

Article

Nitrogen Budget Illustrates the Efficiency of Co-Farming Sea Lettuce (*Ulva fenestrata*) with Cold-Water Fish in a Marine Recirculating Aquaculture System

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Abstract

Integrated multitrophic level aquaculture (IMTA) in land-based recirculating aquaculture systems (RAS) has been proposed as an effective nitrogen bioremediation strategy. It can improve the utilization of added nitrogen through further biomass production, which increases the sustainability and efficiency of the farming system. Few studies have evaluated how co-farming in cold-water land-based marine recirculating aquaculture systems affects the nitrogen utilization; here, we quantify the effects of co-farming sea lettuce (*Ulva fenestrata*) with Atlantic wolffish (*Anarhichas lupus*) and rainbow trout (*Oncorhynchus mykiss*) in such a system. Nitrogen budgets were used to evaluate the efficiency of the system by summarizing all processes, both inputs and outputs. In alternating four-week periods for four months, the recirculating system held either only fish or fish together with sea lettuce. Fish biomass held 90–91% of the system nitrogen, while 9–10% was available as dissolved inorganic nitrogen. The nitrogen uptake by the sea lettuce biomass was quantified to 1.0–1.5% of the total nitrogen input. Extrapolations based on these results show that a wet weight ratio of sea lettuce:fish of 0.7 would be optimal for remediation of all added nitrogen.

Keywords: nitrogen remediation; recirculating aquaculture systems (RASs); IMTA; co-farming; sea lettuce; nitrogen budget



Academic Editor: Xiangli Tian

Received: 1 June 2026

Revised: 9 July 2026

Accepted: 24 July 2026

Published: 30 July 2026

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1. Introduction

The aquaculture sector has been growing rapidly over the past 30 years and is predicted to further increase. However, there are environmental challenges that need to be met to ensure a sustainable expansion, for example to reduce the risk of eutrophication [1,2]. Recirculating aquaculture systems (RASs) on land can provide technical solutions to improve the environmental sustainability of finfish farming. Such developments include refined waste management, improved system performance, and efficient treatment of effluent

water [3,4]. RASs circulate water in a loop between tanks holding the fish and a series of water treatment steps. This allows regulation of water quality parameters in the system to ensure good fish welfare, while also creating a barrier between the internal farming setting and the surrounding environmental ecosystem [5]. It enables wastewater treatment, which typically includes waste capture, conversion, and reduction [6,7]. The treatment steps usually start with particle removal by mechanical filtration, followed by nitrifying biofilters, disinfection, degassing and oxygenation, with certain systems also including denitrification [5]. The water treatment aims to remove dissolved and particulate organic matter (DOM and POM), total suspended solids (TSS), and dissolved nutrients, particularly the nitrogenous compounds that can negatively affect fish health and welfare [3].

Improving methods for nitrogenous waste management is an important focus area in RAS research and development. Nitrogen is usually added to a farming system through the feed and is released as ammonia (NH_3) after digestion, which equilibrates with ammonium (NH_4^+), and both forms are toxic to fish [8,9]. The uptake and conversion of NH_4^+ is therefore one of the primary water treatment objectives in RASs and makes the fate of nitrogen important both for the efficiency and sustainability of a farming system, as well as for animal health and welfare [7]. In aerobic biofilters, nitrifying bacteria oxidize NH_4^+ into nitrite (NO_2^-) and further into the less toxic form nitrate (NO_3^-), which has to be removed through repeated water changes, or by denitrifying bacteria in anoxic biofilters [9]. An alternative strategy is to assimilate the nitrogen into new biomass by adding extractive species to the RAS loop through so-called integrated multitrophic aquaculture (IMTA; [10,11]). When harvesting the new biomass, dissolved nutrients are removed from the water, and the resulting nitrogen output will be as additional biomass [12]. The nutrient cycling is thereby improved, and less dissolved nitrogen remains in the effluent water. The concept of IMTA has successfully been tested in several cold-water open sea settings with various extractive species, most often including blue mussels and macroalgae [13–15]. Macroalgae alone have also proven to provide bioremediation benefits in various aquaculture systems [16,17]. Positive bioremediation effects have been shown for the red macroalgae *Gracilaria* spp. and green macroalgae *Ulva* sp. in co-farming systems with both fish and shrimps held either in RASs, flow-through systems or in isolated laboratory tanks holding effluent waters, at temperatures ranging between 10 and 29 °C [18–21]. For example, IMTA in a flow-through system with red macroalgae pelillo (*Gracilaria chilensis*), coupled in series to Cabinza grunt (*Isacia conteptionis*), Pacific oysters (*Crassostrea gigas*) and sea urchins (*Loxechinus albus*), held at 14–20 °C, were found to remove almost all dissolved nitrogen excreted by the animals from the water [19]. IMTA studies in land-based RASs exist but have mainly been performed on warm-water species. For example, promising bioremediation effects have been found for IMTA in land-based outdoor RASs with *Ulva Lactuca* and *Gracilaria conferta* held with gilthead seabream (*Sparus aurata*) and abalone (*Haliotis discus hannai* or *Haliotis tuberculata*), at cultivation temperatures between 16 and 31 °C [21,22]. Species of the *Ulva* genus thereby have a large potential in assimilating dissolved nutrients in land-based systems [23]. Despite this, however, they have not previously been studied in cold-water RASs.

In Sweden, the environmental laws and increased risks for coastal water eutrophication have led to the implementation of the Environmental Impact Assessments aimed at reducing or eliminating nitrogen release in order to maintain a good environmental status required under the EU Marine Strategy Framework Directive [24]. Consequently, the Swedish aquaculture industry, targeting coastal zones, is considering the land-based RAS for future expansion as it provides the possibility to reduce or abolish nutrient release to the environment. This planned expansion is mainly focused on farming salmonids in cold water, so studies are needed on methods that can optimize the systems' bioremediation,

especially those related to economically viable extractive species such as the macroalgae *Ulva*. The nitrogen usage in an aquaculture system can be estimated through nitrogen budgets, where mass balance equations present how nitrogen added as feed moves through the system [25]. The results from a nitrogen budget can inform on critical processes where nitrogen is lost and highlight where efforts should be focused to improve the system efficiency [9].

