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Ulva spp. performance and biomitigation potential under high nutrient concentrations: implications for recirculating IMTA systems

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Abstract
The growth, tissue content and nutrient removal rates of *Ulva* spp., when exposed to moderate to high nitrogen (0.5–5 mmol L^{-1}) and phosphorus (0.01–0.9 mmol L^{-1}) concentrations, were examined to get a better understanding of recirculating IMTA (Integrated Multi-Trophic Aquaculture) systems with fish and seaweed. It was hypothesized that fish waste effluents might lead to unfavorable nutrient stoichiometry and/or toxic conditions, which might harm seaweeds and, specifically for the present study, reduce *Ulva* spp. performance. Results demonstrate that: (I) the unfavorable N:P stoichiometry (N:P ≠ Atkinson atomic ratio of 30:1) did not restrict *Ulva* spp. growth nor tissue content; this indicates that supply of both nutrients exceeded the minimum requirements; (II) a high orthophosphate concentration (0.9 mmol L^{-1}) was toxic to *Ulva* spp., whereas (III) a high nitrate concentration (5 mmol L^{-1}) did not inhibit phosphorus uptake; (IV) *Ulva*'s growth was not enhanced when nitrate was exchanged for similarly high ammonium concentrations. However, tissue nitrogen content was 1.4 times higher when exposed to ammonium than nitrate, suggesting that the former N-form was stored faster in the seaweed's tissue. Therefore, other factors must have limited growth with the high ammonium concentrations. This study also highlights the importance of relatively long acclimatization periods (one week) when maintenance uptake (V_m) is evaluated, as surge uptake (V_s) may result in considerably different and more variable rates. Results of this study contribute to a better understanding of the application of *Ulva* spp. as extractive component in closed IMTA systems, thus advancing sustainable and circular production techniques.

Keywords Integrated multi-trophic aquaculture · Nitrate · Phosphorus · Ammonium · N:P ratio · Seaweed

Introduction

Green seaweeds belonging to the genus *Ulva* (Chlorophyta) are well known for their high nutrient uptake capacity and biomass productivity (Bruhn et al. 2011; Kang et al. 2011; Luo et al. 2012; Gao et al. 2018, 2020). Furthermore, they can be cultivated in artificial media and wastewater effluents (Guist Jr and Humm 1976; Cohen and Neori 1991; Luo et al. 2012; Kumari et al. 2013). These characteristics make them an ideal model species for small scale laboratory experiments on eco-physiological processes, like nutrient uptake kinetics (Lubsch and Timmermans 2018), but also for land-based integrated aquaculture systems, for biomitigation of the inorganic waste fraction resulting from fish cultures (Krom et al. 1995; Shpigel and Neori 1996; Neori et al. 2003; Schuenhoff et al. 2003; Msuya et al. 2006). Fish excrete inorganic phosphorus as orthophosphate (PO_4) and inorganic nitrogen in the form of TAN (NH_3-N + NH_4-N), which can convert to the less toxic nitrate (NO_3) by bacterial nitrification. Water renewal is minimized in recirculation aquaculture systems (RAS), and the waste nutrients PO_4 and NO_3 may accumulate in the culture water to high concentrations. Potentially toxic levels of up to 20 mmol L^{-1} nitrate and 3 mmol L^{-1} orthophosphate have been reported in various seawater RAS systems (Neori et al. 2007; Tal et al. 2009;

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van Bussel et al. 2013; Yogev et al. 2017). Integrated recirculating fish-seaweed systems can be designed in two ways; either with or without bacterial biofilters. Both designs are characterized by high nutrient concentrations but vary in the form of accumulated nitrogen (TAN or NO_3), with implications to N-removal efficiency (Neori 1996). Nutrient dynamics in integrated aquaculture based on closed systems (*i.e.,* RAS) are different from open-water IMTA systems, where fish wastes are quickly mixed with ambient nutrients (Jansen et al. 2018). Several studies using seaweed as extractive species in integrated aquaculture systems have focussed on biomitigation under low to moderate nutrient conditions (Krom et al. 1995; Al-Hafedh et al. 2012; Kang et al. 2021). These conditions are not always representative for the high nutrient concentrations in RAS effluents.

Molar N:P ratios of inorganic fish waste range between 5 – 35, depending on different factors like fish species and diet type (Wang et al. 2012; Hadley et al. 2014). The Atkinson atomic ratio (N:P 30:1) is considered optimal for seaweeds (Atkinson and Smith 1983), whereas tissue ratios between 16:1 and 24:1 (N:P) have been reported to sustain maximum growth in *Ulva* spp. (Björnsäter and Wheeler 1990; Tremblay-Gratton et al. 2018). Indeed, suboptimal N:P ratios have reduced the growth rate of seaweed (Björnsäter and Wheeler 1990), indicating that one of the nutrients was limiting. The N:P ratios in fish wastes can be well below the Atkinson ratio, suggesting that phosphorus may accumulate in an integrated RAS system creating suboptimal N:P ratios, and growth of seaweeds might be limited under such conditions. However, at the same time, the high nutrient concentrations in RAS effluents may surpass the minimum values required for maximum growth (0.8 µmol L^{-1} P and 6.7 µmol L^{-1} N; Pedersen and Borum 1997, Pedersen et al. 2010). Under this condition, the N:P ratios in the RAS effluent may not limit the uptake of either nutrient and will thus not result in reduced growth of the seaweed.

Apart from potentially limited growth due to unbalanced stoichiometry in fish effluents, high nutrient concentrations may create toxic conditions for seaweeds. As highlighted above, phosphorus may accumulate in integrated RAS systems. Some studies suggest that a high (0.8–3.2 mmol L^{-1}) orthophosphate concentration may reduce seaweed growth (Friedlander and Ben-Amotz 1991; Navarro-Angulo and Robledo 1999). Still, it is unclear whether the cause is the high orthophosphate concentration itself or the low N:P ratios. As far as we know, phosphorus toxicity to *Ulva* spp. has not been defined. There are, however, indications that exposure of *Ulva lactuca* to high nitrate concentrations may inhibit phosphorus uptake and, therefore, growth (Lundberg et al. 1989). The opposite has been reported for *Fucus vesiculosus* (Perini and Bracken 2014), an inconsistency that highlights the limited understanding of the interactions between nitrate and phosphorus uptake kinetics in seaweed. As nitrate concentrations are high in bacterially-biofiltered RAS effluents, these processes are particularly relevant for such systems, resulting in an even higher phosphorus accumulation in the water and potentially reduced seaweed performance.

