$) ICs were compared for this purpose. The pH was well controlled in the range of 6.5~8.5 in the CO_2-IC system, which was suitable for the photosynthetic growth of *T. subcordiformis*. The NH_4^+-N (100 mg/L) was almost completely removed in three days, with the maximum removal rate of 60.13 mg N/L/day and minimal N escape of 19.65% obtained in the CO_2-$NaHCO_3$ system. The CO_2-IC system also restricted the release of extracellular organic matter by preventing stress conditions. The CO_2-$NaHCO_3$ system enabled the highest "normal" starch production suitable for fermentation, while the CO_2-$CaCO_3$/$MgCO_3$ system facilitated high-amylose starch accumulation that was conducive to producing bio-based materials and health-promoting ingredients. The proteins accumulated in *T. subcordiformis* were of good quality for animal feeds.

Keywords: microalgae; ammonium reclamation; pH control; inorganic carbon; starch; protein

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1. Introduction

With the acceleration of urbanization, the demand for metropolitan wastewater treatment is increasing [1]; at the same time, the reclamation of nutrients (mainly nitrogen, phosphorus, and carbon) from the wastewater is also crucial for the development of a sustainable life cycle following the circular economy principle [2,3]. Urban wastewater usually contains moderate amounts of ammonium-nitrogen (NH_4^+-N, 27~100 mg/L) as the dominant nitrogen form [4–7], which has to be removed before discharging for the prevention of eutrophication to the water ecosystem. The current urban wastewater treatment techniques in the wastewater treatment plants (WWTPs) in China mainly include conventional activated sludge treatment, anaerobic-anoxic-oxic (A^2/O), anaerobic-oxic (A/O), sequencing batch reactor (SBR), oxidation ditch, etc., which are efficient for COD removal yet have limited removal capacity for nitrogen and phosphorus [6]. In addition, the excess waste sludge discharge and substantial greenhouse gas (mainly CO_2 and N_2O) emission during the treatment remain a big challenge to meet sustainability standards [8].

Microalgae-based wastewater treatment processes have recently attracted increased attention because of their considerable benefits over traditional techniques, including highly efficient nutrient removal to a very low level with or without limited extra nutrient

supplementation, reduction of greenhouse gas emission with the CO_2 fixation, and nutrient reclamation for generating value-added products [9]. As the main nitrogen source in the urban wastewater, NH_4^+-N can be assimilated and converted to valuable proteins by microalgae, but the efficiency is highly dependent on the maintenance of a favorable environment for the microalgae [7]. The most critical challenge is the pH decrease during the NH_4^+ assimilation owing to the excretion of H^+, which causes the diminished photosynthesis and deteriorates the removal [10,11]. To stabilize the pH, organic buffering agents (e.g., Tricine, TAPS) or on-site pH monitoring/adjustment systems are usually applied, which can introduce extra CODs and increase operation facilities and costs [12–14]. The addition of alkaline inorganic bicarbonate (mainly $NaHCO_3$) is an alternative strategy to abate the acidification of the NH_4^+ removal process by simultaneously providing a carbon source for microalgae to meet the proper C/N ratio in the wastewater treatment [12,15]. However, the utilization of bicarbonate by microalgae tends to release OH^- that exceeds the H^+ excretion caused by the NH_4^+ assimilation because of the inherent high C/N demand (100/14), leading to the alkalization of the water with pH > 9 [5]. A high pH, under which NH_4^+ is prone to be converted to NH_3, is detrimental to the NH_4^+ assimilation by microalgae because of the ammonia inhibition and probably the extreme pH itself [7]. In addition, NH_3 under a high pH is readily volatilized, resulting in a low bioconversion efficiency and potential air pollution [16]. CO_2 injection has been proposed to help control the pH variations and improve the treatment performance [5,6], but to what extent the application of CO_2 contributes to the reduction of NH_3 escape is largely unknown.

Aside from the soluble bicarbonate $NaHCO_3$, insoluble carbonates such as $CaCO_3$ have been shown to efficiently regulate pH for microalgae cultivated in NH_4^+-rich wastewaters [17,18]. The insoluble nature of $CaCO_3$ restricted the unnecessary increase in pH [17]. However, since Ca^{2+} released into the water with the NH_4^+ removal process can be inhibitory to microalgae [19], and the insoluble particles can shelter the microalgae from being exposed to light that is crucial for photosynthesis, the ultimate performance of the insoluble carbonate needs to be evaluated for comparison with traditional $NaHCO_3$.

This study aimed to establish a CO_2-inorganic carbon (CO_2-IC) buffering system to automatically stabilize the pH with the supply of a carbon source for efficient photosynthetic removal and reclamation of NH_4^+-N by a euryhaline microalgal strain *Tetraselmis subcordiformis*. This strain is a euryhaline green microalga capable of acclimating to a wide salinity range (5.4~67.5 g/L NaCl) [20] and is assumed to be resistant to the fluctuations of salinity in urban wastewaters, especially those originated from coastal or island areas where seawater is used for toilet flushing or the intrusion of seawater into the wastewater often occurs [21]. The genus *Tetraselmis* has been reported to tolerate high NH_4^+-N and have an excellent capability of apparent NH_4^+-N removal from varied types of wastewater, including urban wastewater [4,13,22,23], but the processes were generally run without pH control (Table 1), or with a pH stat system or manual adjustment by acid/base, or buffered with organic agents (such as Tricine) [13,23,24]. The proportion of NH_4^+-N that was incorporated into the microalgae biomass or escaped from the culture system, which was the essential index to assess the NH_4^+-N bioconversion efficiency, remained undetermined in these processes. The present study compared different ICs, namely, soluble $NaHCO_3$ and insoluble $CaCO_3$ and $MgCO_3$, under both the IC and CO_2-IC systems in terms of the pH control, photosynthesis, cell growth, NH_4^+-N removal and reclamation efficiency, and organic matter release to comprehensively illustrate the potential of *T. subcordiformis* used for wastewater treatment. In addition, the biomass production ability and the quality of the main components accumulated (starch and protein) were assessed within an algal biorefinery concept.

Table 1. Photoautotrophic removal of NH_4^+-N by microalgae with different inorganic carbon sources and CO_2 supplies in synthetic NH_4^+-N-containing wastewater.

Algae Strain	Water Source	NH_4^+-N (mg/L)	Carbon Source	Air + CO_2	NH_4^+-N Removal Efficiency (%) (Day)	Maximum NH_4^+-N Removal Capacity (mg N/L/Day)	NH_3 Escaped (%)	Extra pH Control	Reference
Chlorella sp. L38	Freshwater	248	NH_4HCO_3	Air	80 (24)	8.67	NA [a]	No	[25]
		370	NH_4HCO_3	Air	55 (27)	10	19 [b]	Acid/base	[26]
		280	NH_4HCO_3	Air	45 (15)	8.31	21.43	Acid/base	[27]
		248	NH_4HCO_3	Air	44 (27)	12	24.2 [b]	Acid/base	[28]
Chlorella sp. L166	Freshwater	247	NH_4HCO_3	Air	87 (36)	13	76	No	[29]
		247	NH_4HCO_3	Air + 5%	52 (18)	10	42	No	
		247	NH_4HCO_3	Air	84 (36)	9	73 [b]	No	
Chlorella vulgaris	Freshwater	120	NH_4HCO_3	Air + 1%	100 (3)	53.4	NA	Acid/base	[30]
Chlorella strains	Freshwater	50	Na_2CO_3	Air	100 (4)	12.5	NA	No	[11]
Haematococcus pluvialis QLD	Freshwater	63	$NaHCO_3$	Air + 1%	95.6 (5)	15.75	NA	No	[15]
Desmodesmus sp. F51	Freshwater	60.2	$NaHCO_3$	Air + 2.5%	100 (1.1)	55.2	NA	No	[12]
		60.2	No	Air + 2.5%	100 (1.3)	50.9	NA	Acid/base	
		60.2	$NaHCO_3$	Air	25.25 (2)	20	NA	No	
Botryococcus braunii	Freshwater	83.15	$CaCO_3$	Air	68.55 (20)	2.85	NA	No	[17]
Arthrospira platensis	Semi-seawater	100	$NaHCO_3$	Air	100 (2)	50	23~40	No	[31]
Tetraselmis chui	Seawater	13	$NaHCO_3$	Air	73 (1)	9.5	NA	No	[32]
Nannochloropsis oculata	Seawater	13	$NaHCO_3$	Air	32 (1)	4.2	NA	No	[32]
Tetraselmis sp.	Seawater	3.8	No	Air	100 (1)	3.8 [c]	NA	No	[33]
Tetraselmis subcordiformis	Seawater	100	$NaHCO_3$	Air	99.6 (4)	49.27	48.60	No	This study
		100	$MgCO_3$	Air	99.6 (4)	44.00	43.70	No	
		100	$CaCO_3$	Air	99.3 (4)	31.69	38.93	No	
		100	$NaHCO_3$	Air + 2%	99.5 (3)	60.13	19.65	No	
		100	$MgCO_3$	Air + 2%	99.5 (2)	54.38	20.33	No	
		100	$CaCO_3$	Air + 2%	99.8 (3)	45.95	26.86	No	

Notes: [a] Not available. [b] Fed-batch mode for NH_4^+-N supply. [c] Immobilized algae cells.

2. Materials and Methods

2.1. Algal Strain and Culture Conditions

Tetraselmis subcordiformis FACHB-1751, obtained from the Marine Bioengineering Group of the Dalian Institute of Chemical Physics, Chinese Academy of Sciences, was previously cultured in artificial seawater (ASW, containing 27 g/L NaCl) [34] with the addition of 0.81 g L^{-1} Tris and 0.33 mL L^{-1} glacial acetic acid. Algae cells were collected in the late exponential phase and washed twice with nitrogen-free artificial seawater (ASW-N) to remove nitrate and organic carbon.