The aim of this study was to investigate the co-farming potential of sea lettuce with fish in a functional, well-established 18 m³ SW-RAS test facility under cold-water conditions. A primary objective was to establish nitrogen budgets for the test facility, when alternatively holding fish with and without sea lettuce in series in the RAS loop. The measurements and calculations were made to estimate and quantify nitrogen circulation and utilization when co-farming fish with sea lettuce, to reveal the potential benefits of co-farming. The rationale for using an already established, medium-scale test RAS facility was to make the results readily applicable for the industry when designing future facilities.

2. Materials and Methods

2.1. Experimental Setup

The experiment was conducted between 4 February 2022 and 9 June 2022, at the animal holding facility, Department of Biological and Environmental Sciences, at the University of Gothenburg (Gothenburg, Sweden, facility number 5.2.18-18639/17; [26]). The study was performed in a cold-water RAS holding approximately 18 m³ artificial seawater (SW). The test facility held 39 individuals of Atlantic wolffish (*Anarhichas lupus*), with a mean start weight of 630.6 g $\pm$ 32.9, making a total start biomass of 28.7 kg. The wolffish were originally wild-caught as fertilized eggs by the Icelandic Marine and Freshwater Research Institute (Hafnarfjörður, Iceland) and hatched at the Aquaculture Research Station of Grindavík (Grindavík, Iceland). The wolffish were transferred to the University of Gothenburg in January 2017 and have been a part of the RAS facility since [27]. The wolffish were held in two rectangular tanks with dimensions of 102 $\times$ 103 $\times$ 70 cm (W $\times$ L $\times$ H), each holding 735 L of water, making a total stocking density of wolffish of 19.52 kg/m³ in the tanks. At the experimental start, the system was stocked with additional fish biomass by introducing 360 recently SW-acclimated rainbow trout (*Oncorhynchus mykiss*; 112.8 g $\pm$ 1.4; mean $\pm$ SEM) from Vänneåns Fiskodling (Knäred, Sweden), corresponding to an additional fish biomass of 41.0 kg. The rainbow trout were introduced to six conical tanks with dimensions of 100 $\times$ 60 cm (D $\times$ H), each holding 430 L of water. The total stocking density of rainbow trout was 15.87 kg/m³. The total fish biomass was 69.7 kg, corresponding to a total system stocking density of 3.87 kg/m³. The rainbow trout were used in the nitrogen budget calculations and were sampled at the end of each experimental period for health and welfare parameters as presented in Andersson et al. [26]. Air stones supplied with pressurized air were placed in all tanks for aeration. Water quality was maintained at an average temperature of 9.5 $\pm$ 0.02 °C, salinity of 32.9 $\pm$ 0.06 ppt, and a dissolved oxygen saturation of 87.7 $\pm$ 0.5%. The photoperiod was kept at 14L:10D throughout the experiment.

The experiment was divided into two phases with each phase having two periods, and each period lasting for four weeks (Figure 1B). Phase one consisted of the first two periods (periods 1 and 2), while phase two consisted of the last two periods (periods 3 and 4). Only fish were held in the SW-RAS during the first period of each phase. During these periods the macroalgae bags were filled with only water (periods 1 and 3). During the second period of each phase (periods 2 and 4), both fish and sea lettuce were held in the SW-RAS. The second phase repeated the first with the same fish maintained in the system during

both phases, whereas new sea lettuce was introduced at the start of period 4. Between the two phases, a larger water change (4.6 m³) was performed over a period of two weeks.

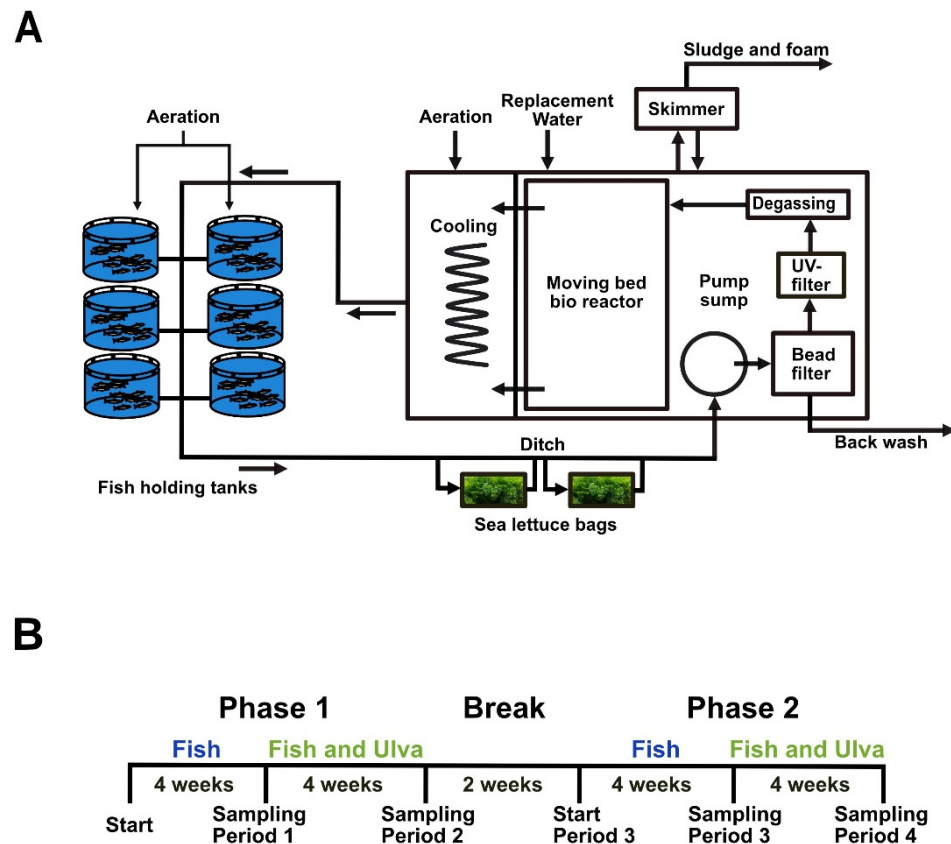


Figure 1. A schematic of the (A) recirculating aquaculture system (RAS) and (B) experimental design. Water was continuously circulated through the sea lettuce bags, which contained sea lettuce only during periods 2 and 4. Modified from Andersson et al. [26].

