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Filter Feeding and Carbon and Nitrogen Assimilation of a Freshwater Bivalve (*Unio douglasiae*) on a Toxic Cyanobacterium (*Microcystis aeruginosa*)

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Abstract: We investigated the possible intake of toxic cyanobacteria (*Microcystis aeruginosa*) as a nutrient resource for a filter-feeder bivalve (*Unio douglasiae*) based on the measurement of feeding and assimilation rates of carbon and nitrogen in a limited space with no current for 72 h using ^{13}C and ^{15}N dual isotope tracers. With high clearance rates, the unionid rapidly removed *Microcystis* cells within 24 h, but only a small amount of carbon and nitrogen were incorporated into the tissues. Even with the low assimilation rates, the mussels showed more favorable uptake of carbon than of nitrogen from toxic *Microcystis* water, and of tissues, the gills and gut accumulated more carbon and nitrogen than the muscle and mantle. Collectively, our findings indicate that although *Unio douglasiae* effectively uptake toxic *Microcystis* cells, they can assimilate only low amounts of nutrients into tissues within three days, despite a non-flowing system.

Keywords: assimilation; carbon; filter-feeder; nitrogen; stable isotope; *Unio douglasiae*; uptake; toxic *Microcystis*

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1. Introduction

Widely distributed in East Asia (China, Japan, and Korea), Unionoida, which includes the genera *Anodonta* and *Unio*, is a group of freshwater mussels, representing a guild of benthic, burrowing, and filter-feeding bivalves [1]. Bivalves are efficient filter feeders of particles suspended in the water column, including bacterioplankton, protists, phytoplankton, zooplankton, and other abiotic particles [2–4]. Thus, in rivers and lakes where they are abundant, unionoids are expected to play pivotal roles in driving ecosystem functions, such as carbon flow and nitrogen recycling.

The bivalve species *Unio douglasiae* is widespread in Asia and has been consumed as food for 500 years in Korea [5,6]. At present, this species is neither threatened nor endangered [7]. Despite the lack of extensive research (i.e., taxonomic and ecological studies) on unionid bivalves, including *Unio douglasiae* in Korea, their biomass and diversity are anticipated to decline due to repetitive cyanobacterial blooms. These are usually triggered by the destruction of habitat, pollution, loss of host-fish for larval development, and outbreaks of invasive species [8]. Algal blooms represent a serious threat during every dry season or early summer (between May and July). Unfortunately, whether *Unio douglasiae* populations in Korea can persist through repetitive cyanobacterial blooms, primarily those of *Microcystis*, *Dolichospermum*, *Oscillatoria*, and *Aphanizomeon*, remains unclear.

Cyanobacterial blooms are common in eutrophic or hypertrophic freshwater ecosystems worldwide [9,10]. These blooms negatively affect the nutritional quality of food

sources in such ecosystems, and toxins secreted by these cyanobacteria (e.g., microcystin or anatoxin) are harmful to aquatic organisms and humans. Unionid mussels are known to filter feed on cyanobacteria, such as colony-forming *Microcystis aeruginosa* and filamentous *Planktothrix agardhii* [11,12]. In addition, *Unio douglasiae* and *Corbicula fluminea* actively graze on both toxic and non-toxic *Microcystis aeruginosa*, while *Anodonta anatina* shows a slight preference for green algal species, such as *Scenedesmus*, although it can still filter feed and ingest both *Microcystis* and *Planktothrix* [13]. Ultimately, these unionoids can reduce the abundance of cyanobacteria in the water column, although cyanobacteria bloom globally reduces the diversity and health of other species [10].

In the present study, stable isotope tracers (^{13}C and ^{15}N) were used to evaluate the accumulation of cyanobacterial (*Microcystis aeruginosa*) carbon and nitrogen in unionid bivalves (*Unio douglasiae*) through laboratory experiments. Isotope labeling techniques based on the enrichment of ^{13}C and ^{15}N stable isotope ratios have been commonly applied to determine the uptake rate of carbon and nitrogen and their allocation to various animal tissues or organs. These techniques can also be used to trace the fate of algal-derived organic matter in natural biota and elucidate energy sources and pathways [14–16]. Using ^{13}C- and ^{15}N-labeled phytoplankton, the assimilation pathways of carbon and nitrogen in aquatic organisms can be directly traced. The inorganic forms of $^{13}C(HCO_3^-)$ and $^{15}N(NO_3^-)$ tracers were assimilated into the *Microcystis* as organic forms through photosynthesis, and synthesized newly organic cells.

The objective of the present study was to evaluate the feeding effect of a unionid bivalve (*Unio douglasiae*) on a toxic cyanobacterium *Microcystis aeruginosa* as a nutrient resource and to determine whether the unionid assimilates *Microcystis* carbon and nitrogen using ^{13}C and ^{15}N dual tracers in a short period of time with no current.

2. Materials and Methods

2.1. Animal Collection and Maintenance

Between 2011 and 2013, freshwater bivalve (*Unio douglasiae* Griffith et Pidgeon; Unionidae) specimens were collected from a small agricultural canal (35°56′02″ to 35°54′13″ N; 126°47′07″ to 126°45′43″ E) in Gunsan, Korea. During preliminary sample collection in 2011, the density of *Unio douglasiae* was approximately 12.5 individuals per m^2, comprising 40% of the total molluscan community. Other species inhabiting the canal included several mussels (*Anodonta woodiana*, *Cristaria plicata*, and *Corbicula leana*), invasive apple snail (*Cipangopaludina chinensis*), and fish (*Carassius auratus* and *Zacco platypus*).