Fish excreta consist, among others, of TAN which is transformed into nitrate by the bacterial biofilters in RAS systems (Krom et al. 1995; Neori et al. 2007). Although *Ulva* spp. can assimilate both nitrogen sources, uptake of nitrate is generally much slower than TAN (Neori 1996; Hadley et al. 2014), which is also reflected in a lower growth (Ale et al. 2011). The different rates of nutrient uptake and growth by *Ulva* spp. with these two nitrogen forms are related to different uptake and assimilation energy requirements (Shahar et al. 2020). Nitrate, unlike TAN, requires metabolic energy for uptake and assimilation into protein (Taylor et al. 2006). Therefore, seaweed growth in the nitrate-rich RAS IMTA systems might be sub-optimal. An additional consideration is the much lower toxicity of TAN to seaweed than to fish (Harrison and Hurd 2001; Moustafa et al. 2014). Thus, replacing the bacterial biofilter with a seaweed unit may improve water quality for fish (low levels of TAN, consumption of acidity and production of O_2) and at the same time improve seaweed production.

In this study, the growth and nutrient assimilation were measured in *Ulva* spp., when exposed to relatively high and moderate nitrogen and phosphorus concentrations. Such conditions have been insufficiently addressed in the literature (Lundberg et al. 1989; Demetropoulos and Langdon 2004; Tremblay-Gratton et al. 2018) and would give a better understanding of biomitigation potential and seaweed performance under high nutrient concentrations. The following four hypotheses were examined: (I) *Ulva* spp. performance is not influenced by N:P stoichiometry in fish waste effluents under high nutrient concentrations, (II) High orthophosphate concentrations typical in RAS effluents are not toxic to *Ulva* spp., (III) High nitrate concentrations typical in RAS effluents can limit the phosphorus uptake, (IV) High TAN concentrations improve the performance of *Ulva* spp. compared to comparably high nitrate concentrations.

Materials & methods

Experimental design

This study consisted of two experiments. The first experiment evaluated maintenance uptake kinetics (V_m, *i.e.*, when internal nutrient concentrations remain constant; Lubsch and Timmermans 2018) in *Ulva* spp. exposed to different nutrient treatments. Separate batches of *Ulva* spp. were continuously exposed to one of six nutrient treatments (treatments A to F) for two weeks. In treatments A, B, C and D, nitrate

was the N-source, while ammonium was the N-source in treatments E and F. The treatments varied in nutrient concentration between 0.7–5.0 mmol L^{-1} nitrogen (either nitrate or ammonium) and 0.013–0.9 mmol L^{-1} orthophosphate, using dilution steps with a factor of 10, and resulting in stoichiometries that were either high (N:P 60–70) or low (N:P 9–10). For a complete description of the treatments, see Table 1. These treatments reflected nutrient conditions expected from fish effluents in a RAS facility, and tested all four hypotheses. In the second experiment, only hypothesis III was tested for surge uptake (V_s, *i.e.,* filling of internal nutrient pools; Lubsch and Timmermans 2018), as the effect of high nitrate concentrations on phosphorus uptake rates might only be visual during surge uptake (V_s). This second experiment studied phosphorus uptake for five hours, during which nutrient-starved *Ulva* spp. was exposed to moderate and high nitrate concentrations (treatment A and B).

Holding conditions

The experiments were conducted in a temperature- and light-controlled room at the Aquatic Research Facility of the Wageningen University (ARF—Carus, Wageningen, The Netherlands). *Ulva* spp. was obtained from the greenhouse facility of the Wageningen University and Research Centre (Nergena, Wageningen, The Netherlands), where it has been cultivated since 2012. It was assumed that the culture stock consisted of *Ulva lactuca*, but recent analysis from the location where initial samples were collected indicates the presence of additional *Ulva* species (Fort et al. 2020). We, therefore, refer to '*Ulva* spp.' for the species investigated in this study.

Before the experiments, seaweeds were maintained in a stock tank (1 m^3) filled with artificial seawater (Reef Crystals, Aquarium Systems, Inc., Mentor, Ohio, USA), to which once a week plant fertilizer (Pokon© Groene Planten Voeding, Veenendaal, The Netherlands) was added. N:P atomic ratio of this plant fertilizer is 11, which is in line with the low N:P ratio of treatments C, D, and F of the current study (Table 1). Light tubes (T5 TL 24 W – AquaBlue Special—ATI) were placed above the tank (~ 400 µmol photons m^{-2} s^{-1}), the temperature was maintained at 17.5 ± 0.5 °C, and aeration was added at the bottom of the tank, to maintain vertical water movement (Msuya and Neori 2008).

Experiment 1: Maintenance uptake (V_m) and growth

Six treatments were formulated varying in nutrient concentrations between 0.7–5.0 mmol L^{-1} nitrogen (either nitrate or ammonium) and 0.013–0.9 mmol L^{-1} orthophosphate, using dilution steps with a factor 10, and resulting in stoichiometries that were either high (N:P 60–70) or low (N:P 9–10) (Table 1). Four experimental systems (header tank plus three associated seaweed cultivation tanks) were available, and treatments were, therefore, divided over two consecutive runs, using new seaweed stocks in each run. The first run included all nitrate-based treatments, while the second run included all ammonium-based treatments. Two identical reference nitrate-based treatments (A1 and A2) were included in both runs to elucidate a potential effect of 'run'. Treatment concentrations were formulated by daily addition of a mix of artificial seawater (29.7 ± 1.8 ‰, Reef Crystals, Aquarium Systems, Inc., Mentor, Ohio, USA), wastewater from a RAS system with sea bass (2L) and stock solutions to header tanks with a total volume of 110 L. The stock solutions were prepared with NaH_2PO_4 and $NaNO_3$ for the nitrate treatments and NaH_2PO_4 and NH_4Cl for the ammonium treatments. Water samples were collected daily in the header tanks, and were directly stored in the freezer (- 20 °C) until nutrient analyses.