The clonal, gametophytic algal material used in this study was obtained from a long-term indoor tank cultivation system maintained at the Tjärnö Marine Laboratory, University of Gothenburg, Sweden (TML; 58°52′36.4″ N, 11°6′42.84″ E). Comprehensive details regarding cultivation parameters and molecular identification of the parental *U. fenestrata* biomass are provided in Toth et al. [28]. Two 2 m tall, cylindric plastic bags containing 200 L were positioned in the beginning of the RAS loop and stocked with sea lettuce during the co-farming periods (periods 2 and 4). No more than two sea lettuce cultivation bags could be used in this study due to spatial limitations. The water inlet was at the top of each bag and had an average flow of 0.6 L/min. This corresponded to a fraction of 1% of the total system turnover supplied to the sea lettuce bags. The outflow was also located at the top of the bag on the opposite side to the inlet. A pressurized air inflow was placed at the bottom of the bags to ensure aeration as well as thorough water mixing. Surrounding LED lights (Lampgrossen, Vellinge, Sweden) generated light at 16L:8D with an average intensity of 50 µmol/m²/s measured inside the bags to ensure optimal conditions for assimilating nitrogen [28]. At the start of period 2, 1200 g of sea lettuce was evenly distributed between the two bags (3.00 g sea lettuce/L). The sea lettuce was completely removed at the end of period 2. At the start of period 4, 900 g of new sea lettuce was evenly distributed between the two bags (2.25 g sea lettuce/L). The relative sea lettuce to fish biomass ratio was 0.017 in period 2 and 0.013 in period 4.

2.2. SW-RAS Loop

The SW-RAS loop was located in two adjacent rooms with one holding the fish tanks and the other holding a 15 m³ pump sump (water basin), the different water treatment filters and the sea lettuce cultivation bags (Figure 1A). The outflow water from the fish tanks was directed to the adjacent room in a concrete “ditch”. A submerged pump in this ditch supplied the sea lettuce bags with water during periods both with and without sea lettuce. Gravity then fed the outflow water into a semi-closed barrel located within the concrete pump sump basin. From the barrel, a submerged pump moved the water into the start of the water treatment loop consisting of a bead filter, followed by a UV-filter, and a degassing unit. No backwash was required for the bead filter during the experiment as the filter pressure remained within optimal levels throughout the study. The water was released from the degassing unit into a moving bed bio reactor consisting of a 1 m³ flow-through bag filled with bio balls (ø3.8 cm propylene bio balls, Aerobic Guard, Waxahachie, TX, USA) positioned inside the pump sump. A stand-alone submerged pump led 5 m³ water/h from the sump into a protein skimmer (Red dragon Bubble king, Royal exclusive, Wesseling, Germany). All skimrate from the skimmer (approximately 0.92 L/d) was collected in a bucket and the volume was measured continuously. Water from the pump sump was finally automatically siphoned into an adjacent basin equipped with a titanium cooling coil and an aeration unit at the bottom with large air stones and pressurized air, aerating the water before returning to the fish tanks via a main pump. The RAS had been running for five years prior to this study, and the Atlantic wolffish were already held there before the rainbow trout were added a few days before the experiment started. When needed, freshly mixed artificial SW was added to the pump sump to compensate for losses due to evaporation, over-flow, and skimrate from the protein skimmer. Water replacements were performed on seven occasions during period 1 (total loss of 6500 L), two occasions during period 2 (1950 L), four occasions during period 3 (2600 L), and on six occasions during period 4 (4550 L). The higher water loss during period 1 was mainly due to initial over-flow problems during the first four days. The losses observed in period 4 were attributed to an increase in turbidity during the first week of this experimental period, likely resulting from feed accumulation after a new bottom enrichment structure was introduced in the wolffish tanks. After extensive tank cleaning and the establishment of repeated cleaning routines, along with the water changes, the water returned to its original quality within seven days.

2.3. Fish Feed

The rainbow trout were handfed 0.75% of the tank biomass every weekday with commercial feed Spirit Trout 4.5 mm from Skretting (Stavanger, Norway). The feed was split between two feeding events, one in the morning at 9 am and one in the afternoon at 3 pm. The feed ration for each tank was adjusted at the beginning of every period (periods 1–4) according to the fish biomass recorded during weighing at the start of each period. The feed was added slowly to the water surface, and fish were left undisturbed for 10 min before tanks were flushed. Flushed water from all rainbow trout tanks led to a common pre-filter where uneaten pellets were captured and counted. The corresponding dry weight was deducted from the amount of added feed to determine the weight of consumed pellets. The wolffish, also housed in the system, were hand fed, slowly to satiation around 9 am on Mondays, Wednesdays, and Fridays with a mixture of Amber Neptun 4 and Amber Neptun 7 from Skretting (Stavanger, Norway). Pellet consumption for wolffish was recorded using the same procedure as described for the rainbow trout. The nitrogen content (% N) of the pellets was provided by the manufacturer and confirmed for the specific feed batches by a certified laboratory performing the Kjeldahl method (Eurofins Food & Agro Testing

Sweden AB, Linköping, Sweden). Spirit Trout contained 6.89% nitrogen while Amber Neptun 7 contained 8.18% nitrogen and Amber Neptun 4 contained 8.90% nitrogen.