To acclimatize the collected mussels to the laboratory environment, we carefully removed the debris attached to their shells and transferred them to an acclimatization system of mussels (ASM) connected serially to three tanks [8]. Each tank was made from plastic and was cylindrical, with volume of 120 L. Water flow in all tanks was regulated by an electric pump. The first tank (T1) continuously received surface water from a eutrophic lake (Lake Ilgam) [8], after being filtered to remove large zooplankton using a net (pore size = 200 μm). Filtered water was then transported to the second tank (T2), which harbored mussels at a density of approximately 120 individuals per m^2. The third tank (T3) received water from T2, and water from T3 was finally returned to Lake Ilgam after aeration to remove ammonia nitrogen. The ASM was automatically controlled at ambient temperature of 20 ± 0.5 °C, flow rate of 0.5–0.6 m^3 d^{-1}, light intensity of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and photoperiod of 14 h. Mussel viability in T2 was assessed daily, and dead animals that were immobile, with completely open shells and no accumulations surrounding the exhalant siphon, were removed immediately. For all mussels used in this study, we put them in the experimental tanks after simply washing their shells with T3 water and removing attached debris. The mussel mortality rate under these conditions was 0.5–1.0 individuals per month.

2.2. Preparation of Experimental Water

To prepare experimental water (M-water) in a laboratory aquarium, we incubated water and sediment samples collected from Lake Ilgam, where *Microcystis* blooms occur every year [17]. Briefly, during the *Microcystis* bloom, water and sediment samples were collected in a boat using a Van Dorn sampler (5 L, General Oceanics, FL, USA) and an Ekman dredge, respectively. A glass aquarium (45 × 120 × 50 cm; 280 L) was filled with 240 L of surface water and 30 L of sediment collected from the lake. The aquarium and its contents were maintained at water temperature of 27 ± 0.5 °C, light intensity of 80 μmol m^{-2}·s^{-1}, and photoperiod of 14 h, with weekly addition (1 L) of BG-11 medium [18,19] for nutrient supply and daily addition (500 mL) of water to compensate for evaporation loss. Three to five small fish (*Carassius auratus*) caught from the lake were introduced to freely mix the water column in the aquarium and to sustain the cyanobacterial blooms. We have previously used this method to maintain M-waters and prepare cyanobacterial bloom samples for other feeding experiments after measuring the phytoplankton biomass or cell density using a light microscope (Olympus, Tokyo, Japan) [8,20]. The prepared M-water predominantly comprised *Microcystis aeruginosa* and *Microcystis wesenbergii* (>97% of total biomass) but did include other phytoplankton, such as *Scenedesmus quadricauda*, *Cryptomonas ovata*, and *Aulacoseira granulata*, corresponding to 124.8–506.7 μg·L^{-1} of chlorophyll-*a* (CHL). Of note is that the M-water used in the present study was toxic, containing microcystin-LR [8,17].

2.3. Labeling and Grazing Experiments

Grazing experiments using *Unio douglasiae* (length, 6.1–7.4 cm) were conducted in the laboratory using an acrylamide quadrate chamber (23 × 12 × 13 cm; 3.6 L) filled with M-water (Figure 1). The experimental conditions are listed in Table 1.

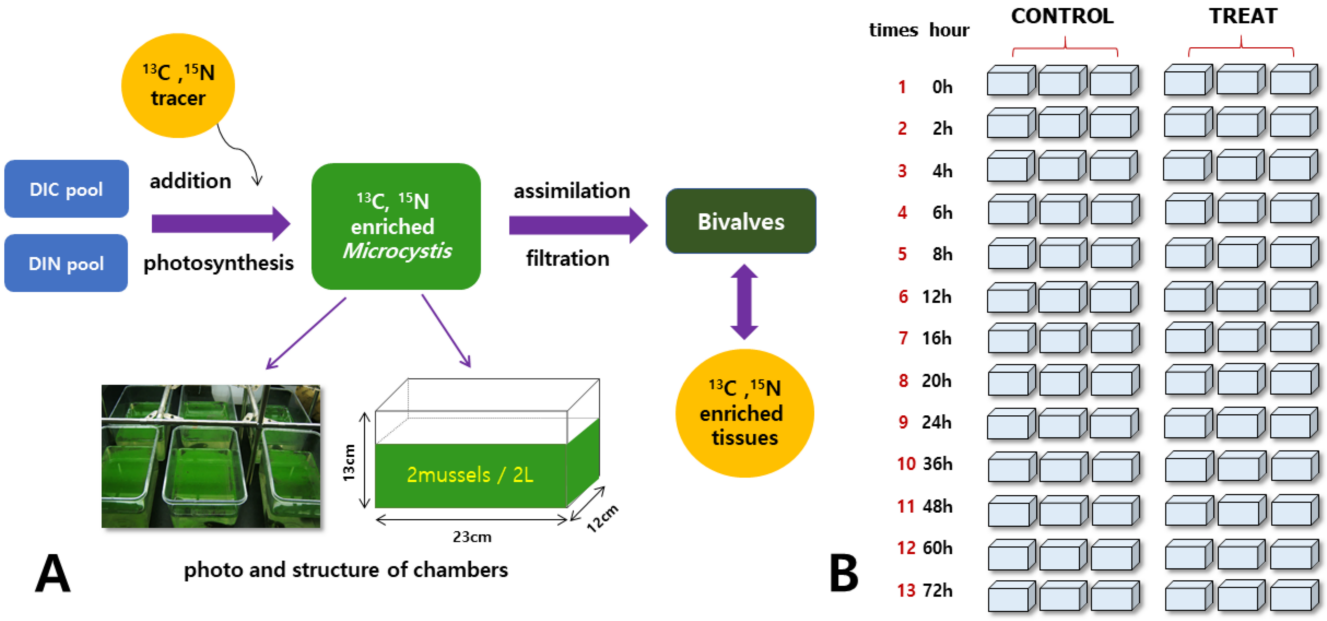

Figure 1. Feeding and assimilation experiments of freshwater bivalve *Unio douglasiae* Griffith et Pidgeon in *Microcystis*-predominating waters for 72 h. (**A**) Enrichment of carbon and nitrogen tracer by mussel feeding: (**B**) 78 trial chambers labeled CONTROL (3 replicates × 13 times) and TREAT (3 replicates × 13 times) were used in this study. Animal density in each TREAT chamber is indiv. L^{-1}.