Seaweed tanks consisted of 15 L white round tanks (0.07 m^2 surface area) with an overflow PVC pipe in the center. Each overflow pipe was covered with an additional perforated

Table 1 Treatment overview including dissolved inorganic nitrogen (DIN) and phosphorus (DIP) concentration, and N:P ratio (molar) in the culture media. Planned values refer to the targeted concentrations before the experiment, and realized values are actual daily average concentrations measured in the header tanks. Values are given as mean ± SD

Treatment	Run	Planned			Realized			
		DIN (mmol L^{-1})	DIP (mmol L^{-1})	N:P	NO_3/TAN (mmol L^{-1})	DIN (mmol L^{-1})	DIP (mmol L^{-1})	N:P
A1	1	5.0 NO_3	0.160	30	4.9 ± 0.9 NO_3	5.0 ± 0.9	0.13 ± 0.02	38 ± 3
B	1	0.5 NO_3	0.016	30	0.8 ± 0.1 NO_3	0.8 ± 0.1	0.013 ± 0.003	62 ± 10
C	1	0.5 NO_3	0.080	6	0.7 ± 0.1 NO_3	0.7 ± 0.1	0.07 ± 0.01	10 ± 1
D	1	5.0 NO_3	0.800	6	5.5 ± 0.4 NO_3	5.5 ± 0.4	0.86 ± 0.17	7 ± 1
A2*	2	5.0 NO_3	0.160	30	4.9 ± 0.6 NO_3	4.9 ± 0.6	0.14 ± 0.01	34 ± 4
E	2	0.5 TAN	0.016	30	0.6 ± 0.1 TAN	1.0 ± 0.2	0.015 ± 0.003	68 ± 13
F	2	0.5 TAN	0.080	6	0.5 ± 0.1 TAN	0.8 ± 0.1	0.09 ± 0.01	9 ± 1

*Treatment A2 is identical to A1 and acts as a reference between both runs

PVC pipe topped with a perforated cap, preventing seaweed pieces from floating out of the tank. Aeration was added via the bottom of each tank, approximately 5 cm from the center, creating sufficient turbulence to suspend the seaweed pieces and move them through the water column. A slow, internal recirculation flow through a UV-light removed potential microalgal and bacterial contaminants. A double perforated bottom allowed the water to pass the UV light, without damaging the seaweed. Light tubes (T5 TL 24 W – AquaBlue Special—ATI) were placed above the seaweed tanks, resulting in irradiance of 396 ± 46 µmol photons m^{-2} s^{-1} (measured by LI-COR LI193R PAR meter, just underneath the water surface). A 12 h light-12 h dark light regime was maintained. All seaweed tanks were connected to a flow-through set-up with a continuous supply of nutrients. Nutrients from one header tank were pumped to three replicate seaweed tanks with a flow rate of 1.6 ± 0.09 l h^{-1}, resulting in an average water exchange rate of 2.5 tank volumes day^{-1}.

Each experimental run consisted of an acclimation week followed by an experimental week. This acclimatization period stabilized the internal nutrient pools (Lubsch and Timmermans 2018). Nutrient removal rates measured in the second week were thus assumed to reflect metabolic controlled uptake rates (V_m). At the start of the acclimation period, each seaweed tank was stocked with 60.3 ± 0.4 g, equivalent to 4.02 ± 0.03 g L^{-1}, fresh *Ulva* spp. material (spin-dried by a lettuce hand-centrifuge). Start samples were collected to determine initial tissue content (n = 3 samples for each run). After the acclimation week, all seaweed material was harvested, spin-dried and weighted to determine fresh weight. Then, 30.5 ± 0.3 g (equivalent to 2.04 ± 0.02 g L^{-1}) of the harvested *Ulva* spp. material from each tanks was returned to the tank, while the remaining material was collected for analysis. At the end of the second week, all seaweed material was collected, spin-dried and weighted to determine fresh weight. Seaweed samples were rinsed with deionized water, spin-dried and thereafter stored in the freezer (-20 °C) until analyses. Specific growth rate (SGR, % day^{-1}) was calculated based on dry weight (DW) for week 1 and week 2 separately, using the following formula:

$$\mathrm{SGR} = \left(\left(\ln\left(\mathrm{W_f}\right) - \ln\left(\mathrm{W_i}\right)\right)/\mathrm{T}\right) \times 100$$

where W_f is the final biomass in g dry weight, W_i the initial biomass in g dry weight, and T the number of experimental days.

The biomass yield was determined for week 2, using the following equation after Revilla-Lovano et al. (2021):

$$\mathrm{Yield}\left(\mathrm{g\ FW\ m^{-2} day^{-1}}\right) = \left(\mathrm{W_f} - \mathrm{W_i}\right)/\mathrm{T}$$

where W_f is the final biomass (g FW m^{-2}), W_i is the initial biomass (g FW m^{-2}), and T the number of experimental days.

All seaweed samples were analyzed for dry matter, ash, N and P tissue content (see biochemical analyses). Nitrogen and phosphorus removal rates were calculated for week 1 and week 2 separately, using the following equation after Kim et al. (2007):

$$\text{Removal rate } (\mu\text{mol g}^{-1}\text{ DW day}^{-1}) = \left(\left(\mathrm{W_f} * \mathrm{TC_f}\right) - \left(\mathrm{W_i} * \mathrm{TC_i}\right)\right)/\mathrm{DW}/\mathrm{T}$$

where W_f is the final biomass in g dry weight, W_i the initial biomass in g dry weight, TC_f the final N or P tissue content (in µmol g^{-1} DW), TC_i the initial N or P tissue content (in µmol g^{-1} DW^1), DW the mean biomass in g dry weight of either week 1 or week 2, and T the number of experimental days.