2.4. Fish Sampling

All fish were lightly anesthetized using metomidate hydrochloride (6 mg/L, Aquacalm, Syndel Laboratories, Ferndale, WA, USA) at the experimental start. Their weight and length were measured before returning to their tanks. The same procedure was performed at the end of period 1 and 2, at the start of period 3 (after two weeks of water changes) and at the end of periods 3 and 4 for the rainbow trout. The wolffish were weighted at the start and end of the experiment and their in-between weight was extrapolated from that data. At the end of each period (1–4) a sub-set of two rainbow trout from each tank ($n = 12$) were anesthetized using an overdose of metomidate hydrochloride (12 mg/L) and euthanized by a blow to the head. These fish were analyzed for additional physiological welfare parameters to assess the effect of co-farming on fish health and welfare and the results can be found in the accompanying paper Andersson et al. [26].

2.5. Ethical Statement

All experimental procedures and husbandry protocols followed the ARRIVE guidelines [29]. The experiment followed regulations set by the Swedish National Board for Laboratory Animals and was approved by Gothenburg's animal ethics committee (ethical permit number 5.8.18-15096/2018).

2.6. Water Quality

Water samples were collected three times per week (Mondays, Wednesdays, and Fridays) from the inflow to the sea lettuce bags during all four periods ($n = 9$ for period 1, $n = 8$ for periods 2 and 3, $n = 9$ for period 4). Water samples were collected at 8 am, before the first feeding event as NH_4^+ , NO_2^- , and NO_3^- levels in RASs are known to fluctuate throughout the day, especially in response to feeding events [30,31]. The tri-weekly samplings were performed to study the long-term effects of co-farming with sea lettuce on the system nitrogen balance. Dissolved inorganic nitrogen (DIN) compounds were measured using commercially available kits and a DR2800 laboratory spectrophotometer (Hach Lange, Dusseldorf, Germany; [32,33]). The ammonia reagent powder pillow method (salicylate method, 8155) was used to measure $\text{NH}_4^+\text{-N}$, which also converts $\text{NH}_3\text{-N}$ to $\text{NH}_4^+\text{-N}$ prior to measurement. $\text{NO}_2\text{-N}$ were measured using the powder pillow method NitriVer 3 (diazotization method, 8507) and the LCK 339 kits were used to measure $\text{NO}_3^-\text{-N}$. Total DIN in the system was calculated as follows:

$$\text{Total DIN} = ([\text{NH}_4^+\text{-N}] + [\text{NO}_2^-\text{-N}] + [\text{NO}_3^-\text{-N}]) \times V_{\text{sys}} \quad (1)$$

where $[\text{NH}_4^+\text{-N}]$ is the $\text{NH}_4^+\text{-N}$ concentration in the water flowing into the sea lettuce bags, $[\text{NO}_2^-\text{-N}]$ is the $\text{NO}_2^-\text{-N}$ concentration, and $[\text{NO}_3^-\text{-N}]$ is the $\text{NO}_3^-\text{-N}$ concentration. V_{sys} is the total water volume in the RAS. The change in DIN over the period was calculated by subtracting the total DIN at the start of the period from the total DIN at the end of the period.

2.7. Sea Lettuce Sampling

Every Friday during co-farming periods 2 and 4, all sea lettuce were carefully netted from each cultivation bag. The fresh weight (fw) of sea lettuce was measured using a standardized method with a salad spinner to gently remove excessive water before returning it to the cultivation bags for continued growth [34]. Additionally, five individuals from each cultivation bag were randomly sampled from each cultivation bag and subsequently

frozen at $-80\text{ }^{\circ}\text{C}$ at the start and end of each period (periods 2 and 4). After the experimental period, samples were freeze-dried, homogenized, and their nitrogen content was analyzed using a GSL elemental analyzer coupled to an isotope-ratio mass spectrometer (EA-IRMS, 20-22, Sercon Ltd., Crewe, UK). The full results on growth and nitrogen content are presented in Andersson et al. [26]. The total nitrogen content in the sea lettuce bags at the start and end of each period (periods 2 and 4) was calculated as follows:

$$N_{\text{tot}} = \%N \times \text{fw} : \text{dw} \times \text{Biomass}_{\text{tot}} \quad (2)$$

where N_{tot} is the total nitrogen content in the sea lettuce bags, $\%N$ is the average nitrogen content in dried sea lettuce, $\text{fw}:\text{dw}$ is the fresh weight:dry weight ratio of the sea lettuce biomass (following the equation from Supplementary Figure S1) and $\text{Biomass}_{\text{tot}}$ is the total fresh weight in the bag. To estimate the total nitrogen assimilated by the sea lettuce over the four-week period, the total sea lettuce nitrogen content at the start was subtracted from the total nitrogen content at the end.

2.8. Nitrogen Budget

A nitrogen budget was made for the SW-RAS for each period (periods 1–4) with all known nitrogen inputs and outputs as follows:

$$N_{\text{budget}} = N_{\text{feed}} - N_{\Delta\text{DIN}} - N_{\text{water loss}} - N_{\text{skimmer}} - N_{\text{fish biomass}} - N_{\text{sea lettuce}} \quad (3)$$

where N_{feed} is the nitrogen input to the system from consumed feed. $N_{\Delta\text{DIN}}$ is the change in DIN (see Equation (1)) over each four-week period (periods 1–4). $N_{\text{water loss}}$ is the nitrogen lost from the system as water losses, for example due to over-flowing. This estimation was calculated by using the DIN concentrations on the day of the water loss and the replacement volume needed to refill the system to its previous level. N_{skimmer} is the nitrogen removed by the protein skimmer. This was calculated based on the total volume of skimmed collected by the protein skimmer for each period (continuously measured) and the average nitrogen content in the skimmed. The nitrogen content in the skimmed was analyzed in five samples collected at random timepoints throughout the study and their average value was used in the calculation (Kjeldahl method; Eurofins Food & Agro Testing Sweden AB, Linköping, Sweden). $N_{\text{fish biomass}}$ is the nitrogen amount assimilated by fish over time according to Yogev et al. [35] as follows:

$$N_{\text{fish biomass}} = Y_{\text{N-fish}} \times N_{\text{feed}} \quad (4)$$

where $Y_{\text{N-fish}}$ is the fraction of nitrogen assimilated by fish and subsequently converted into their biomass, and N_{feed} is the nitrogen input to the system via feed. The rationale behind this model for estimating the nitrogen uptake by fish, rather than directly from the biomass increase, was due to unbalanced mortalities between different periods [26]. The nitrogen retention rate from feed in rainbow trout was set to 41%, according to a recent study by Wang et al. [36] for the closely related species Atlantic salmon (*Salmo salar*). To the authors' knowledge, no nitrogen retention study has been published on Atlantic wolffish or close relatives. However, a feed study on another marine species, the Red Sea Bream (*Pagrus major*), observed a nitrogen retention rate of 33% for standard feed [37]. This retention rate was used for the Atlantic wolffish calculations in the present study. $N_{\text{sea lettuce}}$ is the total amount of assimilated nitrogen by the sea lettuce over the four-week periods in periods 2 and 4 (see Equation (2)).

To calculate the percentage of nitrogen that the budget could account for, the sum of all nitrogen outputs was divided by the nitrogen input. To estimate the sea lettuce uptake efficiency, their nitrogen uptake was divided by the nitrogen input for periods 2 and 4, respectively.

2.9. Statistics

Data analysis was performed in Microsoft Excel version 2606 (Microsoft corporation, Redmond, WA, USA) with visualizations in GraphPad Prism version 10.0.0 (GraphPad Software, Boston, MA, USA) and Affinity Designer 2 version 2.6.0 (Serif Europe Ltd., Nottingham, UK). Statistical analyses were made in SPSS version 30.0.0.0 (IMB SPSS Statistics for Windows, Version 24.0, IMB Corp, Armonk, NY, USA). Linear regression analyses were performed to assess how sea lettuce influenced the system concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$. The analyses were made separately for the two phases with “fish” or “fish and sea lettuce” as independent variables and $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, or $\text{NO}_3^-\text{-N}$ concentrations as the dependent variables, respectively. Alpha was set at 0.05 for all statistical tests. Data is presented as mean $\pm$ standard error of mean (SEM).

3. Results

3.1. Water Quality

The DIN concentrations in the whole system remained stable over time (Figure 2A). There was an increase in $\text{NO}_2^-\text{-N}$ during period 4 compared to period 3 ($R^2 = 0.437$, $F(1,16) = 12.395$, $p = 0.003$; Figure 2C). Co-farming with sea lettuce had no further statistically significant effects on concentrations of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, or $\text{NO}_3^-\text{-N}$ at the system level (Figure 2B–D). The main nitrogen species in the DIN throughout the experiment was $\text{NO}_3^-\text{-N}$ (9.85 ± 0.19 mg/L; Table 1), with lower concentrations of $\text{NO}_2^-\text{-N}$ (0.04 ± 0.00 mg/L) and $\text{NH}_4^+\text{-N}$ (0.08 ± 0.01 mg/L).

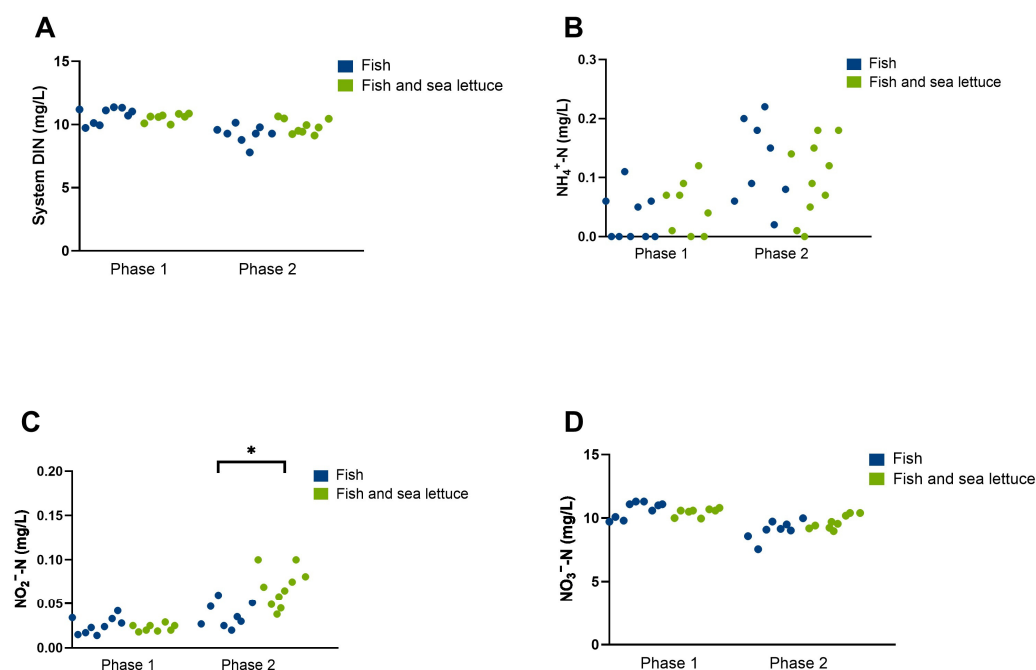


Figure 2. The concentration in the inflow to the sea lettuce cultivation bags of (A) Dissolved inorganic nitrogen (DIN), (B) $\text{NH}_4^+\text{-N}$, (C) $\text{NO}_2^-\text{-N}$, and (D) $\text{NO}_3^-\text{-N}$. Blue colors indicate fish-only periods, whereas green colors indicate co-farming periods with both fish and sea lettuce. Significant differences ($p < 0.05$) between co-farming periods within the phases are annotated with stars.

Table 1. The average ($\pm$ SEM) concentrations of DIN, NH_4^+ -N, NO_2^- -N, and NO_3^- -N in the inflow to the sea lettuce cultivation bags during the four experimental periods (1–4). Significant differences ($p < 0.05$) are annotated with different letters, here only between periods 3 and 4 for NO_2^- -N.