As shown Figure 1, a total of 78 trials (chambers) were performed: 2 groups (control and treatment) × 3 replicates × 13 samples (0, 2, 4, 6, 8, 12, 16, 20, 24, 36, 48, 60, and 72 h). Each chamber was filled with 2 L of M-water filtered using a plankton net (64 μm) to remove zooplankton and maintained at a temperature of 20 °C, light intensity of 50 μmol·m^{-2}·s^{-1}, and photoperiod of 14 h until the end of the experiment. $NaHCO_3$ (Isotech; ^{13}C > 99%) and

$(NH_4)_2SO_4$ (Isotech; $^{15}N > 99\%$) were added to individual chambers as isotope tracers. The ^{13}C content of the dissolved inorganic carbon (DIC) pool increased to approximately 4%, and the ^{15}N content of the dissolved inorganic nitrogen (DIN) pool was stabilized with 1% enrichment over 48 h.

Table 1. Experimental conditions for the study of the freshwater bivalve *Unio douglasiae* Griffith et Pidgeon in *Microcystis*-rich waters.

Parameter	Conditions
Total volume of chamber (L)	3.6
Experimental volume (L)	2 (M-water)
Animal density per chamber (indiv. L^{-1})	1.0
Experimental replications	3
Shell length of mussels (cm)	6.59 ± 0.28 (6.10–7.40)
Ash-free dry matter of mussels (g) *	0.34 ± 0.07 (0.23–0.58)
Water temperature of tank (°C)	22 ± 2
Light intensity (μmol·m^{-2} s^{-1})	40–43
Light:dark cycle (h)	14:10
Total experimental period (h)	72
Sampling time points (h)	0, 2, 4, 6, 8, 12, 16, 20, 24, 36, 48, 60 and 72

* Ash-free dry matter for mussels was calculated based on a regression line obtained from mussels sacrificed before the experiments (n = 29).

All feeding experiment was performed in triplicate for both the control group (CONTROL) and the experimental group (TREAT), and as described in Table 1 and Figure 1. Water quality and bivalve analysis experiments were performed 13 times for 72 h. All TREAT chambers contained 2 *Unio douglasiae* individuals (indiv./L), and 6 chambers (3 CONTROL × 3 TREAT) were used at every sampling time for the two analyses. Before the study, several preliminary experiments were performed to determine the number of mussels to be stocked into each experimental chamber. When three or more mussels were placed in a chamber the size used in the experimental group, a dense resuspension of pseudofeces collected during the feeding process of the mussels. Thus, it was very difficult to measure algal biomass or chlorophyll-a. Then, we decided to use the minimum density of 2 animals and conducted the experiment. All mussels used in the experiment were acclimatized over 2 months in the ASM, selected at random, and starved for 24 h.

The instant clearance rate (CR) of each mussel was calculated as the difference in the concentration of CHL (μg L^{-1}) or *Microcystis* biomass (cells/mL) between the chambers without (CONTROL) and with the mussels (TREAT) at each time point based on the Equation (1) [19].

$$\text{CRs (L g}^{-1}\text{ h}^{-1}\text{)} = V/t \times \ln (C_o/C_t)/w \quad (1)$$

where V is the experimental volume (L), t is the duration of the experiment (h), C_o and C_t are the concentrations of CHL (μg L^{-1}) or *Microcystis* biomass (cells/mL) in the CONTROL and TREAT chambers, respectively, and w is the weight (AFDM) of mussels (g).

CHL concentration was measured as described below [21]. Numerical characteristics (shell length, width, and wet weight) of 29 individuals selected at random from the ASM were directly measured. Of these, ash-free dry-matter (AFDM) was estimated based on closely related physical characteristics of 83 mussels (Figure 2). To obtain AFDM, each mussel (without the shell) was dissected to identify individual organs (gills, stomach, gut, mantle, and muscle) using a fine knife. Each organ was weighed, transferred to a heat-resistant chamber, desiccated at 70 °C for 30 min in a dry oven, weighed, and burned in a furnace at 500 °C for 30 min. The AFDM was calculated as the difference in dry weight before and after burning [21].

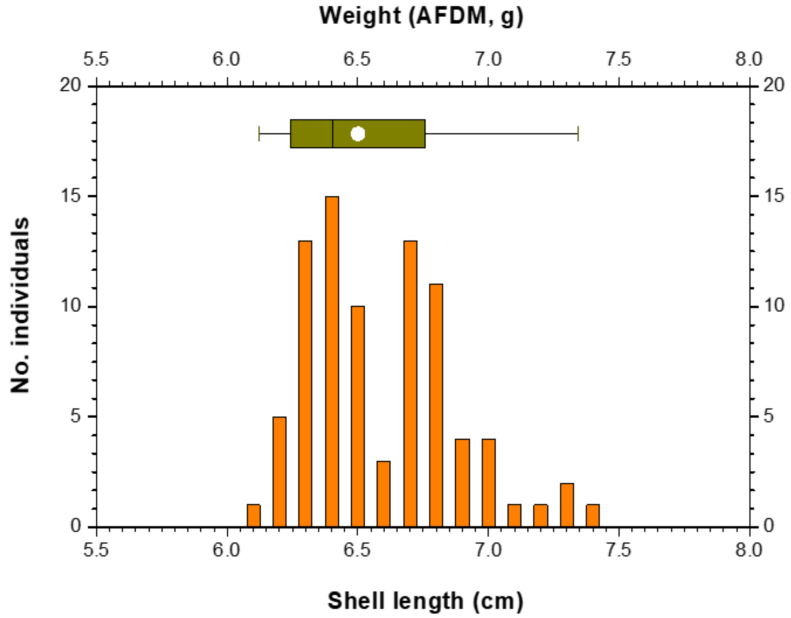

Figure 2. Distribution of shell length and weight (ash-free dry-matter: AFDM) of freshwater bivalve *Unio douglasiae* Griffith et Pidgeon used in this study (n = 83).