Experiment 2: Surge uptake (V_s)

For the measurement of the surge uptake related to hypothesis III, *Ulva* spp. was exposed to either high (5 mmol L^{-1}) or moderate (0.5 mmol L^{-1}) nitrate concentrations, both with a fixed orthophosphate concentration (0.026 ± 0.005 mmol L^{-1}). The solutions were prepared by artificial seawater (29.7 ± 1.8 ‰, Reef Crystals, Aquarium Systems) and NaH_2PO_4 and $NaNO_3$. Surge uptake measurements were based on the method described by Hurd and Dring (1990). Before the experiment, *Ulva* spp. were nutrient-starved for 3 days. At the start of the experiment, pieces of *Ulva* spp. of comparable weights (1.43 ± 0.03 g fresh weight) were placed in 500 mL glass jars, filled with 400 mL of one of the above-described solutions, in four replicates per treatment. Two control jars were added for each treatment, containing nutrient solution without seaweed. The jars were placed in a water bath shaker, creating a constant water movement for mixing and reducing the diffusion boundary layer between seaweed and the medium. Light tubes (T5 TL 24 W – AquaBlue Special—ATI) were placed above the seaweed tanks, resulting in irradiance of 826 ± 14 µmol photons m^{-2} s^{-1} (measured by LI-COR LI193R PAR meter, just underneath the water surface). Water samples (in duplicate; 2 × 1 mL) were taken at t = 0, 10, 20, 30, and 300 min and instantly analyzed as described below for nitrate and orthophosphate concentrations. As seaweed used in experiment 2 was not analyzed for dry matter content, phosphorus uptake rate was calculated on the basis of fresh weight, using the following formula:

$$\text{Uptake rate } (\mu\text{mol g}^{-1}\text{ FW h}^{-1}) = \left(\left(\mathrm{WC_f} * \mathrm{V}\right) - \left(\mathrm{WC_i} * \mathrm{V}\right)\right)/\mathrm{FW}/\mathrm{T}$$

where WC_f is the nutrient concentration in the water (µmol L^{-1}) at Tx, WC_i the initial nutrient concentration in the water (µmol L^{-1}), V is the volume (L), FW is the fresh weight of the *Ulva* spp. (g) and T is the time in hours.

Biochemical analyses

Water samples of the growth experiment (maintenance uptake) were analyzed for TAN, nitrate–N and orthophosphate-P using

an auto-analyzer (SANplusSYSTEM, Skalar). Water samples of the surge uptake experiment were analyzed for nitrate–N and orthophosphate-P using a SmartChem 200 Discrete Analyzer. Freeze-dried seaweed samples were ground with a centrifugal grinding mill (Retsch/Brinkmann ZM 100/w 1 mm sieve, Verder NV, The Netherlands). Dry matter and ash were determined according to ISO-6496 (1983) and ISO-5984 (1978), respectively. Phosphorus content in the seaweed was analysed using inductively coupled plasma-mass spectrometry (ICP-OES) according to the standard NEN 15510 (2007). Carbon and nitrogen content of the seaweed were analysed by combusting the samples with an element analyzer (Flash 2000, Therm Fisher) at 1020 °C in the presence of oxygen, converting carbon and nitrogen to CO_2 and NO_x ($NO_2 + NO_3$), respectively. Thereafter, NO_x was reduced to N_2 in a reduction column.

Statistical analyses

Statistical analyses were performed in R studio 3.4.0. Before the analysis, residuals of the data were checked for homogeneity of variance and normality using the Shapiro–Wilk and Levene test. A two-way analysis of variance (2-way ANOVA) was used to check for a potential interaction effect between the N:P ratio and N-source (treatments B, C, E, F). Since no interaction effect ($p > 0.05$) was observed for uptake and growth performance parameters, comparative treatments related to hypotheses I and IV were tested independently. For each hypothesis, differences in uptake and performance of *Ulva* (*i.e.*, growth, yield, tissue content, V_m nutrient removal rates) in the corresponding treatments (Table 1) were tested by one-way analysis of variance (1-way ANOVA). In addition to hypothesis III, a 1-way ANOVA was used to detect potential differences in phosphorus surge uptake rates (V_s) by *Ulva* spp. exposed to either a high or moderate nitrate concentration. All statistics for the V_m experiments were based on data collected in the second week only. Paired-Samples T-tests tested the difference in growth and tissue content between week 1 and week 2, for each treatment separately to verify the relevance of acclimation.

Results

Realized nutrient concentrations were not always in range with the planned formulated culture media. Especially treatment B and E showed variations and the measured DIN concentrations were almost double the planned ones, resulting in deviations to the N:P ratios (Table 1). Culture media formulated based on NH_4Cl contained not only TAN-nitrogen but also NOx-nitrogen, leading to slightly higher DIN concentrations than expected. The origin of these additional NOx concentrations remains unknown, but seems to fall within the variation observed for the other treatments and may relate to possible NOx contamination in the salt. This was however not analysed. Despite these variations all hypotheses could still be evaluated.

Hypothesis I: *Ulva* spp. performance under contrasting N:P ratios

The two contrasting N:P ratios (9–10 vs 60–70) did not impact growth and yield (Fig. 1) nor tissue content of the seaweed (Fig. 2) (Table 2; 1-way ANOVA; $p > 0.05$ B vs C; $p > 0.05$ E vs F). As a result nutrient removal rates were comparable for the high and low N:P treatments (Fig. 3) (Table 2; 1-way ANOVA; $p > 0.05$ B vs C; $p > 0.05$ E vs F). Tissue N:P ratios varied between 27–28 for the nitrate-based treatments (B and C) and 39–42 for the ammonium-based treatments (E and F), irrespective of the N:P ratio provided in the culture medium.

Hypothesis II: Toxicity of high orthophosphate concentrations

Ulva spp. cultivated under the highest orthophosphate concentration (treatment D; 0.9 mmol L^{-1} P) showed a different, unhealthy, tissue structure compared to the other treatments; tissue was hard, easy to break and felt brittle, suggesting degradation (visual observation). Most of the material of this treatment was lost during the sampling procedure (spin-drying), and we were therefore unable to derive valid measurements. Treatment D was therefore excluded from the statistical analyses (Table 2; Figs. 1, 2 and 3; no bar shown for treatment D). As described for hypothesis I, no significant differences were observed for the *Ulva* spp. in the remaining low (treatment B) and medium (treatment C) orthophosphate concentrations.