The washed cells were inoculated into a synthetic wastewater with a formula comprising the ASW-N medium containing 100 mg L^{-1} of NH_4^+-N provided as NH_4Cl, the concentration of which represented the common upper limit of the NH_4^+-N in urban wastewaters [7]. The algal biomass concentration was adjusted to ensure an inoculation density of 0.9 g/L. In order to stabilize the pH in the process of NH_4^+-N removal by the microalgae, 2% CO_2-rich air was injected into the culture at the rate of 0.4 vvm, with different inorganic carbons ($NaHCO_3$, $MgCO_3$, or $CaCO_3$) added with a carbon concentration of 12 mM (the minimal concentration of inorganic carbon salts required for maintaining favorable pH during the removal, data not shown), which constituted the auto-buffering CO_2-IC system. For reference, the 2% CO_2 was omitted, forming the IC system. As a negative control, both the 2% CO_2 and ICs were deprived from the cultures.

Microalgae cells were cultured in a cylindrical glass bubble photobioreactor (50 mm diameter, 400 mm height) with a working volume of 500 mL, as described by Yao et al. [35] at 25 ± 2 °C. A cold white fluorescent lamp was used to illuminate continuously from one side, providing an incident photosynthetic photon flux density (PPFD) of 150 μmol m^{-2} s^{-1}.

2.1.1. pH, Growth Measurement and Biochemical Component Analysis

The pH was measured using a standard benchtop pH meter (ARK, pHS-4C+, Sichuan, China). $MgCO_3$ and $CaCO_3$ are insoluble particles in the culture, which interfered with the determination of biomass as cell dry weight. Therefore, the growth of microalgae was estimated by the increase in total main organic matter in the cultures (mg/L), calculated as the sum of volumetric concentrations of protein, carbohydrate, and lipid.

The total protein was extracted with 0.5 M NaOH at 80 °C for 10 min [36] and measured following a BCA method (BCA Protein Assay Kit, Beyotime, Nantong, China). The total carbohydrate content was determined with a sulfuric acid-anthrone method according to Yao et al. [37]. The total lipid content was analyzed with a sulpho-phospho-vanillin (SPV) colorimetric method as described in [38].

The starch in the microalgal biomass was extracted with 45% perchloric acid and stained with I_2-KI solution (1:2, *v*/*v*) at 25 °C for 15 min followed by a spectrophotometry analysis under 618 and 550 nm according to the previous study [39], which allowed the simultaneous determination of amylose (Am) and amylopectin (Ap) concentrations [40]. The total starch was estimated as the sum of amylose and amylopectin.

2.2. Photosynthetic Performance Analysis

The photosynthetic performance of the microalgae was measured as the maximum quantum yield of photosystem II with a chlorophyll fluorometer Os30p+ (Opti-sciences, Hudson, NH, USA) after dark adaption for 10 min [34]. The parameter expressed as F_v/F_m was calculated as described by Strasserf and Srivastava [41]:

$$F_v/F_m = (F_m - F_0)/F_m \quad (1)$$

where F_v represents the variation of chlorophyll fluorescence between maximal fluorescence (F_m) induced by saturating pulse and initial fluorescence (F_0).

2.3. Ammonium-Nitrogen (NH_4^+-N) Analysis

The concentration of NH_4^+-N in the culture system was determined by indophenol blue colorimetry [42] after proper dilution. The removal rate of NH_4^+-N (R_N, mg N/L/day) was calculated as follows:

$$R_N = (N_0 - N_t)/t \quad (2)$$

where N_0 and N_t are the concentrations of NH_4^+-N at culture times 0 and t, respectively.

2.4. Nitrogen Distribution and Total Organic Carbon (TOC) Analysis

In order to evaluate the proportion of N assimilated into the biomass (Biomass-N, %) or escaped from the culture system (Escaped-N, %), the total nitrogen (TN) in the water phase and the nitrogen element in the biomass phase were measured with a TN analyzer (TOC-L CPH/CPN, Shimadzu, Tokyo, Japan) and an elemental analyzer (Elemental Vario EL Cube, Hanau, Germany), respectively.

The Biomass-N (%) and Escaped-N (%) were estimated using the following equations:

$$\text{Biomass-N (\%)} = TN_{bimass(t)}/TN_{(0)} \times 100 \quad (3)$$

$$\text{Escaped-N (\%)} = (TN_{(0)} - TN_{water(t)} - TN_{bimass(t)})/TN_{(0)} \times 100 \quad (4)$$

$$TN_{(0)} = TN_{bimass(0)} + TN_{water(0)} \quad (5)$$

$$TN_{bimass} = \omega_N \times C_{biomass} \quad (6)$$

where $TN_{bimass\ (t)/(0)}$ (mg/L) is the total N element in the biomass at time t/0 (day), $TN_{water\ (t)/(0)}$ (mg/L) is the total N concentration in the water at time t/0 (day), $TN_{(0)}$ represents the initial total N (mg/L) in the culture system, ω_N (w/w) is the N content in biomass, and $C_{biomass}$ (mg/L) is the apparent biomass concentration determined gravimetrically according to the previous study [39].

The total organic carbon (TOC) in the water was determined with a TOC analyzer (TOC-L CPH/CPN, Shimadzu, Japan). The carbohydrate concentration in the water was measured with the phenol–sulfuric acid method [43], and protein concentration was assayed following a BCA method (BCA Protein Assay Kit, Beyotime, China).

2.5. Amino Acid Analysis

The freeze-dried microalgae biomass was used for amino acid analysis according to the method described previously [44]. The relative proportion of each amino acid (AA) was expressed as g AA per 100 g of total AA. The essential amino acid index (EAAI) was calculated according to the following equation [45]:

$$EAAI = \sqrt[n]{\frac{aa_1 \times aa_2 \times \ldots \times aa_n}{AA_1 \times AA_2 \times \ldots \times AA_n}} \quad (7)$$

where aa_n represents the percentage of one kind of essential amino acid in the total essential amino acids in the sample; AA_n represents the percentage of one kind of essential amino acid in the total essential amino acids in reference samples (*Penaeus monodon* juvenile and ideal protein for dairy cow, respectively).

2.6. Statistical Analysis

All experiments were performed in duplicate, and SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis. Two group comparisons were performed using a two-tailed distribution Student's paired *t* test. Values of $p < 0.05$ were defined as statistically significant.

3. Results and Discussion

3.1. pH Variation, Photosynthetic Performance, and Cell Growth

Photosynthesis is indispensable for photoautotrophic removal of NH_4^+-N in microalgae, which is affected by carbon availability and pH conditions surrounding the algal cells [11]. In order to verify whether the ICs could provide sufficient carbon and enable a suitable pH to sustain photosynthesis for algal cell growth, 12 mM of $NaHCO_3$, $MgCO_3$, or $CaCO_3$ were applied to the culture system with synthetic 100 mg/L NH_4^+-N; simultaneously, the culture with no IC supply was set as a negative control. As shown in Figure 1a, the pH in the control group without the IC supply dropped sharply from 6.56 to 3.56 within three days, which was typically observed in the microalgae cultivation using ammonium as the sole nitrogen source owing to the release of H^+ by algal cells after the assimilation of NH_4^+-N [12]. In contrast, the supply of ICs led to a considerable increase in pH up to 9.0~9.6 in the first two days, which was indicative of the assimilation of soluble bicarbonate by the microalgae that generated hydroxyl ions in the medium. The increase in pH was previously reported in the freshwater microalgae *Desmodesmus* sp. F51 and *Haematococcus pluvialis* QLD adding $NaHCO_3$ as the inorganic carbon source for NH_4^+-N utilization [12,15]. Here, it was similar in the marine microalga *Tetraselmis subcordiformis*. Because of the high C/N ratio in microalgae ($CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$) [46], the bicarbonate consumption rate could be much higher than the NH_4^+-N; therefore, the alkalization from the former exceeded the acidification in the latter process, causing an elevated pH. For the insoluble IC ($MgCO_3$ or $CaCO_3$) groups, the increased pH also suggested the partial solubilization of carbonate into the medium in the form of soluble bicarbonate as a carbon source for *Tetraselmis subcordiformis*. Similarly, the leach of Ca^{2+} into the medium was recorded in the *Botryococcus braunii* culture with $CaCO_3$ addition for the photoautotrophic NH_4^+-N removal [17]. The release of H^+ during the NH_4^+-N assimilation could be the trigger for this solubilization. Accordingly, the photosynthetic performance revealed by the maximum quantum yield of photosystem II (F_v/F_m) decreased sharply (from 0.73 to 0.29) within four days in the control group without IC supply, while the groups with IC addition maintained above 0.6 (Figure 1c), which indicated the alleviation of the low-pH stress caused by the H^+ release from the NH_4^+-N assimilation. Consequently, the biomass accumulation as assessed by the sum of the main organic matter (proteins, carbohydrates, and lipids) also showed significant improvement in the IC groups compared with the control group, with the former reaching 1.0~1.2 g/L against the latter being only 0.30 g/L (Figure 1e). These results suggested that the bicarbonate and insoluble carbonate could be used as a pH regulator as well as a carbon source for NH_4^+-N removal and biomass production.

It should be noted that the pH in the groups with IC addition fluctuated during the cultivation, and a high pH of up to 9.5 was reached, which could be unfavorable for the photosynthetic growth and NH_4^+-N removal in *Tetraselmis subcordiformis* because the suitable pH for this alga is 6.5~8.5 [24,47], and the free ammonia (NH_3) generated under such a high pH could be toxic as well [16]. In fact, the F_v/F_m exhibited a temporary decline in the first two days when the pH increased to above 9 in the IC groups (Figure 1a,c), demonstrating a high-pH or ammonia stress present therein. To avoid the instability of the pH as well as the stress caused by the high pH, air enriched with 2% CO_2 was supplied to the IC groups (CO_2-ICs). As shown in Figure 1b, the pH levels in all the cultures with CO_2 and IC addition were maintained in the range of 6.5~8.5 throughout the cultivation, which matched the suitable pH range for *T. subcordiformis*. Correspondingly, the F_v/F_m also stayed at a relatively high level without significant decrease in the first four days (Figure 1d), and the biomass accumulation was enhanced to 1.7~2.1 g/L, which was 73~81% higher than the cultures without CO_2 addition (ICs, Figure 1e). These results demonstrated that the acidic CO_2 could form a buffering system with the alkaline bicarbonate/carbonate that was able to stabilize the pH and facilitate biomass accumulation in *T. subcordiformis* when NH_4^+-N was used as the nitrogen source. It was reported that the addition of 1–2.5% CO_2 in the bicarbonate-NH_4^+-N system was beneficial for the pH stabilization and biomass

production in freshwater microalgae *Desmodesmus* sp. F51 and *Haematococcus pluvialis* QLD [12,15], which coincided with the case of *T. subcordiformis* herein.