Simultaneously, the production rates of pseudofeces and feces (PRs) of *Unio douglasiae* in M-water were measured based on the Equation (2). Fecal pellets forming masses were carefully harvested using a capillary pipette and passed through a GF/F filter. The dry weight of pseudofeces and feces per individual or dry weight of mussels was quantified [19]. PR was calculated as the difference in the dry weight of fecal pellets between two sampling times.

$$\text{PRs (L g}^{-1}\text{ h}^{-1}\text{)} = \text{V/t} \times \ln (\text{P}_\text{o}/\text{P}_\text{t})/\text{w} \quad (2)$$

where V is the experimental volume (L), t is the duration of the experiment (h), P_o and P_t are the dry weights of the fecal pellets (g L^{-1}) before and after sampling, respectively, and w is the weight (AFDM) of mussels (g).

2.4. Stable Isotope Analysis

We first analyzed the natural abundance and isotopic composition of energy sources (carbon and nitrogen) in the *Microcystis aeruginosa* and *Unio douglasiae* samples. We then examined the assimilation of ^{13}C- and ^{15}N-enriched sources derived from the plankton food web during laboratory incubation with *Unio douglasiae*. When both carbon and nitrogen isotope tracers were added to M-water, inorganic forms of both ^{13}C and ^{15}N tracers were assimilated into cyanobacteria through photosynthesis and newly biosynthesized phytoplankton cells. Increase in the atom% of filter feeders indicates the assimilation of newly biosynthesized phytoplankton cells into mussels through feeding activity.

Briefly, the bivalve samples were dissected to separate the digestive gland, stomach, muscle (abductor), gill, and mantle (three samples per tissue). All tissue samples were freeze-dried and ground to a fine powder using a grinder (FRITSCH-Planetary Mono Mill, Pulverisette 6, Germany). Homogenized powder samples of each tissue were decalcified with 1 N HCl for at least 24 h to remove the remaining carbonates. However, subsamples for δ^{15}N analysis were not treated with acid because HCl treatment may affect the δ^{15}N value [22,23]. Following acid treatment, the samples were re-dried using a freeze drier, ground to a fine powder, and thoroughly mixed prior to analysis. Stable carbon and nitrogen isotopic ratios were measured using a continuous-flow isotope-ratio mass spectrometer (Isoprime; GV Instrument, UK) coupled to an elemental analyzer (Euro EA 3000-D, Italy).

Isotopic ratios are presented as δ values (‰) relative to the Vienna Pee Dee Belemnite (vPDB) standard and atmospheric N_2 for carbon and nitrogen, respectively. The reference materials were IAEA-CH6 ($\delta^{13}C = -10.45 \pm 0.04$‰) and IAEA-N1 ($\delta^{15}N = 0.4 \pm 0.2$‰). The analytical precision was within 0.2‰ and 0.3‰ for carbon and nitrogen, respectively. Isotopic ratios were expressed as per mil (‰) using the standard delta (δ) notation (Equation (3)):

$$\delta X = \{(R_{sample} - R_{std})/R_{std}\} \times 1000\ (‰) \quad (3)$$

where X = ^{13}C or ^{15}N, R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$, and std (standard) = vPDB for carbon or atmospheric N_2 for nitrogen. The δ-values were converted to atom%, which is more appropriate for labeled samples [24]. The conversion was performed using Equation (4):

$$A\ (atom\%) = 100/[1/\{(\delta\ sample/1000 + 1) \times a_{ns}\} + 1] \quad (4)$$

where the a_{ns} values for carbon and nitrogen were 1.118×10^{-2} and 3.677×10^{-3}, respectively.

The assimilation rate (AR) of *Unio douglasiae* of labeled phytoplankton in M-water was calculated using the method described by Hama et al. (1987) [25] (Equation (5)):

$$ARs\ (\mu g\ C\ mg^{-1} \cdot h^{-1}) = \{(C_{is} - C_{ns})/(C_{ic} - C_{ns})\} \times C/t \quad (5)$$

where C_{is} is the atom% of ^{13}C in the incubated sample, C_{ns} is the atom% of ^{13}C in a natural (non-incubated) sample, C_{ic} is the atom% of ^{13}C in inorganic carbon, t is the incubation time (h), and C is the concentration of particulate organic carbon (POC) (μg C mg^{-1} h^{-1}).

2.5. Water Quality Analysis

To measure the concentration of chlorophyll-*a*, water samples were collected from each chamber. First of all, 100–200 mL of the samples were filtered using GF/C filter paper (Whatman™. GE Healthcare Life Science, Piscataway, NJ, USA) and extracted with 90% acetone for 24 h at 4 °C under dark and refrigerated conditions. After 20 min of centrifugation (Labogene 1248R, Hanil SME, Korea), the optical density was measured from the supernatant using a spectrophotometer (SP-2700i, Youngwoo, Korea) at 750 nm, 663 nm, 645 nm, and 630 nm, and the concentration of chlorophyll-*a* was calculated [21].

The concentration of total suspended particulate matter (SPM, $mg \cdot L^{-1}$) was determined using the following methods. SPM includes suspended solids in the M-water and fecal pellets excreted by the mussels. SPM concentration was measured as the difference in the weight of GF/F papers before and after drying at 70 °C in an oven [21]. Similarly, particulate organic matter (POM, $mg \cdot L^{-1}$) concentration was measured as the difference in the weight of GF/F papers before and after burning at 450 °C for 1 h [21].

For inorganic nutrient analysis, water samples were passed through Whatman GF/F filters and frozen at −10 °C until use. The concentration of dissolved inorganic nitrogen (NO_2-N, NO_3-N, and NH_4-N) and phosphate (PO_4-P) was measured using the phenate, colorimetric, cadmium reduction, and ascorbic acid methods. Total nitrogen (TN) and phosphate (TP) content was determined using the cadmium reduction and ascorbic acid methods, respectively, after digestion with persulfate [21].

2.6. Data Analysis

Data of water quality were log-transformed, and a Mann–Whitney U-test was used to compare differences in abiotic factors (water quality of chamber) between the CONTROL and TREAT chambers, and differences in biotic factors (clearance and assimilation rates of mussels) between pre- and post-sampling time points. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using SPSS ver. 12.0.1 [26].