Hypothesis III: Inhibiting effects of high nitrate concentration

Phosphorus removal during maintenance uptake (V_m; experiment 1) was approximately 60% higher (Table 2; 1-way ANOVA; $p < 0.05$) for *Ulva* spp. cultivated under high nitrate (treatment A) compared to moderate nitrate concentrations (treatment B) (Fig. 3). This difference was not the result of growth, since SGR and yield did not differ between the treatments (Fig. 1; Table 2; 1-way ANOVA; $p > 0.05$). More likely is that the difference in phosphorus removal was driven by differences in tissue content, as phosphorus content in *Ulva* spp. of the high nitrate treatment was higher by approximately 25% (Table 2; 1-way ANOVA; $p < 0.0001$) compared to the moderate nitrate

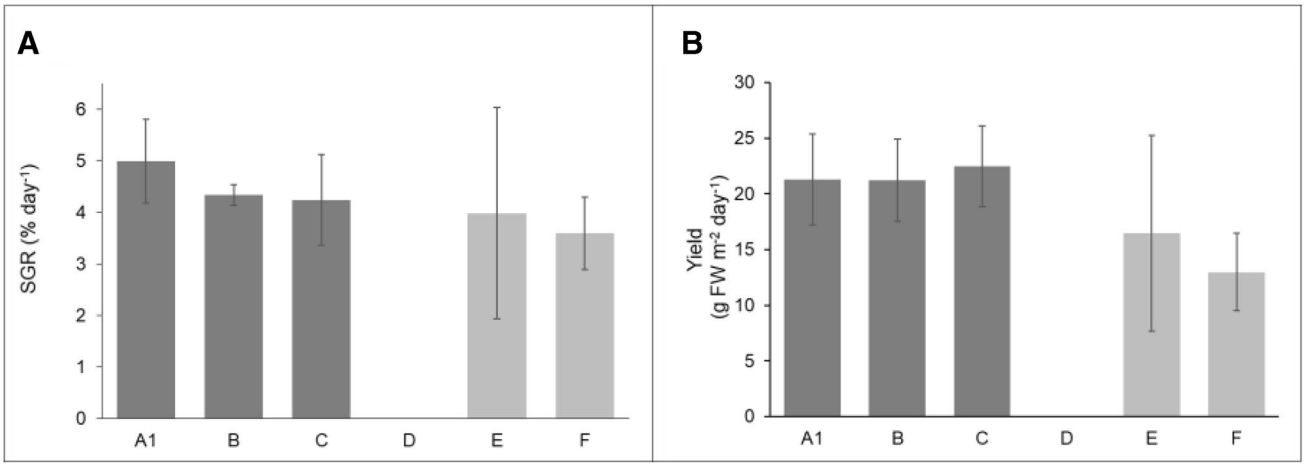

Fig. 1 Specific Growth Rate (**A**) and biomass yield (**B**) of *Ulva* spp. cultivated in the six experimental treatments. For treatment description, see Table 1, and for a description of the hypotheses tested and the statistical results, see Table 2. Dark grey bars represent nitrate-based treatments, and light grey bars represent ammonium-based treatments. Bars represent mean values (n = 3 tanks treatment^{-1}), and error bars represent standard deviations. Due to degradation of the *Ulva* spp. in treatment D, we were unable to derive valid measurements, resulting in no bar shown for treatment D

treatment (Fig. 2). This P difference coincided with a nearly 25% lower tissue N:P ratio for *Ulva* spp. in the high nitrate treatment, compared to *Ulva* spp. cultivated in the moderate nitrate treatment (Fig. 2; Table 2; 1-way ANOVA; $p = 0.0002$). No differences were observed in tissue nitrogen content, tissue C:N ratio and nitrogen removal rates (Figs. 2 & 3; Table 2; 1-way ANOVA; $p > 0.05$). Similarly, surge uptake (V_s; experiment 2) of phosphorus did not vary between the moderate (0.60 ± 0.09 µmol g^{-1} FW h^{-1}) and high (0.51 ± 0.04 µmol g^{-1} FW h^{-1}) nitrate treatments (Fig. 4; 1-way ANOVA, $p > 0.05$).

Hypothesis IV: Effect of N-source (nitrate or ammonium)

The lack of a significant difference in growth between reference treatments A1 and A2 (Table 1; A1 and A2) (1-way ANOVA; $p = 0.076$), validates a comparison between nitrate-based treatments tested in run 1 and ammonium-based treatments tested in run 2. While SGR and tissue phosphorus content were not affected by the type of N-source (Figs. 1 & 2; Table 2; 1-way ANOVA; $p > 0.05$ B vs E; $p > 0.05$ C vs F), tissue nitrogen content (Fig. 2) was higher for *Ulva* spp. in the ammonium-based treatments (5.2% DM) compared to the nitrate-based treatments (3.8% DM) (Table 2; 1-way ANOVA; $p < 0.0001$ B vs E; $p < 0.0001$ C vs F). This was also reflected in a significant higher tissue N:P ratio, but lower tissue C:N ratio for *Ulva* spp. provided with ammonium-N (Fig. 2). Interestingly, nitrogen removal (in µmol g^{-1} DM day^{-1}) did not differ between nitrate and ammonium treatments (Fig. 3; Table 2; 1-way ANOVA; $p > 0.05$). Numerically, the highest nitrogen removal rates were obtained in the ammonium-based treatments (Fig. 3).

The relevance of acclimatization

Except for treatment B, a significant increase in tissue nitrogen content was observed between the first and second weeks in all treatments, while tissue phosphorus content remained similar over time (Table S1; Paired-Samples T-tests; $p > 0.05$). However, the increased nitrogen content did not result in a significant difference in tissue N:P ratio between the two weeks. The C:N ratio decreased significantly in the second week only for the *Ulva* spp. that was cultivated in treatment A1 and treatment F (Table S1; paired-samples T-test; $p < 0.01$). Interestingly, growth seemed to increase over time when nitrogen was provided in the form of ammonium (treatment E & F), while growth decreased in most cases when nitrate was provided (treatment B & C) (Table S1). Nevertheless, a significant time effect for growth was only observed for treatment C (Paired-Samples T-test; $p < 0.05$).

Discussion

Our data suggest that *Ulva* spp. growth is not influenced by (unfavorable) stoichiometry under moderate to high nutrient concentrations, and high nitrate concentrations do not limit phosphorus uptake. This result is promising for closed IMTA systems, where marine fish in RAS are integrated with seaweed. Nevertheless, our data also suggest that high nutrient concentrations (0.9 mmol L^{-1} orthophosphate) in (simulated) fish waste effluents may, in specific cases, lead