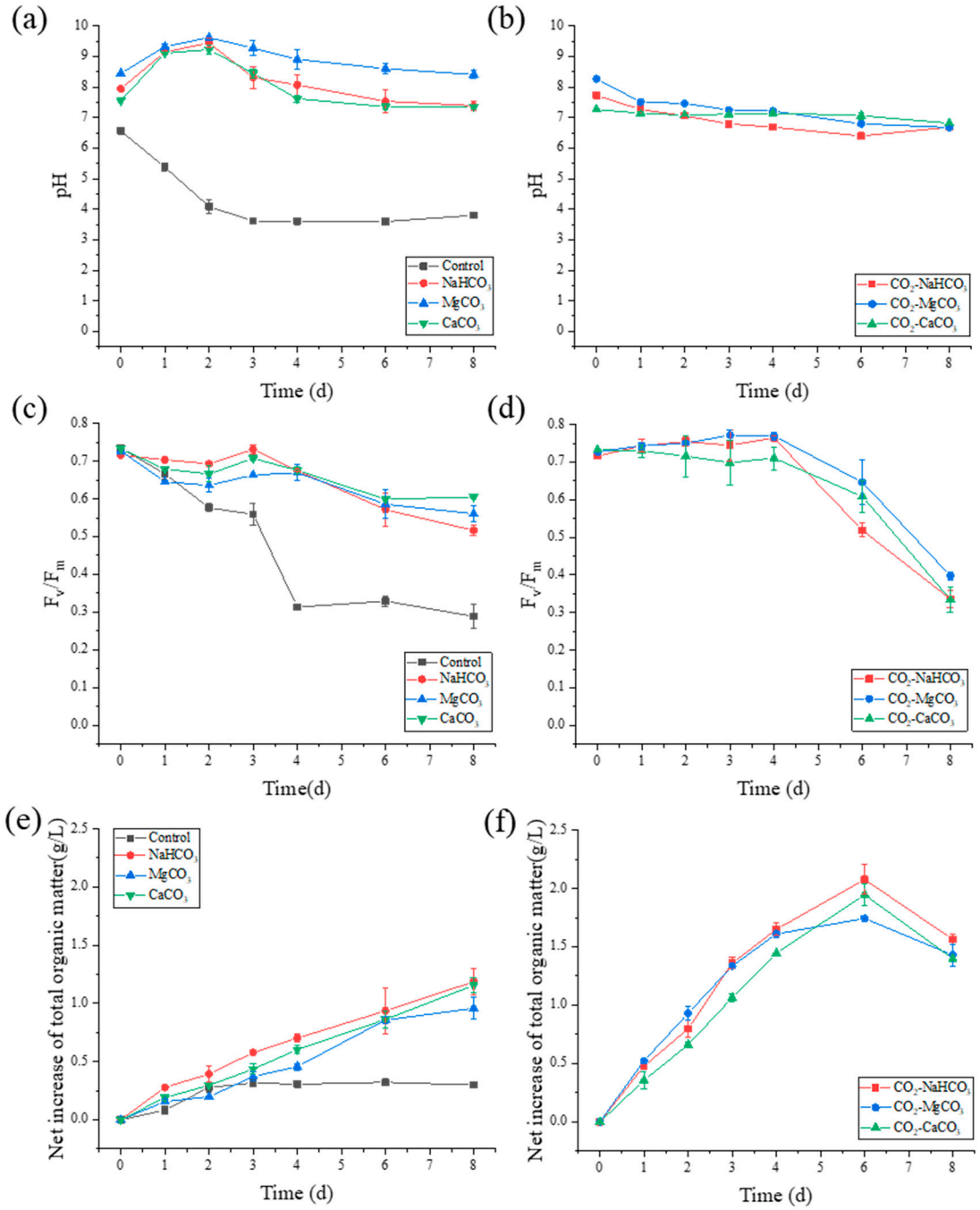

Figure 1. pH variation (**a**,**b**), photosynthetic performance indicated by F_v/F_m (**c**,**d**), and biomass accumulation indicated as total main organic matter (**e**,**f**) in *Tetraselmis subcordiformis* under IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) (**a**,**c**,**e**) or CO_2-IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) conditions (**b**,**d**,**f**) during the NH_4^+-N removal process.

Different ICs enabled diverse chemical environments, which in turn influenced the biomass accumulation and NH_4^+-N removal. As shown in Figure 1e,f, the biomass accumulation in the cultures with $MgCO_3$ addition was generally inferior to the $NaHCO_3$ counterpart, regardless of the application of CO_2 buffering. Specifically, when no CO_2 was applied for buffering, the culture with $MgCO_3$ addition resulted in an overall higher pH

(8.5~9.6) than $NaHCO_3$ and $CaCO_3$ (7.6~9.3, Figure 1a), and a lower F_v/F_m was obtained during the first three days (Figure 1c), indicating that the algal cells were exposed to severer high-pH or ammonia stress in the $MgCO_3$ culture, which finally led to a 25% decrease in biomass accumulation (Figure 1e) compared with the $NaHCO_3$ and $CaCO_3$ counterparts. *Tetraselmis* was reported to have a significantly higher growth rate under a pH of 7.5 rather than 8.5 [48], which was consistent with the results here. This indicated that the pH of the culture should be considered when choosing ICs as the carbon source for the photosynthetic removal of NH_4^+-N. The addition of $CaCO_3$ did not affect the final biomass accumulation relative to the $NaHCO_3$ culture, but it caused an attenuation of biomass accumulation in the early phase (0–4 days) of the cultivation (Figure 1e,f). Considering that the pH levels of these two cultures were almost identical during this period, the decreased biomass production could not be ascribed to the pH difference but might be due to the inhibitory effect of Ca^{2+} released from the $CaCO_3$. In fact, the Ca^{2+} concentration increased from the initial level of 528 mg/L to 800 mg/L in the $CaCO_3$ culture when buffering with CO_2 (Figure S1), which can exert stress on *T. subcordiformis*. A high Ca^{2+} concentration in the medium can reduce the growth and photosynthesis in microalgae [19,49]. The lower F_v/F_m in the $CaCO_3$ culture was also observed compared to $NaHCO_3$ (Figure 1c,d), which supported this hypothesis. In addition, the insoluble nature of $CaCO_3$ could have partially impeded the light penetration in the culture, leading to relatively lower light exposure to the algae cells, compared with the $NaHCO_3$ counterpart, and consequently reduced biomass production. In all, the CO_2-ICs buffering system was efficient for *T. subcordiformis* to stabilize the pH and maintain photosynthesis along with the carbon supply for biomass accumulation in the synthetic NH_4^+-containing wastewater, with the soluble $NaHCO_3$ performing the best.

3.2. NH_4^+-N Removal and N Distribution

3.2.1. NH_4^+-N Removal

To verify the NH_4^+-N removal efficiency in the IC and CO_2-IC systems, the NH_4^+-N concentration in the medium was tracked during the cultivation. As shown in Figure 2a, the addition of ICs significantly improved the NH_4^+-N removal efficiency compared to the control group without IC addition, with more than 99% of NH_4^+-N being removed within four days in the former as against only 38% achieved in the latter. The maximum NH_4^+-N removal rates of 49.27, 44.00, and 31.69 mg N/L/day were obtained on the first day in the cultures with $NaHCO_3$, $MgCO_3$, or $CaCO_3$ addition, respectively, which was 1.7~3.3-fold higher than that in the control culture (Figure 2c). The supply of CO_2 to the IC cultures further accelerated the NH_4^+-N removal, with very trace NH_4^+-N detected in the culture medium on Day 3 (Figure 2b). The maximum NH_4^+-N removal rate in the CO_2-IC cultures also showed 22~45% improvement relative to the IC counterparts (Figure 2d). The enhanced NH_4^+-N removal efficiency in the IC system compared with the control and the further improvement in the CO_2-IC system were in accordance with the higher photosynthetic performance and biomass accumulation therein (as discussed in Section 3.1), demonstrating the effectiveness of pH control and carbon supply for the photosynthetic NH_4^+-N removal by *T. subcordiformis* with these strategies. However, the NH_4^+-N removal efficiency did not seem exactly the same when different ICs were applied. The addition of $CaCO_3$ led to a slower NH_4^+-N removal (Figure 2a,b) and reduced the NH_4^+-N removal rate ($p < 0.05$, Figure 2c,d) in both the IC and CO_2-IC systems compared with the $NaHCO_3$ counterparts. The maximum NH_4^+-N removal rates in the $CaCO_3$ culture reached 31.69 mg and 45.95 mg N/L/day in the IC and CO_2-IC systems, respectively, which accounted for a 24–36% reduction relative to the cultures with $NaHCO_3$ addition (Figure 2c,d). The diminished NH_4^+-N removal in the $CaCO_3$ culture was in accordance with the reduced photosynthesis and biomass accumulation (Figure 1c–f), which could be ascribed to the inhibitory effects of the high Ca^{2+} load as well as limited light penetration resulting from the insoluble nature therein, as discussed in Section 3.1. The highest maximum NH_4^+-N removal rate reached 60.13 mg N/L/day in the CO_2-$NaHCO_3$ culture. In addition,

the phosphorus (P) concentration was reduced from the initial level of 14.3 to less than 0.1 mg/L within one day in all the cultures (Figure S3), which perfectly met the P discharge standard (Grade I–A) for urban wastewater treatment in China [50], demonstrating the excellent ability of *Tetraselmis subcordiformis* for simultaneous NH_4^+-N and P removal.