3. Results

3.1. Mussel Grazing

When *Unio douglasiae* were stocked in experimental chambers filled with M-water, SPM was completely removed within a short period (12 h), while POM and CHL levels were decreased by 51% (at 8 h) and 66% (at 16 h), respectively (Supplement-1). The CR of mussels for SPM (1.74 L g^{-1} h^{-1}), POM (0.64 L $dw^{-1}h^{-1}$), and CHL (0.39 L g^{-1} h^{-1}) peaked during the initial stages of the experiment (within 24 h) but sharply declined thereafter (Figure 3).

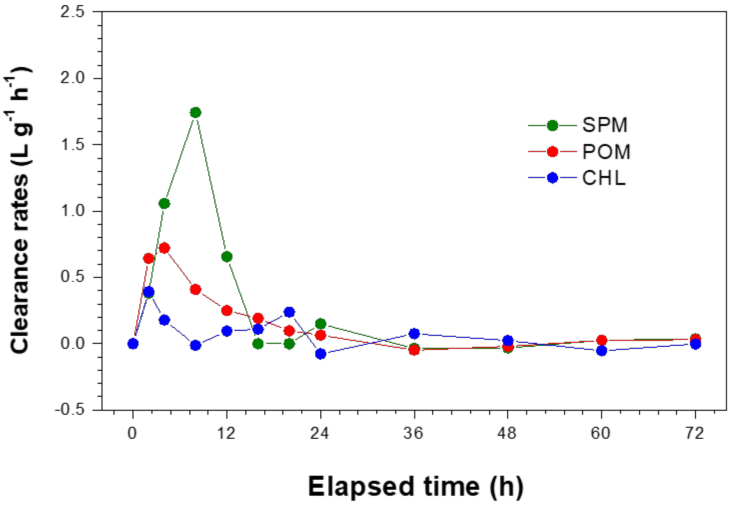

Figure 3. Clearance rate (L g^{-1} h^{-1}) of *Unio douglasiae* for suspended particulate matter (SPM), particulate organic matter (POM), and chlorophyll-*a* (CHL). Each value is the average of three subsamples (standard errors are presented in Supplement-1).

3.2. Atom% of ^{13}C and ^{15}N in POM and Mussel Samples

The atom% of ^{13}C and ^{15}N in the POC and PON of POM (mostly *Microcystis*) in each chamber showed similar trends (Figure 4a). Within 1 d of the addition of the tracers, the atom% of ^{13}C and ^{15}N in POM was enriched through active phytoplankton assimilation, peaking at 16 h (max. 4.15%, 1.07–4.15%) and 12 h (max. 1.10%, 0.37–1.10%), respectively. However, the values became saturated and were slightly decreased by the end of the experiment. The atom% of ^{13}C and ^{15}N in feces and pseudofeces (FECAL) peaked at 20 h (max. 2.81%, range of 2.03–2.81%) and 60h (max. 1.26%, 0.77–1.26%), respectively. The atom% of pseudofeces was 1.26~2.81% throughout the 72 h of the experiment, which was higher than the background value of 1.01%. This indicates that the bivalve fed on ^{13}C-enriched phytoplankton (*Microcystis*), assimilated them into the body, and then discharged them in the form of pseudofeces (Figure 4b). Unlike POM and FECAL samples, *Unio douglasiae* samples showed continuous enrichment of ^{13}C and ^{15}N atom% in mussel tissues (BODY), ranging from 1.083–1.102% and 0.369–0.373% for ^{13}C and ^{15}N, respectively (Figure 4c). Overall, the slope of enrichment for carbon was slightly higher than that for nitrogen throughout the experimental period.

The accumulation of carbon and nitrogen in the muscle and digestive tissues of *Unio douglasiae* indicates that these components were newly synthesized by the organisms through dietary assimilation; however, a major portion of these components was not assimilated and discharged in the form of fecal pellets (Figure 4). Of tissues, the gut accumulated the highest amount of carbon and nitrogen over a similar time (^{13}C atom% of 1.138% at 60h and ^{15}N atom% of 0.379% at 72 h, respectively), but the values were generally low for the remaining tissues. Overall at 72 h, carbon and nitrogen accumulation in tissues was in the order of gut> gill>stomach>mantle>muscle, regardless of the isotope kind (Figure 4, Supplement-2).