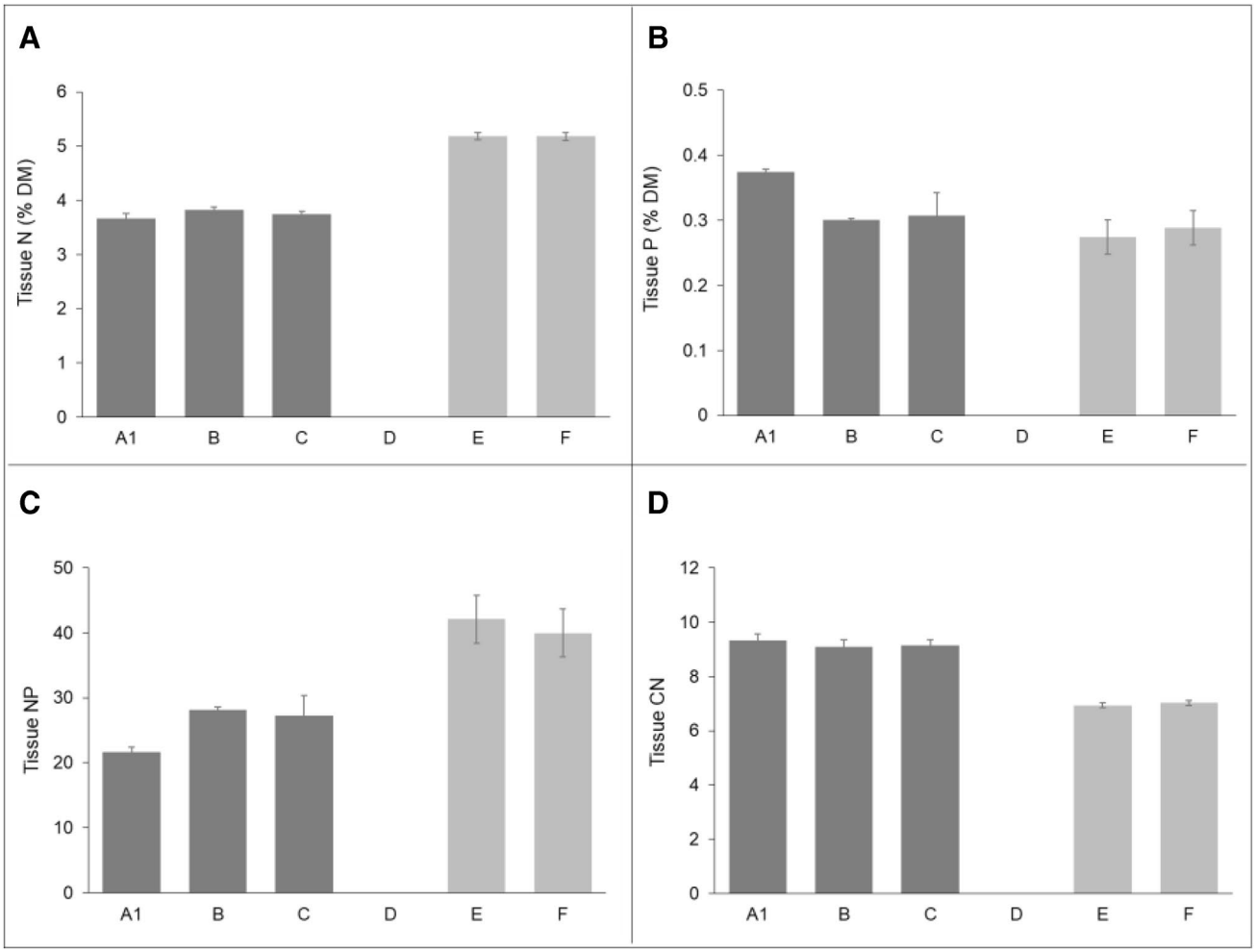

Fig. 2 Tissue content (tissue nitrogen and phosphorus, % of dry matter, and tissue N:P and C:N molar ratio) of *Ulva* spp. cultivated in the six experimental treatments. For treatment description, see Table 1, and for a description of the hypotheses tested and the statistical results, see Table 2. Dark grey bars represent nitrate-based treatments, and light grey bars represent ammonium-based treatments. Bars represent mean values (n = 3 tanks treatment^{-1}), and error bars represent standard deviations. Due to degradation of the *Ulva* spp. in treatment D, we were unable to derive valid measurements, resulting in no bar shown for treatment D

to reduced *Ulva* spp. performance. Therefore, the design of IMTA including seaweed as extractive unit should not allow such conditions by matching the uptake capacity of the seaweed unit to the waste production rate (Neori et al. 2001; Neori and Guttman 2017).

Limiting nutrients

Under nutrient limiting conditions, the ratio between macroelements (N:P) regulates growth and nutrient uptake in seaweeds (Björnsäter and Wheeler 1990; Fan et al. 2014; Perini and Bracken 2014), highlighting the importance of studying nutrient interactions, rather than a single nutrient at a time. Under the high nutrient concentrations in the current study, nutrient removal and growth rates were not different for the two contrasting N:P ratios (9–10 vs 60–70; B vs C; E vs F). This observation suggests that neither of the nutrients was limiting at any time. Both nitrogen and phosphorus tissue contents were above the critical tissue values required to sustain maximum growth reported for *Ulva* spp. (0.20% P of DW and 2.17% N of DW; Pedersen and Borum 1997; Pedersen et al. 2010). This observation must have derived from the non-limiting concentrations that prevailed for both nitrogen and phosphorus in the current study. Steffensen (1976) also reported for *U. lactuca* that maximum growth could be achieved under a wide variety of N:P ratios in the medium (N:P ratios of 1:48 – 1:2), demonstrating that variations from the Atkinson ratio do not necessarily reduce seaweed growth. When absolute nutrient concentrations are above the saturation threshold, other factors could be limiting seaweed growth. Phosphorus tissue contents measured in the current study were below the highest phosphorus tissue content

Table 2 Overview of hypotheses and associated treatment comparisons, including statistical results of treatment comparisons. Treatments within a row lacking a common letter differ significantly ($p < 0.05$) for the parameter(s) indicated in the first column. When treatments are compared separately within a hypothesis (i.e. Hyp I & Hyp IV), the compared treatments share either lower case letters or capital case letters. The 'na' (not applicable) denotes missing data due to algal fragmentation in treatment D.