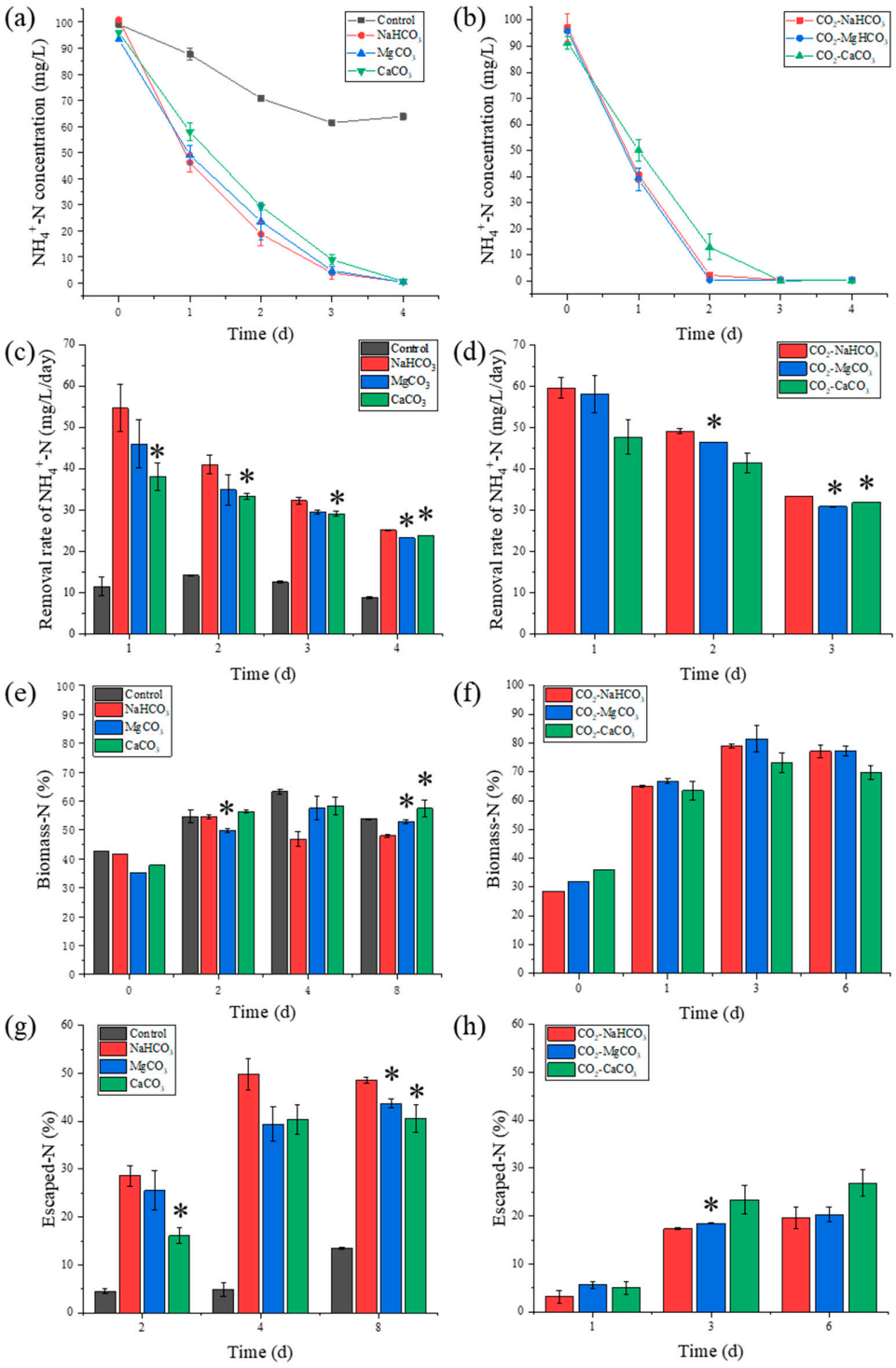

Figure 2. The NH_4^+-N concentration in the medium (**a**,**b**), NH_4^+-N removal rate (**c**,**d**), percentage of total N in the biomass (**e**,**f**), and percentage of N escaped from the culture system (**g**,**h**) under IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) (**a**,**c**,**e**,**g**) or CO_2-IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) conditions (**b**,**d**,**f**,**h**) during the NH_4^+-N removal by *Tetraselmis subcordiformis*. The asterisk (*) indicates a significant difference ($p < 0.05$) compared with the corresponding $NaHCO_3$ culture.

3.2.2. N Distribution

The removed NH_4^+-N from the medium could have either been assimilated by the microalgae or stripped out of the culture system as NH_3 [26,27]. In order to gain further insight into the efficiency of NH_4^+-N reclamation by the microalgal cultivation systems, the N balance was analyzed to explore the distribution. As shown in Figure 2e,f, the percentage of total N in the biomass generally increased during the first four (ICs) or three (CO_2-ICs) days when NH_4^+-N was almost completely removed from the medium, yet with the maximum proportion of 55~58% achieved in the former and 73~81% reached in the latter. It suggested that although NH_4^+-N was removed from the medium, only a part of it could be stored in *T. subcordiformis*, and the application of CO_2 significantly improved the bio-reclamation of NH_4^+-N. Accordingly, N balance analysis showed that 39~50% of the initial N escaped from the culture system when only ICs were applied, whereas the N escape level was reduced to 20~27% when CO_2-IC systems were used (Figure 2g,h). It demonstrated that the supply of CO_2 in the IC systems could partially avoid the stripping of N from the culture and improve the NH_4^+-N assimilation by the microalgae, which could be attributed to the pH reduction because of the acidic CO_2 supply and the enhanced photosynthetic activity that enabled a more efficient bio-assimilation of NH_4^+-N. A higher pH was reported to result in more NH_3 escape in the cultivation of microalgae *Chlorella* sp. L38 [26] and *Arthrospira platensis* [31]. The pH of the IC systems reached 9~9.5 during the first two days (Figure 1a), under which the NH_4^+ was very prone to be converted to ammonia (NH_3) in view of the dissociation constant (pK_a) of 9.25 for the reaction $NH_4^+ \leftrightarrow NH_3 + H^+$, and it was easily stripped out of the culture system with the aeration [16,31]. Instead, in the CO_2-IC systems, the pH was kept at 6.5~8.5 throughout the cultivation, under which NH_4^+ was dominant and hence greatly reduced the probability of the stripping. In fact, the estimation of free ammonia (NH_3) relative to the total ammonia (NH_3 and NH_4^+) in the medium according to the formula of NH_3 (%) = $100/(1 + [H^+]/K_a)$, where K_a is the dissociation constant of ammonia (4.36×10^{-10} at 25 °C in seawater) [13,51], showed that free ammonia accounted for maximally 42~65% in the ICs as against less than 8% in the CO_2-ICs (Figure S2a). Therefore, the CO_2-IC system allowed much less N escape than the IC systems. Meanwhile, the NH_3 generated under such a high pH in the ICs could be toxic to *T. subcordiformis*, which would diminish the photosynthesis and adversely affect the N bio-assimilation ability [16]. The estimated free ammonia (NH_3-N, mg/L) calculated as $[NH_4^+]/(1 + [H^+]/K_a)$ reached as high as 18~23 mg/L in the ICs, which exceeded the reported maximum EC_{50} (1258 μM, 17.6 mg/L in *Scenedesmus obliquus*) of NH_3 inhibition in microalgae [16], whereas it was less than 0.5 mg/L in the CO_2-ICs except for the initial day of 0.7~7.1 mg/L (Figure S2b). Higher photosynthetic activity was also recorded in the CO_2-ICs compared with ICs, as discussed in Section 3.1, which could enable a higher N bioconversion efficiency and less N escape. For the control group without IC addition, the percentage of total N in the biomass also showed a significant increase, and the maximum proportion reached 63% on Day 4, which was even slightly higher than that of the IC groups yet inferior to the CO_2-IC groups (Figure 2e,f). The N escape was less than 5% before four days because of the acidic environment (Figure 2g), which suggested that the removed NH_4^+-N was predominantly assimilated by the microalgae. However, because of the low-pH stress, as discussed in Section 3.1, the overall NH_4^+-N removal was impeded (Figure 2a). Collectively, the CO_2-IC auto-buffering system exhibited not only faster and complete NH_4^+-N removal but also enabled the better ability of N bio-reclamation in *T. subcordiformis*.

It should be noted that different ICs caused different NH_4^+-N bioconversion efficiency and N escape rates. Under the IC system on Day 4 when NH_4^+-N was completely removed, $MgCO_3$ or $CaCO_3$ addition led to 23% higher N stored in the biomass than that of $NaHCO_3$ (58% vs. 47%), which was mirrored by the 22% decrease in N escape (39% vs. 50%) therein ($p < 0.05$, Figure 2e,g). The decreased N escape in the $MgCO_3$ or $CaCO_3$ culture could not be ascribed to the pH effect as discussed above. For instance, the $MgCO_3$ culture held the highest pH and free ammonia (Figure 1a and Figure S2b) but had a reduced N escape rate

relative to the $NaHCO_3$ culture (Figure 2g). Instead, the higher N escape in the $NaHCO_3$ culture under ICs might be attributed to the N release from the biomass rather than the direct stripping of the NH_3 from the medium. It can be seen in Figure 2e that the percentage of N stored in the biomass decreased from Day 2 (55%) to Day 4 (47%) in the $NaHCO_3$ culture, suggesting a N release from the biomass, which was not observed in the $MgCO_3$ or $CaCO_3$ culture. Considering that ICs caused a high-pH environment and alkaline stress (Figure 1a,c), it can be speculated that metabolic changes including some deamination reactions (e.g., purine deamination [52]) were induced in the culture with $NaHCO_3$ addition that generated extra NH_3, which was further stripped out. Nevertheless, the reason for the mitigation of N escape in the $MgCO_3$ or $CaCO_3$ culture remained unclear. In contrast to the case under the IC system, the addition of $NaHCO_3$ under the CO_2-IC system led to the lowest N escape (19.7%) with the highest N stored in the biomass (77.2%) (Figure 2h). Comparatively, the addition of $CaCO_3$ herein caused an enhancement of N escape (26.9%) and reduction of N storage in the biomass (69.8%), which could be ascribed to the lower photosynthetic activity originated from the relatively less light exposure caused by the insoluble nature and the high-Ca^{2+} stress, as discussed in the previous sections.