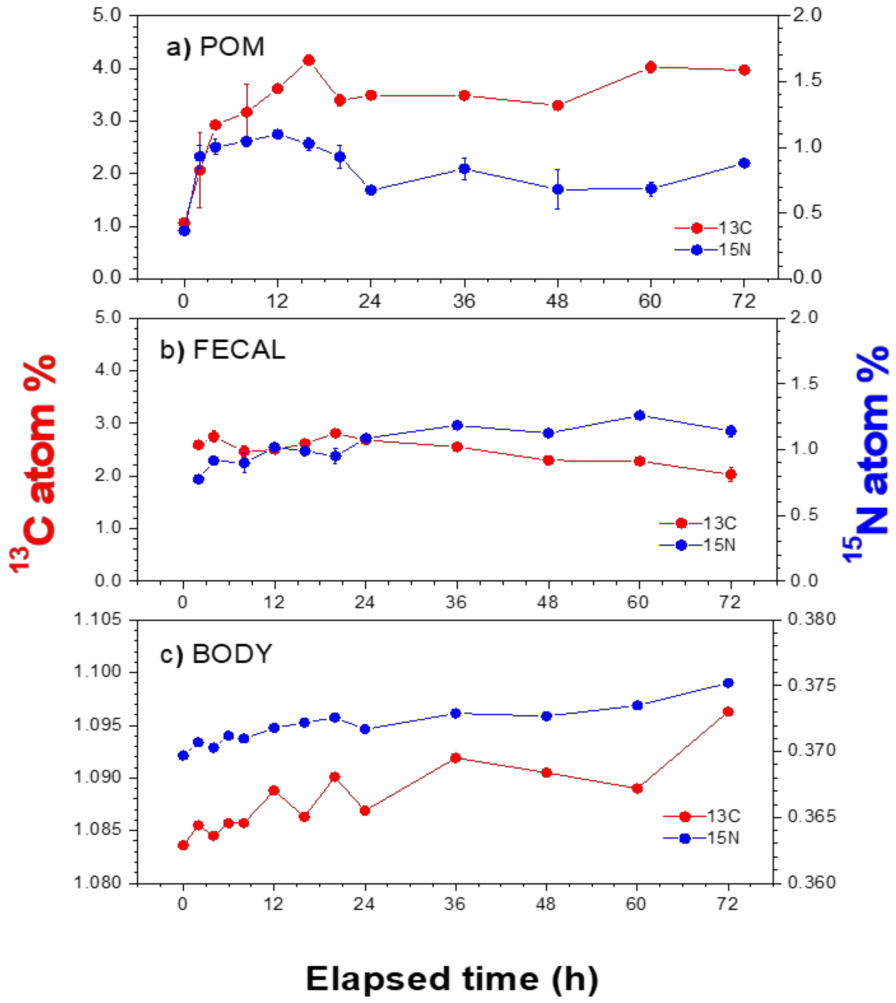

Figure 4. Timely variations of atom% of ^{13}C and ^{15}N in particulate organic matter (POM) (**a**), feces and pseudofeces (FECAL) (**b**), and whole body (BODY) (**c**) samples of *Unio douglasiae* in experimental chambers. The values for BODY are presented as the sum of values for the gills, stomach, gut, mantle, and muscle (standard errors are not shown).

3.3. Assimilation Rates in Mussel Tissues

After 2 h of incubation with ^{13}C- and ^{15}N-enriched *Microcystis* substrates, ^{13}C and ^{15}N enrichment was detected in all major organs of *Unio douglasiae*, with assimilation rates (ARs) of 0.3–39.8 μgC mg tissue^{-1} h^{-1} for carbon and 0.08–5.9 μgN mg tissue^{-1} h^{-1} for nitrogen, respectively (Figure 5). As described in Section 2.4, the unit means the amount of newly generated organic carbon and nitrogen matter at one mg tissue per hour.

During the initial stages of the experiment, *Unio douglasiae* exhibited high assimilation efficiency for cyanobacterial cells through feeding activity. The assimilation pattern of carbon was similar to that of nitrogen, peaking after 2 h and then decreasing throughout the remaining experimental period. However, the declining pattern of nitrogen was less profound than that of carbon. Moreover, *Unio douglasiae* showed different assimilation rates across tissues, with higher values in the gut and stomach but lower values in the muscle, mantle, and gills (Figure 6).

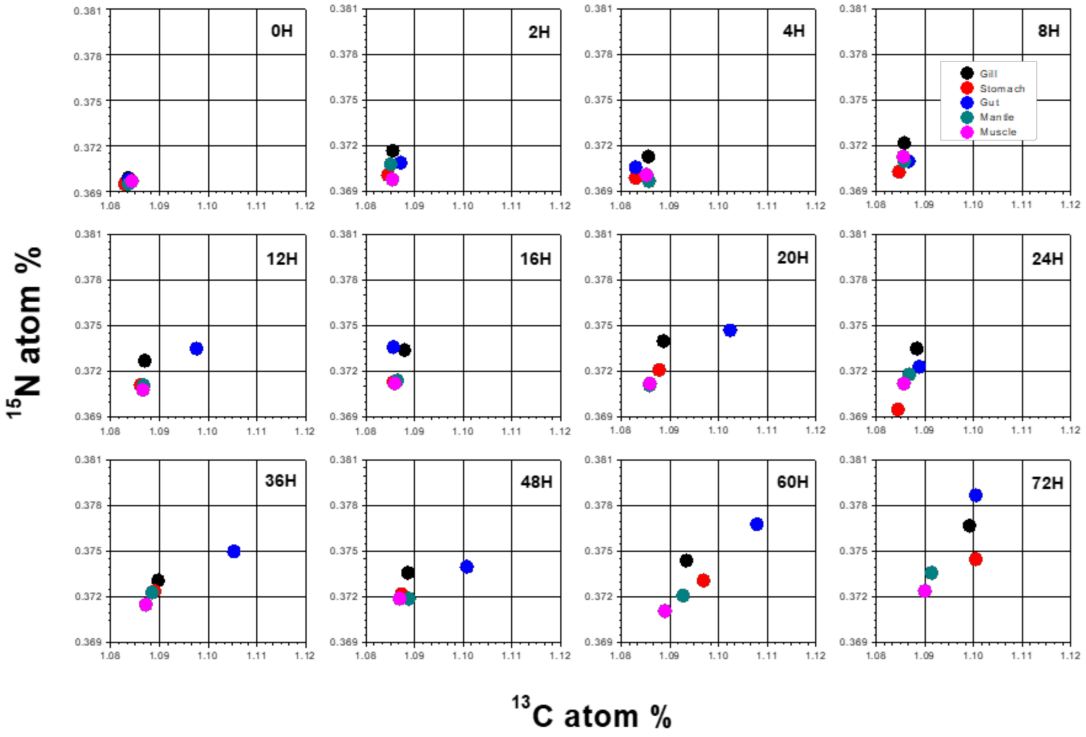

Figure 5. Timely variation of atom% of ^{13}C and ^{15}N in various tissues of *Unio douglasiae* in experimental chambers over 72 h. Each value is the average of three subsamples (Supplement-2).