	DIN (mg/L)	NH_4^+ -N (mg/L)	NO_2^- -N (mg/L)	NO_3^- -N (mg/L)
Period 1	10.73 $\pm$ 0.20	0.03 $\pm$ 0.01	0.03 $\pm$ 0.00	10.67 $\pm$ 0.20
Period 2	10.54 $\pm$ 0.11	0.05 $\pm$ 0.01	0.02 $\pm$ 0.00	10.47 $\pm$ 0.10
Period 3	9.24 $\pm$ 0.24	0.13 $\pm$ 0.02	0.04 $\pm$ 0.00 ^a	9.08 $\pm$ 0.25
Period 4	9.41 $\pm$ 0.44	0.10 $\pm$ 0.02	0.07 $\pm$ 0.01 ^b	9.24 $\pm$ 0.44

3.2. Sea Lettuce Sampling

The sea lettuce biomass increased by 43% and 40% during the four weeks of periods 2 and 4, respectively (1200 to 1720 g for period 2 and 900 to 1261 g for period 4; [26]). The highest stocking density throughout the experiment was therefore 4.3 g/L for period 2 and 3.15 g/L for period 4. The crude nitrogen content of sea lettuce increased with 29% dry weight (dw) during period 2 and with 35% dw during period 4. The total nitrogen content held by the sea lettuce increased from 6.3 to 11.7 g during period 2 and from 4.6 to 8.6 g for period 4.

3.3. Nitrogen Budget

Fish biomass accounted for the main part, 90–91%, of the system nitrogen, while DIN contributed with 9–10% (Figure 3). The sea lettuce held a smaller part of the system nitrogen, 0.5%, at the end of periods 2 and 4.

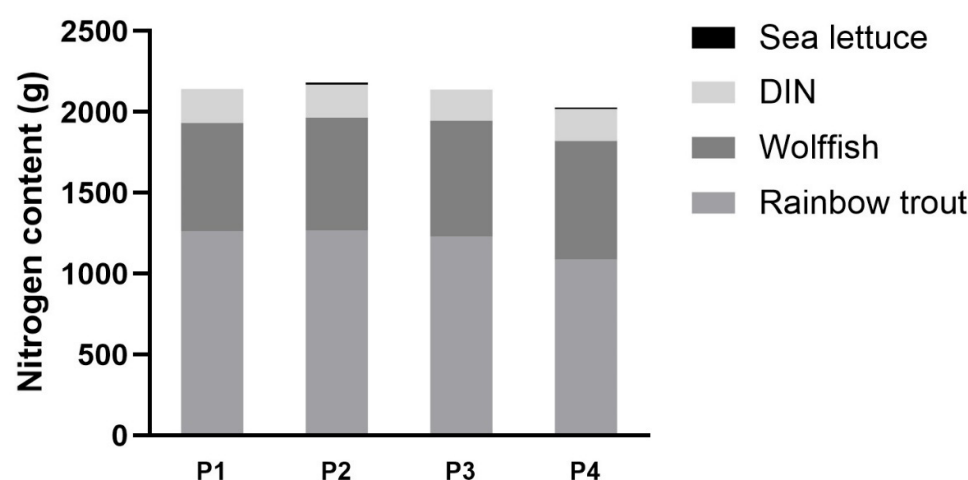


Figure 3. The distribution of total nitrogen in the RAS at the end of each experimental period.

A nitrogen budget was made for each of the four periods (Table 2). The nitrogen input through feed varied with total fish count and biomass in the system, as did the nitrogen output in the form of fish biomass. The sea lettuce nitrogen uptake reflected both the relative increase in nitrogen content of the seaweed and the increase in total biomass. DIN changes ($N_{\Delta\text{DIN}}$) showed changes between the start and end of each period and increased for all periods 1–4. The increase was smaller during the co-farming periods compared to the fish-only periods. Water loss reflects the nitrogen leaving the system due to over-flow events. The loss was highest during period 1 due to initial over-flowing problems during the first four days of the experiment and during period 4 due to high turbidity for one week. The skimmer had a stable and relatively low nitrogen output throughout the experiment. The overall nitrogen budget could account for between 49.7 and 72.1% of the nitrogen input

depending on period. Sea lettuce uptake during periods 2 and 4 amounted to 1.5 and 1.0%, respectively, of the nitrogen input.

Table 2. The nitrogen budget in the RAS with and without sea lettuce for periods 1–4. The nitrogen that is not accounted for is assumed to have left the system through denitrification or nitrogen trapping.

	Period 1	Period 2	Period 3	Period 4
Nitrogen input (g)				
Feed	278.9	370.1	446.9	401.7
Nitrogen output (g)				
Fish	106.6	142.9	173.4	156.3
Sea lettuce	-	5.4	-	4.1
DIN change	27.5	14.5	25.5	23.4
Water loss	65.5	20.0	24.0	46.7
Protein skimmer	1.4	1.4	1.4	1.4
Nitrogen budget (%)				
Nitrogen accounted for	72.1	49.7	50.2	57.7
Denitrification/N-trapping (assumed values)	27.9	50.3	49.8	42.3
Sea lettuce uptake efficiency		1.5		1.0

4. Discussion

In this study, we calculated nitrogen budgets, an important tool to assess the efficiency of aquaculture systems, with the aim to quantify and compare the efficiency of co-farming of fish and sea lettuce in a SW-RAS. We used the same 18 m³ SW-RAS holding either only fish or fish together with sea lettuce. The nitrogen budget of a farming system shows its production efficiency by following the fate of nitrogen from feed into fish biomass and various biodegradation pathways [25]. A mass balance equation, as made here, is an established method to evaluate the nitrogen dynamics in a system and the results can be used for optimization purposes, including the design and dimensioning of species in a co-farming system, effluent treatment processes and overall system performance [9]. The nitrogen budgets in the present study showed and quantified how co-farming with sea lettuce improved the nitrogen usage efficiency by converting 1.0–1.5% of the initially added nutrients into valuable and edible biomass. The calculations on how much sea lettuce biomass would be needed to remediate the nitrogen build up in the system can be of importance when members of the Swedish aquaculture industry design their future farming systems.