The above results highlighted the CO_2-IC buffering system as an efficient strategy for the bio-reclamation of NH_4^+-N by *Tetraselmis subcordiformis*. The NH_4^+-N was completely removed from the medium within three days, with a maximum NH_4^+-N removal capacity of 46~60 mg N/L/day, which was among the top performances compared with other microalgae under similar conditions reported in the literature (Table 1); meanwhile, the limited N escape rate (19.7~26.9%) in this auto-buffering system was also comparable to those obtained in *Chlorella* where an acid/base pH control system was applied [26–28] (Table 1). Among the three CO_2-ICs tested, the CO_2-$NaHCO_3$ culture system was particularly outstanding in the aspect of NH_4^+-N removal, N bioconversion, and biomass production, which demonstrated the feasibility of eliminating pH real-time monitoring without affecting operation efficiency and could reduce instrument investment and operating cost. In addition, according to the emission standard for ammonia in China (GB 14554-93), the emission rate (G, kg/h) should not exceed 4.9 kg/h for each plant. Accordingly, in the context of influent NH_4^+-N (N_{in}) of 100 mg/L with the minimum N escape of 19.65% obtained in this study, the maximum wastewater treatment capacity (Q, m^3/day) has to be less than 5985 m^3/day, as calculated with the formula $Q < G \times 1000 \times 24/(N_{in} \times 19.65\%)$. This treatment scale can be classified as "large scale" [53], although the capacity is relatively small in China yet still acceptable [50]. Considering that the N_{in} in the urban wastewater from most of the cities in China was below 50 mg/L [50], the treatment capacity could be doubled. These analyses further signified the importance of the reduction of NH_3 release by using the established CO_2-IC systems during the wastewater treatment process to enable higher treatment capacity.

3.3. Extracellular Organic Matter Release during NH_4^+-N Removal

The photoautotrophic cultivation of microalgae is always accompanied by extracellular organic matter (EOM) release, especially when algal cells are exposed to stressful conditions such as low light intensity, low nutrient availability, and extreme temperatures or pH [30,54–56]. The release of EOM is generally unfavorable to the wastewater treatment process since it increases the COD in water. Therefore, the variation of the total organic carbon (TOC) in the medium was investigated under IC or CO_2-IC systems. As shown in Figure 3a, the TOC in the medium increased throughout the cultivation in all the cultures, with the control group reaching the highest TOC release of 117.6 mg/L on Day 8. The addition of ICs remarkably reduced the TOC release, with the ΔTOC range at 67~77 mg/L (Figure 3a). Comparatively, the supply of CO_2 dramatically suppressed the release of TOC, with less than 20 mg/L of ΔTOC obtained in all the cultures (Figure 3b). Considering that stress is usually an important trigger for the EOM release [56], it could be inferred that the high ΔTOC in the control and IC cultures was ascribed to the extreme (low in the former and high in the latter) pH. It was reported that the dissolved organic carbon (DOC) was

significantly augmented when the pH increased from 7.5 to 8.5 during the NH_4^+-N removal by *Chlorella vulgaris*, and it was deemed to be raised by the free ammonia stress under the high-pH environment [30], a situation that could be applied in the present study when ICs led to a high pH and increased free ammonia concentration, as discussed in the previous section. The extremely high ΔTOC in the control group could be attributed to the low-pH stress, as was also recorded in the microalga *Scenedesmus* sp. LX1 exposed to a low pH of 5 [54]. It is worth noting (Figure 3b) that the CO_2-$NaHCO_3$ culture exhibited the lowest ΔTOC (1.3 mg/L), followed by CO_2-$MgCO_3$ (8.5 mg/L) and CO_2-$CaCO_3$ (15.3 mg/L). In view of the higher stress that the CO_2-$CaCO_3$ culture was subjected to compared with the CO_2-$NaHCO_3$ culture (discussed in Section 3.1), it was reasonable to have this higher ΔTOC. A high Ca^{2+} load was reported to improve extracellular secretions in the the NH_4^+-N removal system by microalgae [19], which coincided with the present study.

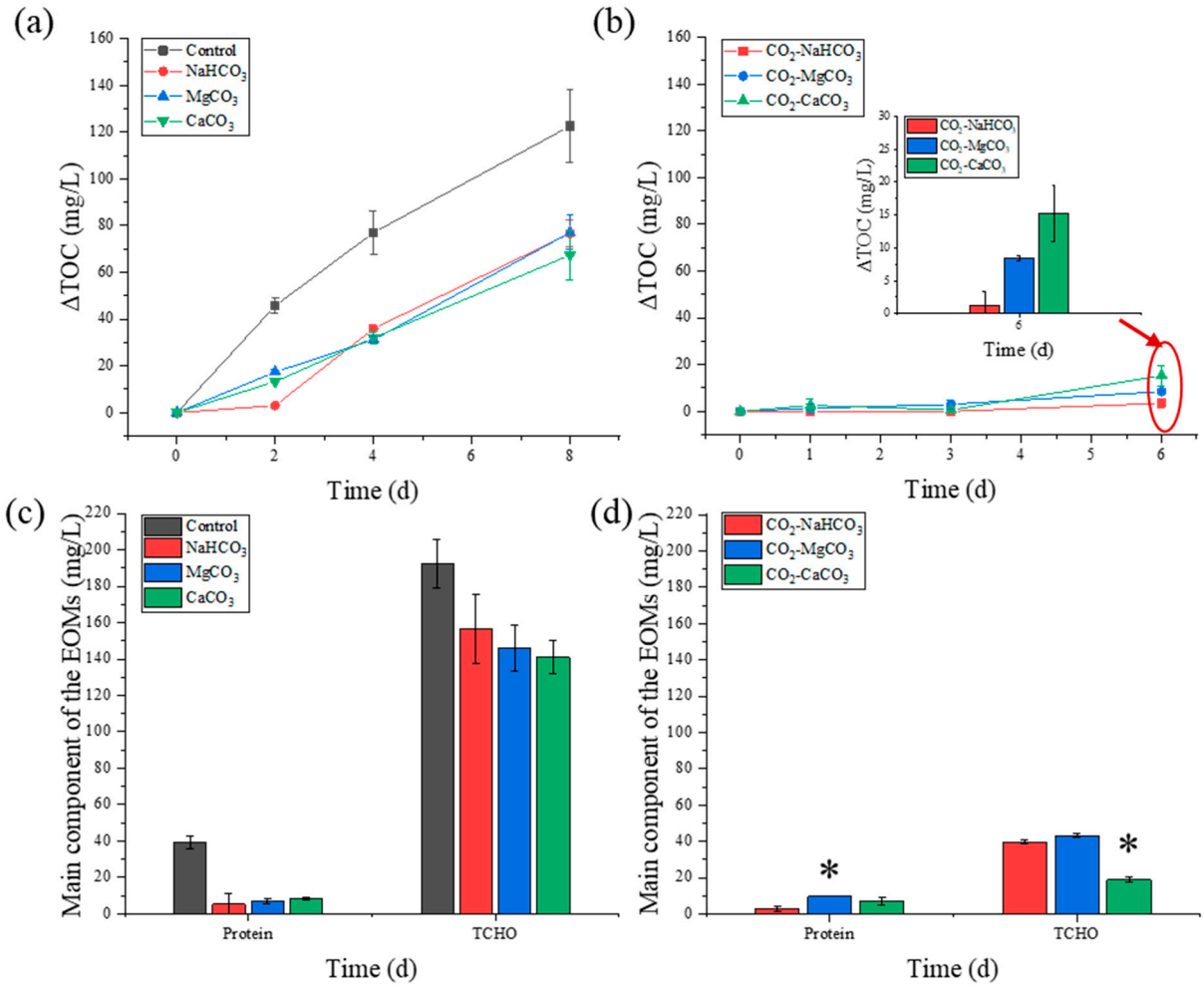

Figure 3. The release of EOMs as assessed by the increase in total organic carbon (ΔTOC, mg/L) (**a**,**b**) and the main component of the EOMs (**c**,**d**) under IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) (**a**,**c**) or CO_2-IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) conditions (**b**,**d**) during the NH_4^+-N removal by *Tetraselmis subcordiformis*. The ΔTOC on the sixth day of each group in Figure 3b (circled) is magnified in the bar chart directed with an arrow. The asterisk (*) indicates a significant difference ($p < 0.05$) compared with the corresponding $NaHCO_3$ culture.

In general, carbohydrate and protein are regarded as the main components in EOM [57]. Therefore, these two components were determined in IC or CO_2-IC systems. It was clear that the carbohydrate accounted for the dominant proportion of the EOM as against protein in all the cultures (Figure 3c,d), and the carbohydrate concentration in the IC cultures was

2.4~6.4 times higher than those in the CO_2-ICs cultures, which was in agreement with the TOC concentration (Figure 3). Interestingly, for the CO_2-IC system, while the ΔTOC was the highest in the CO_2-$CaCO_3$ culture, the carbohydrate concentration was the lowest therein ($p < 0.05$, Figure 3d), indicating that some other kinds of EOM, e.g., organic acids (humic acid, fulvic acid, glycolic acid, etc.), hormonal substances, or pigments [55,57], were present in this culture. Collectively, these results further highlighted the advantage of the CO_2-IC buffering system, especially the CO_2-$NaHCO_3$ culture, for enabling a favorable pH environment to avoid EOM generation, which benefited the maintenance of water quality during the NH_4^+-N removal process. In particular, the ΔTOC of 1.3 mg/L in the CO_2-$NaHCO_3$ culture could be considered as a very low or even the lowest level, compared with other microalgae cultivation results reported previously [57].