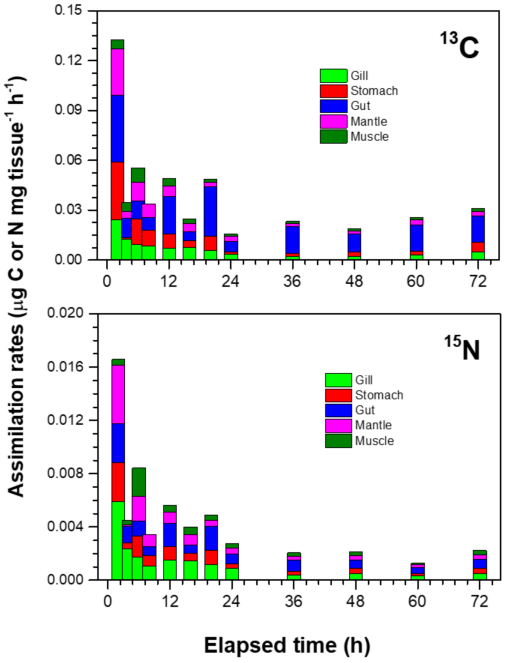

Figure 6. Assimilation rate of carbon and nitrogen in various tissues after introduction of *Unio douglasiae* in *Microcystis*-predominating waters for 72 h. Each value is the average of three subsamples (standard errors are not shown).

3.4. Changes in Water Quality

Following the introduction of *Unio douglasiae* to the experimental chambers containing M-water, ammonia (NH_4) levels increased remarkably but nitrite (NO_2), nitrate (NO_3), total nitrogen (TN), dissolved phosphate (PO_4), and total phosphate (TP) levels did not

(Figure 7). Regardless of the mussel stocking, NH_4 concentration in M-water gradually decreased until 6 h. However, after 6 h, NH_4 concentration in M-water in the CONTROL chamber continuously decreased, while that in the TREAT chamber quickly increased until the end of the experiment. At 60 h, NH_4 concentration in the TREAT chamber peaked to 197.2 μg L^{-1}, whereas that in the CONTROL chamber dropped to 3.5 μg L^{-1}. Throughout the experiment, NH_4 concentration in the TREAT chamber was 1.1–86.3 times higher than that in the CONTROL chamber after 6 h of mussel stocking (Figure 7; Supplement-2).

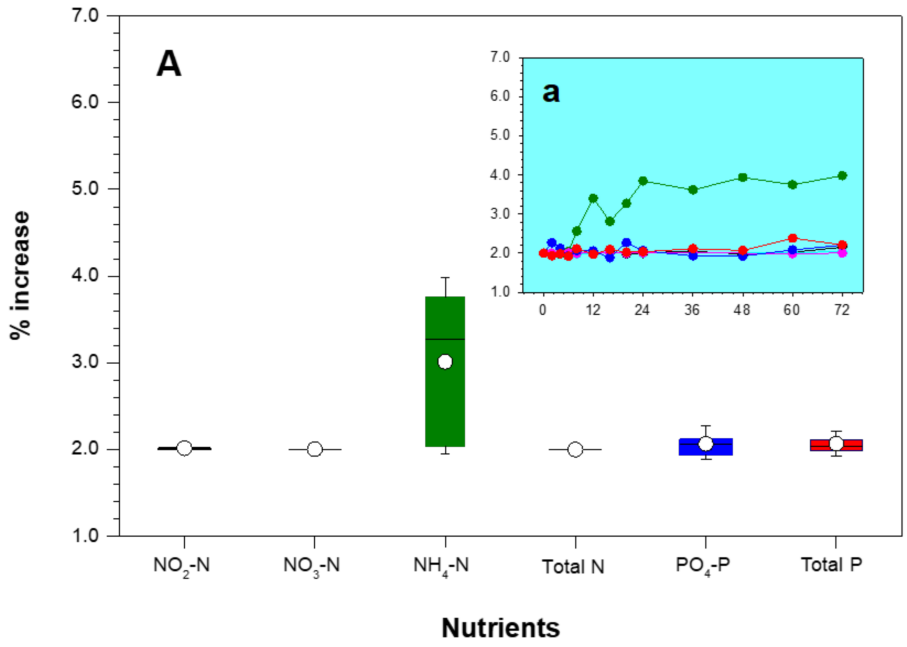

Figure 7. Relative increase of nutrient levels (**A**) and their variations in the experimental chambers (**a**) after introduction of *Unio douglasiae* to *Microcystis*-predominating waters for 72 h (Supplement-3).

4. Discussion

Filter feeders such as unionid bivalves filter large amounts of phytoplankton, including the harmful cyanobacterium *Microcystis aeruginosa* [27–34], thereby improving the water clarity of eutrophic lakes and rivers [35–37] and seawater [38]. In experimental chambers containing water with a high density of *Microcystis aeruginosa* (M-water), the freshwater bivalve *Unio douglasiae* exhibited strong filtering activity on the suspended particles over a short period (~12 h). However, after peaking, this filtration activity of the mussels sharply declined and was constrained toward the end of the experiment, perhaps due to repetitive filtration and resuspension (RFR) in limited experimental space with no current (Figure 2). These high filtering and low assimilation rates of *Unio douglasiae* in M-water may be explained by the high fecal production and nutrient (DIN and DIP) release, which can revive the cyanobacterial blooms. Meanwhile, in experiments using ^{13}C- and ^{15}N-labeled *Microcystis aeruginosa*, mussels did not actively consume cyanobacteria as a food source. The CHL levels were controlled by the photosynthesis and RFR of mussels, while there was some evidence that cyanobacterial blooms could be regenerated from the feces of aquatic animals, including unionid bivalves and planktivorous fish [39,40]. We suspect that pseudofeces or feces of the unionid bivalve *Unio douglasiae* might play a role to revive *Microcystis*.