The 1.2 kg sea lettuce in period 2 and 0.9 kg in period 4 assimilated 1.5% and 1.0%, respectively, of the total nitrogen addition to the system, thus demonstrating the potential of sea lettuce as a nitrogen remediator. The percentage uptake corresponds to a nitrogen uptake of 0.16 g per day and kg stocking biomass, reflecting an accumulation of nitrogen from the RAS water into sea lettuce biomass. No effects were seen, however, on the overall DIN levels, as concentrations of NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N all remained stable throughout the experiment. Most of the DIN was in the form of NO₃⁻-N with considerably lower concentrations of NH₄⁺-N and NO₂⁻-N. Utilizing bacteria to convert toxic NH₄⁺ from the fish farming tanks into NO₂⁻ and further to NO₃⁻ is an important step in the RAS water treatment loop [7,9]. These bacteria cultures can take a few weeks to months to establish in marine biofilters before reaching sufficient rates to fully oxidize all

NH_4^+ [38]. Once established, the bacteria cultures will maintain NH_4^+ and NO_2^- at a low levels and the main nitrogen species, as observed here, will be NO_3^- [39]. This indicates that the current medium-scale test RAS had a well-functioning and mature biofilter system. The slight increase in NO_2^- -N during period 3 compared to period 4 was likely due to a temporary imbalance between the activities of the ammonia oxidizing bacteria and the nitrite oxidizing bacteria, which could have led to the small NO_2^- -N accumulation. The increase was low (0.03 mg/L) and within the frame of natural fluctuations that occur in bacterial communities in RASs over time [40]. The overall lack of direct effects of co-farming with sea lettuce on DIN is likely due to the relatively low sea lettuce to fish biomass ratio. Phosphate levels (PO_4^{3-}) were measured throughout the study but were not reported due to very high variation in the results, but the lowest observed level was 15 mg/L. This concentration is considerably higher than what is considered limiting for *U. fenestrata*, thus indicating that phosphate was not a limiting nutrient in this experiment [28]. It would be interesting to compare our relative fish: sea lettuce densities with previous IMTA studies on macroalgae, but unfortunately the system setups tend to vary too much for making comparisons possible. For example, Neori et al. [22] studied the bioremediation capacity of sea lettuce in ponds, and the stocking density was therefore reported in kg/m². Other studies have reported the stocking density as individuals per tank or studied their removal capacities in still effluent waters rather than adding them directly to a farming system [20,41]. Comparisons to other relative fish: macroalgae stocking densities were therefore not possible, but the results suggest that a higher relative sea lettuce biomass could have reduced the DIN concentrations.

The nitrogen uptake by sea lettuce was 1.5% during period 2 and 1.0% during period 4, with an initial sea lettuce:fish biomass ratio (g wet weight) of 0.017 and 0.013, respectively. Extrapolations of the empiric data from this study were performed to estimate the optimal biomass of sea lettuce versus fish in the current system, as spatial restrictions prevented us from implementing more sea lettuce in the trials. If the sea lettuce would absorb all nitrogen that remained non-utilized by the fish after feeding, given the present conditions (69.7 kg fish with rainbow trout feeding at 0.75% daily), it would require a stocking density of approximately 51–54 kg sea lettuce. This corresponds to a sea lettuce:fish biomass ratio of 0.7, i.e., about 40–50 times higher than the present system. An important consideration is that these calculations are based on the specific fish biomass and environmental parameters presented here and could be different for other systems. Since these calculations required a large degree of extrapolation, it would be useful to validate them through further experimental trials with this suggested biomass ratio. Factors such as feeding regime, temperature, dissolved oxygen, and fish size can all affect the subsequent nutrient release of the fish, and thereby the nitrogen available for the sea lettuce [36,42,43]. Calculations were performed to illustrate the effect of increased feed from 0.75% to 1.4% on rainbow trout. Such an increase would require 81 kg sea lettuce to assimilate all nitrogen from the feed waste and excretions (calculated from period 2 data).

By using nitrogen budgets, the bioremediation potential and nitrogen assimilation efficiency of sea lettuce was evaluated beyond using only NH_4^+ , NO_2^- , and NO_3^- levels. The present budgets could overall account for 50–72% of the total nitrogen input depending on the period, which is similar to nitrogen budgets made for other RASs [9,44,45]. For an IMTA RAS with milkfish (*Chanos chanos*), sea cucumber (*Holothuria scabra*), and sea purslane (*Sesuvium portulacastrum*), for example, the nitrogen budget described approximately 69% of the nitrogen input [44]. In a flow-through IMTA system with abalone (*Haliotis tuberculata*) and two macroalgae (*Ulva lactuca* and *Gracilaria conferta*), 24 h nitrogen budgets were set up on five occasions over one year and the authors found a large variability in nitrogen accounted for, with values between 31 and 110% [21]. There are also nitrogen budgets for

systems with only fish. A nitrogen budget for an enclosed farm on lake Tai Hu (China) with silver carp (*Hypophthalmichthys molitrix*) and Nile tilapia (*Oreochromis niloticus*) could account for 43–57% of the nitrogen input [45], whereas a nitrogen budget for a RAS farm with tilapia could account for 86% of the total nitrogen input [9]. Nitrogen budgets for grouper (*Epinephelus areolatus*) held either in laboratory flow-through systems, or in open sea cages, could account for 66% and 48% of nitrogen input, respectively [46]. The nitrogen budget of the current study falls within these previously reported ranges and accounted for 50–72% of the nitrogen input. The nitrogen that was unaccounted for, 28–50%, was likely due to a combination of several nitrogen sinks that were not measurable in the current study. These sinks could include nitrogen loss by solid waste captured in the bead filter, sedimentation of nitrogen containing particles in different compartments of the RAS loop, and unmeasurable denitrification by bacteria residing in low-oxygen or anoxic parts of the system.