3.4. Biomass Component Production

Microalgae assimilate NH_4^+-N and convert it primarily to protein; at the same time, carbohydrates and lipids are the major products of the carbon fixation during the algal cell growth [58]. The accumulation of the biomass components can be varied depending on the environment that the microalgae are exposed to, especially when stress factors are present [59]. *Tetraselmis subcordiformis* mainly accumulated protein, starch, and lipid as the nitrogen and carbon reserves, which can be used for biorefinery and value-added products [44]. Therefore, the accumulations of these three components were assessed during the NH_4^+-N removal process under IC or CO_2-IC systems.

As shown in Figure 4a, the protein accumulated mainly during the first four (ICs) or three (CO_2-ICs) days when NH_4^+-N was assimilated (Figure 2e,f), demonstrating the conversion of the NH_4^+-N into protein by the microalgae. The supply of CO_2 improved the protein production because of the favorable pH environment therein, with the maximum net increase in protein concentration reaching around 0.62 g/L in all the CO_2-IC cultures on Day 6, which represented 44% of the enhancement relative to the IC counterparts (Figure 4a). The protein accumulation generally showed no difference between the three ICs ($NaHCO_3$, $MgCO_3$, or $CaCO_3$) used under both IC and CO_2-IC systems, although a lag phase was observed in the $MgCO_3$ culture under the IC system (Figure 4a), probably because of the high pH and free ammonia inhibition in the first two days that suppressed the photosynthetic activity required for protein synthesis (Figures 1a,c and S2b). Lower protein production was observed in *Chlorella* sp. L38 when the pH increased from 7 or 8 to 9 during the NH_4^+-N removal, which was consistent with the present study [26].

The starch accumulation generally occurred on Day 4 and Day 2, respectively, in the ICs and CO_2-ICs systems (Figure 4b), which correlated to the exhaustion of NH_4^+-N in the medium (Figure 2a,b), indicating a nitrogen limitation-triggered starch synthesis, as had been widely recognized in microalgae, including *Tetraselmis subcordiformis* [39,60]. Similar to the protein accumulation, the starch production was markedly improved in the CO_2-IC cultures compared to the IC cultures. However, different ICs led to enormous variations in terms of starch accumulation, with $NaHCO_3$ exhibiting the most prominent ability to induce starch synthesis in *Tetraselmis subcordiformis*, followed by $CaCO_3$ and $MgCO_3$; the maximum net increase in starch reached 1.3 g/L in the CO_2-$NaHCO_3$ culture on Day 6, which was 32% ($p < 0.05$) and 1.72-fold higher ($p < 0.05$) than that obtained in the CO_2-$CaCO_3$ and CO_2-$MgCO_3$ cultures, respectively (Figure 4b). The reduced starch accumulation in the CO_2-$CaCO_3$ and CO_2-$MgCO_3$ cultures could be attributed to the limited light exposure originated from the insoluble $CaCO_3$ and $MgCO_3$ particles. Light intensity is an important factor affecting starch production in microalgae; higher light exposure usually results in enhanced starch accumulation, especially under nutrient deprivation conditions [34,60,61]. In fact, the slower decline of F_v/F_m (Figure 1d), a sensitive indicator of photoinhibition, after the nitrogen deprivation from Day 4 in the $CaCO_3$ and $MgCO_3$ cultures relative to the $NaHCO_3$ culture implied the reduced light intensity per cell therein.

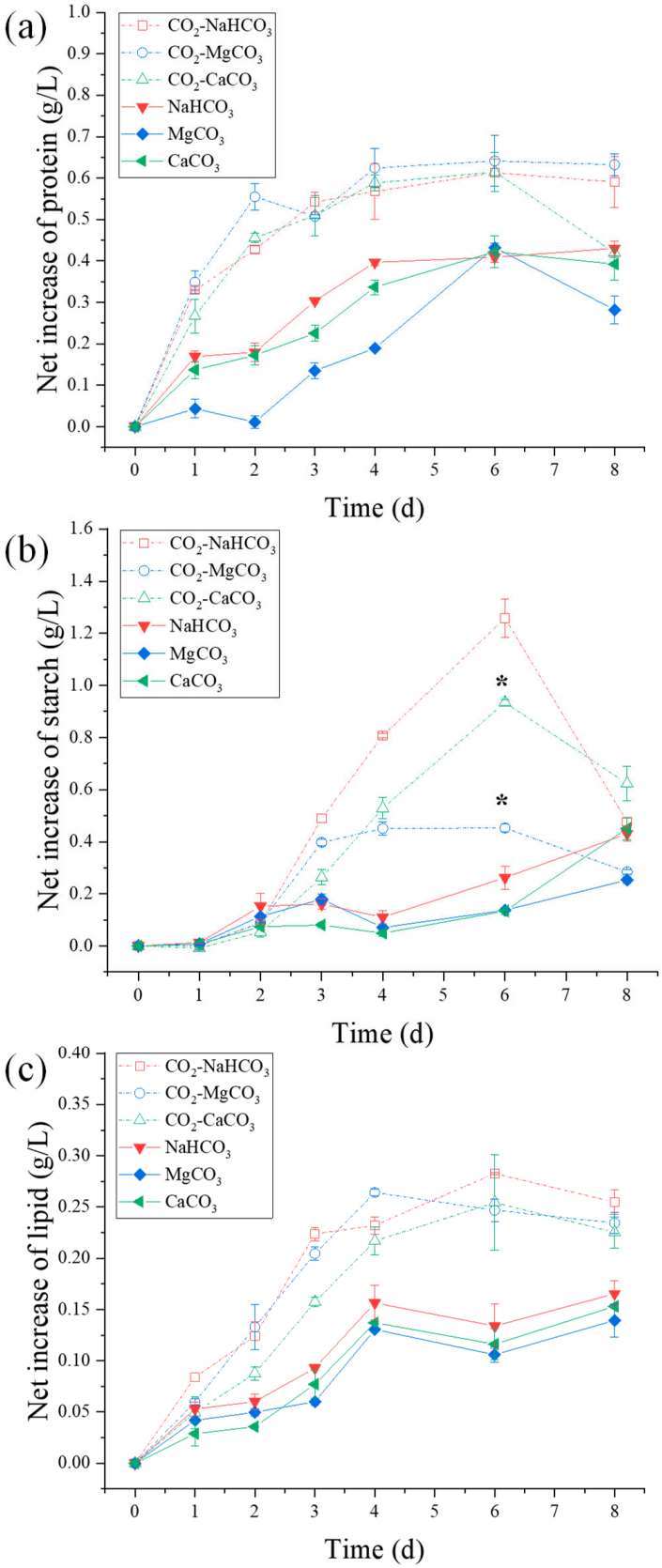

Figure 4. The protein (**a**), starch (**b**), and lipid (**c**) accumulation as assessed by the net increase under IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) or CO_2-IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) conditions during the NH_4^+-N removal by *Tetraselmis subcordiformis*. The asterisk (*) indicates a significant difference ($p < 0.05$) compared with the corresponding $NaHCO_3$ culture.

The lipid production, on the whole, mirrored the profile of protein, in which the accumulation started with the NH_4^+-N assimilation and lasted until NH_4^+-N was completely removed (Day 4), and there was no significant difference between the ICs applied in both cultivation systems (Figure 4c). It indicated that the accumulated lipid were largely polar lipids such as phospholipids and glycolipids, which were responsible for photosynthetic cell growth under nitrogen-replete conditions [62]. The maximum net increase in lipids reached 0.25~0.28 g/L in the CO_2-IC cultures and 0.13~0.16 g/L in the IC cultures, which showed a relatively minor contribution compared with the starch and protein accumulation (Figure 4). In all, *Tetraselmis subcordiformis* was able to assimilate NH_4^+-N and store the N as protein, while starch and lipid could be simultaneously produced under both IC and CO_2-IC systems, with CO_2-$NaHCO_3$ culture showing the best overall biomass component production capacity, which manifested the superiority of this strategy for NH_4^+-N removal and reclamation from wastewater.

3.5. Biomass Quality Evaluation

The main products obtained in the NH_4^+-N removal process by *Tetraselmis subcordiformis* were starch and protein (Figure 4), as discussed above. Since the amylose/amylopectin ratio (Am/Ap) in starch and the amino acid profile of the protein are crucial indexes for the biomass quality of the microalgae that could determine the downstream applications, these two items were further evaluated in detail.

3.5.1. Starch Composition

In general, from the structural perspective, two types of starch, namely, amylose (Am), which is linear polymerized glucose linked by α-1,4 glycosidic bonds, and amylopectin (Ap), which has additional branching at the α-1,6 positions, can be synthesized by microalgae, including *Tetraselmis subcordiformis* [44,47,63]. As shown in Table 2, under the IC culture system, Ap constituted most of the starch stored in the algae, with a proportion of around 60% in all the IC conditions; Am/Ap ranged from 0.6 to 0.7, which was consistent with the starch obtained by adding the same dosage of $NaHCO_3$ under N starvation for four days (0.62) as reported previously in this microalga [47]. A similar starch composition was obtained under the CO_2-$NaHCO_3$ condition with a Am/Ap reaching 0.64 (Table 2), which could be classified as the "normal" starch that is prevalent in plants and algae [64,65]. Starch with a higher Ap proportion is considered to facilitate enzymatic hydrolysis for glucose release and hence improves the fermentation efficiency [66]. In view of the highest starch production under the CO_2-$NaHCO_3$ condition in this study, it was reasonable to apply this strategy for the NH_4^+-N removal coupled with starch production that could be used as the feedstock for fermentation to manufacture biofuels (such as bioethanol) and bio-based chemicals [67]. Interestingly, the addition of $MgCO_3$ or $CaCO_3$ under the CO_2 supply significantly enhanced the relative Am proportion in starch ($p < 0.05$), with Am accounting for 50~60% and Am/Ap of 1~1.5 obtained (Table 2). Tracking the Am and Ap accumulation during the cultivation revealed that the increased Am/Ap could be largely ascribed to the stronger inhibition of Ap production relative to the Am (Figure 5b,d). For instance, in the CO_2-$CaCO_3$ culture, the Am production reached a similar level (0.48~0.49 g/L) as in the CO_2-$NaHCO_3$ culture (Figure 5b), but the Ap accumulation was remarkably reduced (0.47 for CO_2-$CaCO_3$ vs. 0.78 for CO_2-$NaHCO_3$, $p < 0.05$, Figure 5d), which accounted for the decrease in total starch accumulation (Figure 4b). In fact, the increase in Am/Ap in the CO_2-$MgCO_3$, CO_2-$CaCO_3$, and $MgCO_3$ cultures seemed to be correlated with the decrease in total starch accumulation (Table 2 and Figure 4b), suggesting that the low-light-caused reduction of starch accumulation (as discussed in Section 3.4) was manifested more in the Ap rather than the Am synthesis. Low light could have caused a reduced activity of the starch-branching enzyme responsible for the Ap synthesis [68], leading to the increased Am/Ap ratio. The starch obtained under the CO_2-$MgCO_3$ and CO_2-$CaCO_3$ conditions herein, with more than 50% of Am contained in the total starch (Am/Ap > 1), could be classified as high-amylose starch (HAS), which would be of high