In this study, we performed isotope tracer analysis with ^{13}C and ^{15}N to assess the energy flow from *Microcystis aeruginosa* to *Unio douglasiae* though feeding activity. The assimilation rate of carbon of mussels was remarkably higher than that of nitrogen within the first 2 h, indicating a high removal rate of sestons in M-water (Figure 5). Thereafter, however, the assimilation rate of each organ of *Unio douglasiae* decreased until the end

of the experiment. Despite a high atom% value of *Microcystis aeruginosa* cells, relatively few changes were noted in the whole body of the bivalve over time (Figure 3). These findings indicate that very few *Microcystis* cells were incorporated into the mussel body via filter-feeding. The atom% of carbon and nitrogen and assimilation rate of mussels remained relatively low across all tissues but slowly increased toward the end of the experiment. The lowest accumulation was detected in the gills and mantle and the highest in the digestive organs such as gut and stomach (Figures 4 and 5). This may be because the digestive organs experience higher turnover rates than other organs such as mantle and muscle, therefore reflecting short-term assimilation [41,42]. Our finding is consistent with previous studies, in which the muscle, gills, and mantle presented low atom% values and assimilation rates of both carbon and nitrogen due to relatively low turnover and long-term integration of energy sources [42–44].

The present study demonstrated that although the total content of SPM and CHL was remarkably decreased, the relative atom% of the FECAL samples was higher than that of the BODY samples (Figure 2). Results suggest that a very small portion of the given biomass of *Microcystis aeruginosa* was incorporated into mussel organs, and highly toxic *Microcystis* was rapidly expelled in the form of pseudofeces prior to digestion. Notably, M-water used in the present study was toxic (i.e., microcystin-LR), as it had been used many times in previous studies [8,18]. The higher production of pseudofeces in the presence of toxic *Microcystis* explains the higher atom% of pseudofecal samples and the lower atom% of *Unio douglasiae* organs. McIvor (2004) [45] found that three unionid species, namely *Anodonta anatina, Unio pictorum*, and *Unio tumidus*, removed 45% of the suspended solids and produced feces and pseudofeces. No mussel died during the study period. Our previous studies reported that no *Unio douglasiae* died in experimental chambers during daily administration of toxic cyanobacterial water for 3 months [8]. Thus, we present that *Unio douglasiae* did not prefer *Microcystis*-rich water as a nutrient or an energy source, due to its low nutritional value, and strong toxicity of *Microcystis aeruginosa*.

Previous studies have demonstrated that filter feeders, such as fish and mussels (i.e., *Corbicula fluminea*), are likely to disorganize particulate organic nitrogen and phosphorus and improve the biodegradability of POM through filter-feeding, digestion, and excretion [46–48]. In particular, excretion of a large amount of NH_4 is expected following the introduction of unionid mussels to M-waters with cyanobacterial blooms [8,40,49–51]. The lethal NH_4 concentration (LC50) for juvenile unionids (*Lampsilis cardium*) is 93–140 μg L^{-1} for 10 days [52] or 157–372 μg L^{-1} for 96 h [53]. Similarly, we observed remarkable increase in NH_4 concentration after mussel stocking (Figure 7). At 60 h, NH_4 concentration peaked to reach 198 μg L^{-1}, being 25 times higher than the control value; nonetheless, no mussel exposed to this M-water died over the experimental period (72 h). In SPM, BODY, and FECAL samples, the levels of newly biosynthesized carbon and nitrogen primarily increased during the initial 24 h of the experiment, with simultaneous increase in isotope accumulation into tissues. After 24 h, the uptake and accumulation of both ^{13}C and ^{14}N isotopes declined rapidly and did not increase until the end of the experiment. Thus, in mussels, nutrient uptake and accumulation may be accompanied by NH_4 excretion. Additionally, our findings can help elucidate the mechanisms through which unionid mussels detect or reject *Microcystis aeruginosa*, and this information may be beneficial to overcome the hazards of ammonia and microcystin toxicity of water in small closed systems. Overall, indigenous mussel populations in Korea can be conserved and sustained even in streams and lakes with cyanobacterial blooms.

There have been concerns about negative changes in water quality and biodiversity in aquatic ecosystems due to the outbreak of harmful cyanobacteria in rivers and estuaries in Korea [8]. The bivalve *Unio douglasiae* has been used for food [5], and is surviving excellently even in situations where there are large outbreaks of cyanobacteria bloom. In this study, mussels showed to have potential of an effective controller exerting a rapid feeding on toxic *Microcystis aeruginosa*. On the other hand, pseudofeces produced after mussel feeding sink to the bottom of the water body and may provide food source for other benthic organisms. However, a toxic cyanobacterium would not be used as food in

practice; rather, adverse effects on other organisms are expected due to the release of high concentrations of toxins and ammonia. Additionally, an accumulation of toxins through the food chain is also expected to cause direct or indirect damage to animals and humans.

5. Conclusions

The present study demonstrates that the unionid bivalve *Unio douglasiae* actively feeds on toxic *Microcystis aeruginosa* with a high clearance rate, but this does not correspond with its direct diet use. These bivalves exhibit a low carbon and nitrogen assimilation capacity for toxic cyanobacterial cells in their diet, resulting in the rapid transformation and release of toxins through fecal matter, or vice versa. Simultaneously, the mussels excrete nutrients (i.e., high ammonia concentration), which can promote the growth of cyanobacteria. From the viewpoint of conserving indigenous bivalve species, even repetitive interactions with toxic cyanobacterial blooms do not seem to kill unionid mussels, presumably due to their detoxifying capacity and dilution of toxins in ambient waters. To the best of our knowledge, the present study is the first to use stable isotope tracers to evaluate the assimilation efficiency of endemic unionid bivalve on the toxic cyanobacteria.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11199294/s1, Supplement-1: Timely concentrations of suspended particulate matter (SPM), particulate organic matter (POM), and chlorophyll-a (CHL) after the introduction of the freshwater bivalve *Unio douglasiae* Griffith et Pidgeon to *Microcystis*-rich waters for 72 h, Supplement-2: Timely concentrations of stable isotope carbon and nitrogen in various tissues after the introduction of the freshwater bivalve *Unio douglasiae* Griffith et Pidgeon to *Microcystis*-rich waters for 72 h, Supplement-3: Relative variations of nutrients NO_2 (μg/L), NO_3 (μg/L), NH_4 (μg/L), TN (mg/L), PO_4 (μg/L), and TP (μg/L) after the introduction of the freshwater bivalve *Unio douglasiae* Griffith et Pidgeon to *Microcystis*-rich waters for 72 h.

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