Hypothesis I: Ulva spp. performance is not influenced by stoichiometry in fish waste effluents under high nutrient concentrations: Comparison of high and low N:P ratios (B versus C; E versus F)

	Treatment B High NO_3:P ratio	Treatment C Low NO_3:P ratio	Treatment E High TAN:P ratio	Treatment F Low TAN:P ratio
SGR, Yield, Tissue N, Tissue P, Tissue NP, Tissue CN, N removal, P removal	a	a	A	A

Hypothesis II: Orthophosphate concentrations in RAS effluents are not toxic for *Ulva* spp.: Comparison between different orthophosphate concentrations (low = B, moderate = C, high = D)

	Treatment B low PO_4 conc	Treatment C moderate PO_4 conc	Treatment D high PO_4 conc
SGR, Yield, Tissue N, Tissue P, Tissue NP, Tissue CN, N removal, P removal	a	a	na

Hypothesis III: High nitrate concentrations in RAS effluents will limit the phosphorus uptake: Comparison between high nitrate (A) and moderate nitrate (B) concentrations

	Treatment A High NO_3 conc	Treatment B Moderate NO_3 conc
SGR, Yield, Tissue N, Tissue CN, N removal	a	a
Tissue P, P removal	a	b
Tissue NP	b	a

Hypotheses IV: High TAN concentrations will result in better performance of *Ulva* spp. in comparison to comparably high nitrate concentrations: Comparison between nitrate and ammonium conditions (B versus E; C versus F)

	Treatment B NO_3 (N:P ratio of 62)	Treatment C NO_3 (N:P ratio of 10)	Treatment E TAN (N:P ratio of 68)	Treatment F TAN (N:P ratio of 9)
SGR, Tissue P, N removal, P removal	a	A	a	A
Yield	a	A	a	B
Tissue N, Tissue NP	b	B	a	A
Tissue CN	a	A	b	B

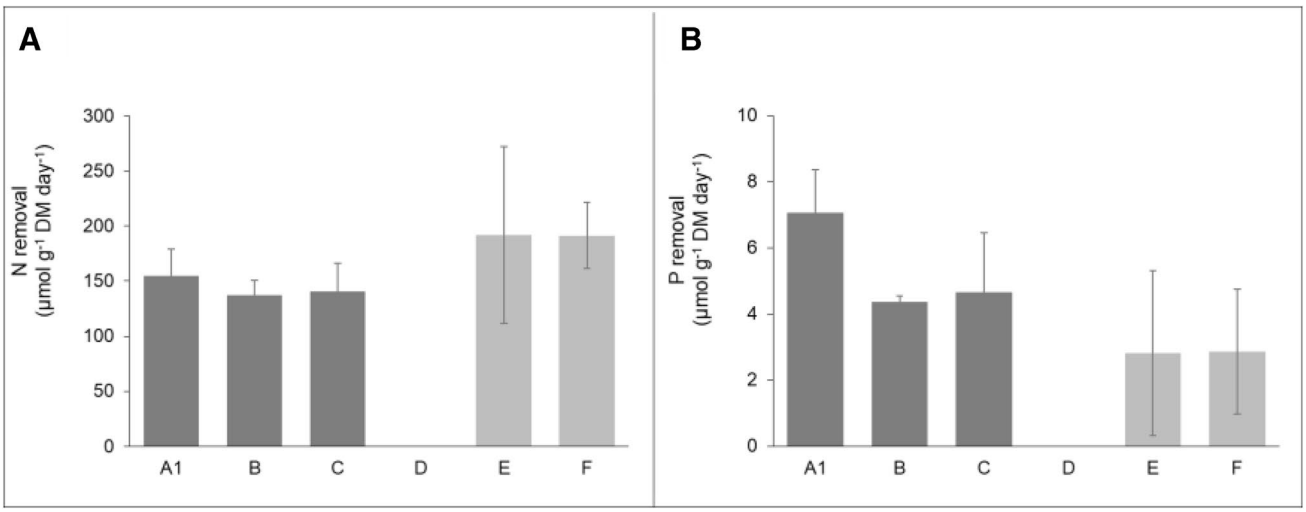

Fig. 3 Nitrogen (**A**) and Phosphorus (**B**) removal rate (µmol g^{-1} dry matter day^{-1}) of *Ulva* spp. cultivated in the six experimental treatments. For treatment description, see Table 1, and for a description of the hypotheses tested and the statistical results, see Table 2. Dark grey bars represent nitrate-based treatments, and light grey bars represent ammonium-based treatments. Bars represent mean values (n = 3 tanks $treatment^{-1}$), and error bars represent standard deviations. Due to degradation of the *Ulva* spp. in treatment D, we were unable to derive valid measurements, resulting in no bar shown for treatment D.

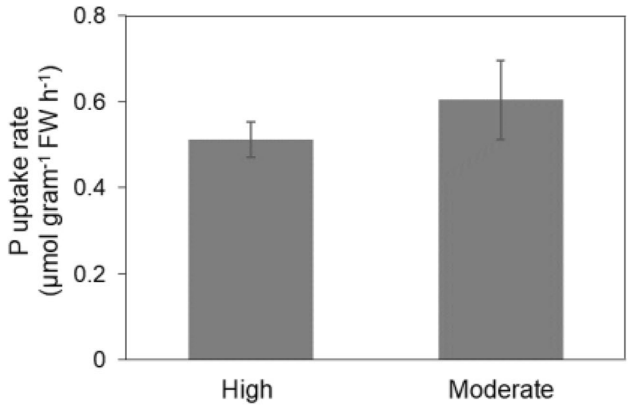

Fig. 4 Phosphorus surge uptake (µmol g^{-1} fresh weight $hour^{-1}$) for *Ulva* spp. exposed to either high (5 mmol L^{-1}) or moderate (0.5 mmol L^{-1}) nitrate concentration. Bars represent mean values (n = 4 tanks $treatment^{-1}$) and error bars standard deviations. Treatments did not differ significantly ($p < 0.05$).

values reported in the literature for *Ulva* spp. (0.4 – 1.5% P of DW; Pedersen et al. 2010; Runcie et al. 2004; Lubsch and Timmermans 2018). Tremblay-Gratton et al. (2018) also reported relatively low tissue N and P content under moderate to high nutrient conditions. They suggested that low tissue reserves might have resulted from a deficiency of trace elements in the media. Trace elements are required to stimulate the phosphate transport system of algae (Lobban and Harrison 1994). Both ASW and RAS water, as used in the present experiments, contain trace elements, but their absolute concentrations and potential limiting effects were not analyzed. Therefore, trace elements might have limited phosphorus uptake in our study.

The results of the treatments used to test hypothesis I indicate that nutrients were supplied in concentrations above the minimum requirements for growth. Under these conditions, unfavorable stoichiometry in fish wastes (N:P ≠ Atkinson ratio) did not limit seaweed growth. The possible adverse effect of such high nutrient concentrations on seaweed performance is discussed below.