value and applied in bio-based material production (such as films, coatings, textiles, paper, medical devices, and biodegradable flexible packaging) because of the excellent material properties [64,69]; meanwhile, it could serve as the resistant starch supplemented in the feeds for promoting health in aquaculture [70]. The above application of starch produced in *Tetraselmis subcordiformis* during the NH_4^+-N removal could improve the economic benefits for the wastewater treatment.

Table 2. Amylose (Am) and amylopectin (Ap) proportion in the starch of *Tetraselmis subcordiformis* under IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$, Day 8) or CO_2-IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$, Day 6) conditions for the NH_4^+-N removal. The asterisk (*) indicates a significant difference ($p < 0.05$) compared with the corresponding $NaHCO_3$ culture.

Culture System	Bicarbonate/Carbonate Supply	Am (% of Total Starch)	Ap (% of Total Starch)	Am/Ap
IC	$NaHCO_3$	39.46 ± 0.01	60.54 ± 0.01	0.65 ± 0.03
	$MgCO_3$	41.76 ± 0.01	58.24 ± 0.01	0.72 ± 0.03
	$CaCO_3$	37.10 ± 0.02	62.90 ± 0.02	0.59 ± 0.05
CO_2-IC	$NaHCO_3$	38.90 ± 0.01	61.10 ± 0.01	0.64 ± 0.02
	$MgCO_3$	59.57 ± 0.00 *	40.43 ± 0.00 *	1.47 ± 0.00 *
	$CaCO_3$	50.54 ± 0.00 *	49.46 ± 0.00 *	1.02 ± 0.01 *

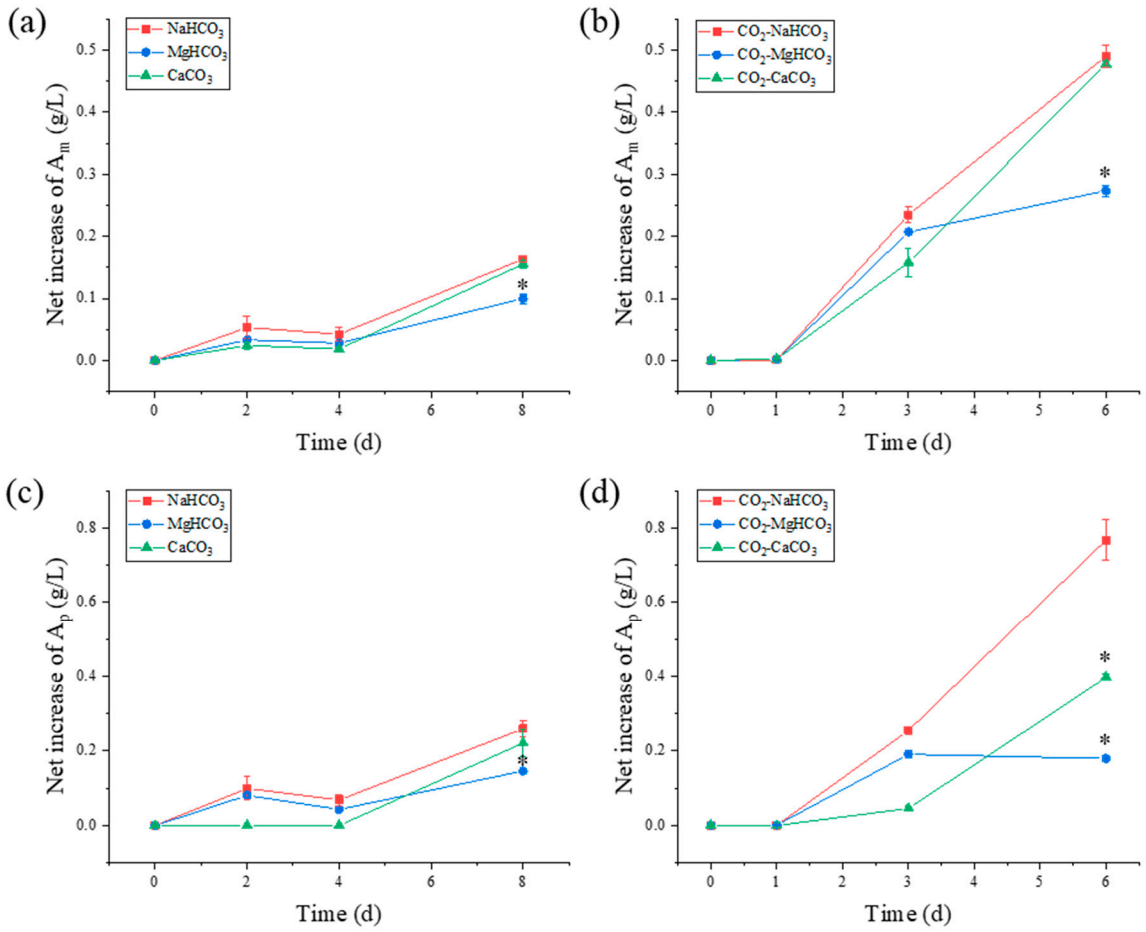

Figure 5. The amylose (**a**,**b**) and amylopectin (**c**,**d**) accumulation as assessed by the net increase under IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$, (**a**,**c**)) or CO_2-IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$, (**b**,**d**)) conditions during the NH_4^+-N removal by *Tetraselmis subcordiformis*. The asterisk (*) indicates a significant difference ($p < 0.05$) compared with the corresponding $NaHCO_3$ culture.

3.5.2. Amino Acid Profile

Table 3 lists the amino acid (AA) profile (g AA/100 g total AA) in *T. subcordiformis* under IC (Day 8) or CO_2-IC (Day 6) conditions for the NH_4^+-N removal. Generally, the AA profiles showed little variation among all the culture conditions. The dominant AAs were registered as glutamic acid, aspartic acid, leucine, and alanine, which accounted for more than 8% of the total AAs; the main essential amino acids (EAAs) were leucine, lysine, threonine, and valine, with the proportion being more than 6% of the total AAs. The AA profile character in *T. subcordiformis* cultivated in ammonium herein was similar to that cultivated in nitrate as nitrogen source [44] and was also consistent with other *Tetraselmis* species cultivated in urban wastewater that contained both nitrate and ammonium [71]. The most evident difference in the AA profiles among different ICs was the methionine, which was markedly increased in the $MgCO_3$ culture under the IC system and $CaCO_3$ under the CO_2-IC system compared with the respective $NaHCO_3$ cultures (Table 3). Methionine in protein was considered as an endogenous antioxidant in cells [72]. The increase in methionine indicated the oxidative stress that the microalgae were exposed to, which was in agreement with the reduced F_v/F_m in the $MgCO_3$ culture subjected to high-pH/ammonia inhibition and in the CO_2-$CaCO_3$ culture with high-Ca^{2+} stress, as discussed in the previous sections.

As for the nutritional concerns, compared to soybean meal, a widely used feed for animals and aquaculture [73], *T. subcordiformis* produced a higher proportion of EAAs (more than 47%) in both the IC and CO_2-IC systems with the removal of NH_4^+-N (Table 3). In particular, the CO_2-IC system resulted in overall higher EAAs than the IC system (except for the $MgCO_3$ culture where methionine contributed to the atypically high EAAs), which could be caused by the enhancement of the histidine and lysine (Table 3), exemplifying the advantage of the CO_2-IC system for providing better AA nutrition for animals. Calculation of the essential amino acid index (EAAI) for prawn showed that the proteins synthesized in *T. subcordiformis* under the IC and CO_2-IC systems had EAAI scores higher than 0.90 (except for the CO_2-$MgCO_3$ culture), which could be classified as "good-quality" protein according to Peñaflorida [74]; the high proportion of valine (6.3~6.7) in *T. subcordiformis* replenished the insufficiency of this AA in soybean meal (actual/ideal = 3.85/5.16) and fish meal (actual/ideal = 5.07/5.16) for *Penaeus monodon* juvenile, demonstrating the good potential to be applied in aquaculture. Notably, the EAAI scores for the ruminant in this study were higher than 1.00 (except for the CO_2-$MgCO_3$ culture), which could be categorized as "high-quality" protein [45] and were even superior to that of ruminant diets (0.97). The shortage of phenylalanine (actual/ideal = 2.9/4.4), leucine (actual/ideal = 6.0/8.6), and threonine (actual/ideal = 3.8/5.2) in the ruminant diets could be completely compensated for by the proteins of *T. subcordiformis* (5.7~6.1 for phenylalanine, 8.9~9.4 for leucine, and 6.1~6.8 for threonine) with the removal of NH_4^+-N under all the culture conditions herein, which manifested the great potential for livestock graziery. Collectively, together with the consideration of protein production ability (Figure 4a), the *T. subcordiformis* biomasses obtained under CO_2-$NaHCO_3$ and CO_2-$CaCO_3$ conditions were preferable for providing excellent alternative protein sources in livestock graziery and aquaculture, which could realize the bio-reclamation and valorization of waste NH_4^+-N.