Nitrogen in suspended particles retained in a bead filter are only released during backwashing. Since no backwashing occurred during the current experiment, the nitrogen remained in the filter. The amount of nitrogenous waste captured by the bead filter is hard to estimate, as it depends on the solid load and the solids' nitrogen content [11]. Suspended solids sediment and remain in pipe bends, the pump sump and other areas of the system with low water flow rates, which are difficult to quantify in a running RAS. Another probable source of unaccounted nitrogen is denitrification, where NO_3^- is converted to dinitrogen gas (N_2) and released to the air [9]. Denitrification often occurs throughout RASs, particularly in mature systems, within anaerobic pockets, sludge compartments, and in the deeper layers of the bio films on the bio balls, even in systems lacking a dedicated reactor/loop [47–49]. Reported nitrogen removal efficiencies include 9–21% of the total nitrogen input in simple RASs, and 27% in more complex RASs with dedicated denitrification treatment loops [25,35]. In addition to heterotrophic denitrification, the anaerobic ammonium oxidation (anammox) process may contribute to nitrogen removal by converting NH_4^+ and NO_2^- directly into N_2 gas under anaerobic conditions. While the anammox process is a promising alternative for engineered nitrogen removal in RASs [50], evidence suggests that this pathway can also occur spontaneously in local anoxic zones within the system [51,52]. Collectively, denitrification, anammox and particulate nitrogen retention in the bead filter likely account for a considerable part of the unaccounted nitrogen (28–50%) in this study's budgets. Previous studies have found that adding macroalgae to biofilters can shift microbial communities that influence the ammonia and nitrite oxidation processes [53]. Bacterial communities were not directly analyzed in the present study but would provide an interesting future research direction.

This is the first study to evaluate the bioremediation effects of sea lettuce in a cold-water SW-RAS with temperate fish species, though it has previously been evaluated in other farming systems with other fed species. The calculations extrapolated from the present study suggest that the optimal ratio for sea lettuce:fish biomass is 0.7. Comparisons with previously published nitrogen removal efficiencies with sea lettuce are difficult since different studies report the biomass of the extractive species in other units, for example as nitrogen removal per m^2 rather than per m^3 as used here [54–56]. Instead, we can compare with aquaponic systems, where nutrients from the fish tanks are extracted and utilized to fertilize and improve plant growth [57]. There are more nitrogen budgets available for aquaponic systems than for IMTA RASs and comparing the relative nitrogen removal capacity between water-based and land-based extractive organisms is also interesting. Macroalgae are generally considered efficient nitrogen assimilators as their lack of a vascular system and true roots allows for a larger surface area for absorption, and larger surface area:volume ratios correlate with higher uptake rates [58]. When compared to the vascular

plant eel grass (*Zostera* spp.), which usually co-exists in the same environment as macroalgae, the results vary depending on local conditions. For example, parallel experiments on the ammonium and nitrate uptake rates of *Ulva pertusa* and *Zostera japonica* reported higher removal rates by the *Ulva* [59]. Another laboratory study on nitrogen surges, however, found higher nitrogen uptake rates of the seagrass species *Zostera marina* and *Zostera noltei* compared to several macroalgae species, including *Ulva rotundata* [60]. Previous aquaponic studies with other vascular plants have reported optimal plant:fish biomass ratios for Koi carp (*Cyprinus carpio*) and water celery (*Oenanthe javanica*) at 0.95 [61], and for Nile tilapia and amaranthus (*Amaranthus Hybridus*), the optimal plant:fish biomass ratio was 0.46–0.53 [62]. For a system with goby (*Oxyeleotris marmorata*) and water spinach (*Ipomoea aquatica*) the volume of the spinach cultivation needed to be at least three times as high as the volume of the fish tanks [63]. The present estimated ratio of 0.7 is towards the upper range compared to the cited aquaponics systems. However, an important consideration is that for algae the entire biomass can be consumed as food, whereas there are parts of green plants, such as stalks and roots, that cannot be eaten. The present results show that a marine macroalgae like sea lettuce can be at least an equally efficient nitrogen remediator as the previously mentioned vascular plants.

5. Conclusions

This study showed how the fate of nitrogen in a cold-water RAS changed when holding either only fish, or fish and sea lettuce together. While adding the sea lettuce did not directly reduce the dissolved nutrient levels in the RAS, the nitrogen budgets could nonetheless reveal how much nitrogen was assimilated by the sea lettuce during co-farming. With an initial sea lettuce:fish biomass ratio of 0.013 and 0.017, the sea lettuce assimilated between 1.0% and 1.5%, respectively, of the nitrogen input. Extrapolations of these results indicated that a wet weight ratio of sea lettuce:fish of 0.7 would be required to maximize the bioremediation benefits of co-farming. These results can be applied when designing future co-farming systems in cold-water RASs, which are becoming increasingly common both in the Nordic countries and worldwide.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/w18151850/s1>, Figure S1: Dry weight (DW): fresh weight (FW) relationship for *U. fenestrata* farmed at the Tjärnö Marine Laboratory. In average the DW:FW ratio was 6.20.

Author Contributions: All authors contributed to the study conception, design and methodology. Investigation, M.A., K.S., S.S., J.A.C.R., H.P. and K.S.S.; formal analysis, M.A., I.D., K.S. and K.S.S.; visualizations, M.A.; project administration and funding acquisition, M.A. and K.S.S.; resources, K.S.S. and H.P.; supervision, I.D. and K.S.S.; first draft of the manuscript; M.A. All authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Blue Food—Center for Future Seafood, with contributions from Formas—a Swedish Research Council for Sustainable Development (grant number 2020-02834) and Region Västra Götaland (grant number RUN 2020-00352) and Helge Ax:son Johnsons stiftelse (grant number F22-0103).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

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

Abbreviations

The following abbreviations are used in this manuscript:

RAS	Recirculating aquaculture system
DOM	Dissolved organic matter
POM	Particulate organic matter
TSS	Total suspended solids
IMTA	Integrated multitrophic aquaculture
SW	Salt water
SEM	Standard error of mean
DIN	Dissolved inorganic nitrogen

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