Table 3. Amino acid (AA) profiles (g AA/100 g total AA) in *Tetraselmis subcordiformis* under IC ($NaHCO_3$, $MgCO_3$, or $CaCO_3$, Day 8) or CO_2-IC ($NaHCO_3$, $MgCO_3$, or $CaCO_3$, Day 6) conditions for the NH_4^+-N removal and comparison with traditional animal feed proteins in terms of the essential amino acid index (EAAI).

Animo Acids	IC			CO_2-IC			Soybean Meal [c]	Fish Meal [c]	*Penaeus monodon* Juvenile [c]	Ruminant Diets [c]	Ideal Protein for Dairy Cow [c]
	$NaHCO_3$	$MgCO_3$	$CaCO_3$	$NaHCO_3$	$MgCO_3$	$CaCO_3$					
Essential AA (EAA)											
Arginine	4.65 ± 0.21	4.35 ± 0.12	4.92 ± 0.65	4.99 ± 0.26	4.50 ± 0.05	4.94 ± 0.17	8.30	6.75	8.00	14.8	
Histidine	1.69 ± 0.06	1.94 ± 0.03	1.75 ± 0.02	2.23 ± 0.19	1.88 ± 0.04	2.03 ± 0.15	3.04	2.54	2.48	4.8	
Isoleucine	4.22 ± 0.30	4.32 ± 0.09	4.39 ± 0.17	4.34 ± 0.12	4.49 ± 0.24	4.30 ± 0.01	5.22	4.32	4.46	3.6	4.8
Leucine	9.12 ± 0.23	9.16 ± 0.08	9.13 ± 0.27	9.16 ± 0.05	9.40 ± 0.23	8.91 ± 0.33	8.30	8.06	7.66	6.0	8.6
Lysine	7.39 ± 0.13	7.15 ± 0.01	7.45 ± 0.06	8.54 ± 0.57	8.31 ± 0.35	8.56 ± 0.28	6.95	8.76	7.58	7.6	6.7
Methionine	1.05 ± 0.62	2.14 ± 0.01	1.19 ± 0.07	0.92 ± 0.14	0.43 ± 0.20	1.45 ± 0.25	1.97	3.23	2.85	1.3	2.0
Phenylalanine	5.94 ± 0.01	5.86 ± 0.05	5.72 ± 0.12	5.71 ± 0.08	6.10 ± 0.23	5.75 ± 0.17	4.88	4.21	4.20	2.9	4.4
Threonine	6.66 ± 0.04	6.78 ± 0.33	6.33 ± 0.06	6.14 ± 0.06	6.15 ± 0.29	6.08 ± 0.04	4.09	5.12	3.96	3.8	5.2
Tryptophan [a]	-	-	-	-	-	-					
Valine	6.56 ± 0.18	6.67 ± 0.02	6.63 ± 0.04	6.25 ± 0.67	6.71 ± 0.12	6.30 ± 0.03	3.85	5.07	5.16	5.1	5.3
TOTAL EAA	**47.27**	**48.39**	**47.51**	**48.05**	**48.54**	**48.69**	**46.60**	**48.06**	**46.34**		
Non-essential AA (NEAA)											
Alanine	8.16 ± 0.03	7.89 ± 0.04	8.15 ± 0.08	7.31 ± 0.00	7.29 ± 0.45	7.75 ± 0.23	4.61	6.45	6.11		
Aspartic acid [b]	11.40 ± 0.34	11.24 ± 0.56	11.17 ± 0.39	11.85 ± 0.47	11.09 ± 1.32	11.15 ± 0.26	11.91	10.63	10.20		
Cysteine	0.60 ± 0.06	0.63 ± 0.16	0.91 ± 0.11	0.60 ± 0.10	0.91 ± 0.23	0.63 ± 0.10	2.20	1.08	1.03		
Glutamic acid [b]	13.74 ± 0.45	12.92 ± 0.30	13.94 ± 0.09	13.52 ± 0.22	13.18 ± 1.27	13.00 ± 0.33	18.07	15.38	16.07		
Glycine	5.48 ± 00.35	5.51 ± 0.03	5.33 ± 0.06	5.23 ± 0.20	6.07 ± 0.54	5.70 ± 0.10	3.99	5.68	8.12		
Proline	4.96 ± 0.15	5.00 ± 0.03	4.98 ± 0.21	4.83 ± 0.08	4.97 ± 0.59	5.02 ± 0.32	4.37	4.21	4.02		
Serine	5.38 ± 0.13	5.45 ± 0.33	5.13 ± 0.23	5.16 ± 0.24	5.04 ± 0.11	5.18 ± 0.07	5.04	5.10	4.16		
Tyrosine	3.00 ± 0.14	2.97 ± 0.02	2.87 ± 0.14	3.21 ± 0.15	3.46 ± 0.35	3.25 ± 0.08	3.22	3.41	3.94		
EAAI for prawn	**0.90**	**0.97**	**0.92**	**0.91**	**0.83**	**0.94**	**0.98**	**1.01**			
EAAI for ruminant	**1.01**	**1.09**	**1.03**	**1.02**	**0.93**	**1.06**				**0.97**	

Notes: [a] Tryptophan was destroyed by acid hydrolysis. [b] Asparagine and glutamine were hydrolyzed to aspartic acid and glutamic acid, respectively. [c] Data acquired from Xiang et al. [44].

3.6. Future Works

The present study demonstrated a high NH_4^+-N removal rate along with low N escape and high-quality biomass production in *T. subcordiformis* cultivated in synthetic wastewater under the CO_2-IC system. In real urban wastewater, although normally low contents of toxicant were present [6], the performance of the algae might be challenged because of the complexity of the composition of the wastewater, e.g., the variations of N/P ratio that could influence the photosynthetic growth and nutrient removal as well as the biomass component produced [6,9]. In addition, the implications of the microorganisms contained in the real wastewater might also be considered, although they could be both positive and negative [9]. Further investigation is needed to evaluate the feasibility of the established microalgal culture system herein for real urban wastewater treatment.

It should be noted that the present study performed the NH_4^+-N removal under batch mode. For the best performance under the CO_2-$NaHCO_3$ culture system, the removal constant (k_N) was calculated to be 1.76 day^{-1} when fitted with the first-order removal kinetics model (Table S1, [75]). It could be extrapolated to a continuous NH_4^+-N removal process where a constant flow of wastewater is treated under a steady state. In this operation mode, the hydraulic retention time (HRT) could be predicted according the formula of $(N_i - N_e)/HRT = k_N \times N_e$ [76], where N_i and N_e are the influent and effluent NH_4^+-N concentrations, respectively. For the N_i of 100 mg/L used herein and the N_e of 5 mg/L that meets the minimum discharge standard (Grade I–A) for urban wastewater treatment in China [50], the HRT has to be set at no less than 10.8 day. Further study needs to be conducted to dissect the effect of HRT on the NH_4^+-N removal and biomass accumulation under the continuous treatment mode to make the process more practical. Moreover, for practical operations, flue gas could be used for the CO_2 supply with a circulation system to further improve the economic feasibility and sustainability of the treatment process.

4. Conclusions

In the present study, the continuous sparging of air containing 2% CO_2 along with inorganic bicarbonate/carbonate addition was used to achieve pH stabilization and maximize algae growth and NH_4^+-N removal by *Tetraselmis subcordiformis*. The application of this culture system also significantly reduced the NH_3 escape into the air and restricted the extracellular organic matter release into the medium by the microalgae, which minimized the secondary pollution during the NH_4^+-N removal process. Specifically, the addition of 12 mM $NaHCO_3$ with 2% CO_2-containing air sparging was demonstrated to exhibit the best performance, in which more than 98% of NH_4^+-N was removed within two days and the maximum NH_4^+-N removal capacity of 60.13 mg N/L/day achieved was the highest level compared with other microalgae under similar conditions reported hitherto. The NH_3 escape rate of 19.7% in this auto-buffering system was also comparable to that obtained in the acid/base-based pH monitoring algal culture system, which can comparatively reduce the instrument investment and operating cost; the total organic carbon release of 1.3 mg/L was also the lowest compared with other microalgae cultures reported previously. The starch accumulated in *T. subcordiformis* with the NH_4^+-N removal was suitable for fermentation or bio-based material production and use as a health-promoting ingredient for aquaculture, and the proteins in the biomass were of good quality for animal feeds, both of which would valorize the NH_4^+-N-containing wastewater treatment. Further study is needed to confirm the performance of the established CO_2-inorganic carbonate system for real urban wastewater treatment under both batch and continuous operation modes in terms of NH_4^+-N removal and algal biomass production to make the process practical.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15091671/s1, Figure S1: Ca^{2+} concentration in the CO_2-$NaHCO_3$ and CO_2-$CaCO_3$ culture system; Figure S2: The estimated free ammonia (NH_3) relative to the total ammonia (NH_3 and NH_4^+) (**a**) and the free ammonia concentration (NH_3, mg/L) in the medium (**b**) during the NH_4^+-N removal process. The asterisk (*) indicates a significant difference ($p < 0.05$)

compared with the corresponding $NaHCO_3$ culture; Figure S3: The phosphorus (P) concentration in the medium under IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) or CO_2-IC ($NaHCO_3$, $MgCO_3$ or $CaCO_3$) conditions during the NH_4^+-N removal by *Tetraselmis subcordiformis*; Table S1: The calculated NH_4^+-N removal constant (k_N) fitted with the first-order removal kinetics model $N = N_0 \times e^{-kN \times t}$ [75] and the estimated hydraulic retention time (HRT) with the formula of $(N_i - N_e)/HRT = k_N \times N_e$ [76] where N_i and N_e were the influent (100 mg/L) and effluent (5 mg/L) NH_4^+-N concentration, respectively under continuous cultivation mode.

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