Toxicity of high orthophosphate concentrations

This study's highest orthophosphate concentration (0.9 mmol L^{-1}; treatment D) was sub-optimal for the *Ulva,* which degenerated. Salinity did not vary between treatments and could thus not explain such results. Other studies reported inhibited growth for *Gracilaria cornea* at 824 µmol L^{-1} P (Navarro-Angulo and Robledo 1999), *Gracilaria conferta* at 3.2 mmol L^{-1} P (Friedlander and Ben-Amotz 1991), *Palmaria mollis* at 83.3 µmol L^{-1} P (Demetropoulos and Langdon 2004) and *Porphyra columbina* at 120 µmol L^{-1} P (Frazer and Brown 1995). It remains unclear whether inhibited growth in these studies was caused by high orthophosphate concentrations or low N:P ratios. To the best of our knowledge, it is unknown at what concentration phosphorus becomes toxic for *Ulva* spp. However, Tremblay-Gratton et al. (2018) showed high growth rate of *U. lactuca* at orthophosphate concentrations up to 291 µmol L^{-1}, which is approximately 2.5 times higher than the moderate orthophosphate concentration (0.1 mmol L^{-1}) used in the current study. The exact levels of phosphorus toxicity for *Ulva* spp. therefore need to be further elucidated, but will likely fall within the range of 0.3–0.9 mmol L^{-1}.

Inhibiting effects of high nitrate concentration

The maintenance (V_m) uptake of phosphorus was 1.6 times higher under high nitrate concentrations in comparison to moderate nitrate concentrations, and surge uptake (V_s) was similar for both nitrate concentrations. Although growth did not differ between the treatments, the higher phosphorus removal rate and tissue content in the high nitrate treatment was associated with higher orthophosphate concentrations in the culture media. Despite of the lower orthophosphate concentration supplied in the moderate nitrate treatment, saturating orthophosphate concentrations (0.8 µmol L^{-1}; Pedersen et al. 2010, 7 µmol L^{-1}; Lubsch and Timmermans 2018) were assumed for both treatments.

Unlike in the studies of Lundberg et al. (1989) and Kumari et al. (2013) the present results show no inhibiting effects of high nitrate levels on phosphorus uptake by *Ulva* spp. Similar results were reported by Lubsch and Timmermans (2018) who studied phosphorus uptake kinetics of *U. lactuca* under saturating nitrate concentrations (5 mmol L^{-1} NO_3). The higher than expected DIN concentration and subsequent high N:P ratio in treatment B are not expected to have influenced the results, as nitrogen concentrations were still contrasting between treatment A and treatment B. It remains unclear why in some studies high nitrate level seem to have an inhibiting effect on phosphorus uptake, while in other studies this is not observed.

Effect of N-source (nitrate or ammonium) under high nitrogen concentrations

Seaweed provided with ammonium or nitrate as a nitrogen source grew at similar rates. Admittedly, Ale et al. (2011) showed that *U. lactuca* grew about 70% better with ammonium than with nitrate, in batch cultures supplied with 50 µmol L^{-1} of N. As in these experiments, the culture medium was not replaced or resupplemented, it is likely that these results resemble surge uptake (V_s) rather than maintenance (V_m). In that respect they seem to differ from the results in our acclimatization week, which could be regarded as V_s, and where lower

growth was observed for the ammonium-based treatments. Both Neori (1996) and Shahar et al. (2020) found better growth of *U. lactuca* with ammonium than with nitrate for non-starved *Ulva*, representing V_m, and attributed this difference to the different uptake and assimilation pathways that are involved for the two N-sources. Not surprisingly, even though growth rates were comparable between nitrate and ammonium treatments in our study, higher tissue nitrogen contents were achieved in the ammonium based treatments, suggesting an accumulation of nitrogen which is not used for growth. Due to the high tissue nitrogen content, 1.4 times higher nitrogen removal rates are estimated for the ammonium-based treatments.

It is largely unknown what levels of nitrogen are toxic to *Ulva* spp. Waite and Mitchell (1972) suggest that TAN is toxic to *U. lactuca* at concentrations > 65 µmol L^{-1}, whereas other studies (e.g. Fujita 1985; Neori et al. 1991; Ji et al. 2019; Shahar et al. 2020) applied higher concentrations and did not report reduced growth or degenerating seaweed. It seems however unlikely that the high nitrogen concentrations (ammonium nor nitrate) in our study were toxic, since no mortality or debilitation was observed, as seen for the *Ulva* cultured under the high orthophosphate concentration.

General patterns on the biomitigation potential and seaweed performance in recirculating IMTA systems

One of the aims of integrated cultures is to remove excess nutrients from the water and improve its quality. Clean water is essential for the health of the cultured organism and, similarly crucial, to the environment that receives the discharged wastewater. In RAS, the receiving environment is the culture itself. Nutrient removal rates observed in the current study were in line with or lower than other studies on different *Ulva* species (summarized in Table 3). This literature describes variable nutrient uptake and removal rates. Besides differences in species, or even strains (Jansen et al. 2022), a potential explanation for the variation might be the method used. Studies either define nutrient removal as a function of biomass increase and nutrient tissue content, while others determine uptake rates based on depletion of nutrients in the medium. Results from both methods may vary, and it was shown that the nutrient depletion method results in a 2 – 4.5 times higher nutrient uptake (Tremblay-Gratton et al. 2018). The nutritional state of the seaweeds may also play a role in the observed variation in literature since nutrient uptake by seaweeds is, among others, a function of their internal nutrient storage (Lobban and Harrison 1994; Hadley et al. 2014). Our study specifically addressed nutrient uptake for maintenance (V_m). Unfortunately, some studies do not define rigorously whether their measurements are V_m or V_s (surge uptake). This difference can considerably change the interpretation of the data because V_s uptake rate is much higher than V_m (Neori et al. 2003).

Besides the capacity to remove excess nutrients from the system, growth performance and quality (*i.e.*, protein content) also determine extractive species' success in integrated systems. Growth rates in the current study ranged between 3.6 and 5.0% per day, while biomass yield ranged between 13 and 22 g FW m^{-2} day^{-1} under continuous high nutrient concentrations. As for nutrient uptake rates, highly variable growth rates are reported in the literature for *Ulva* species (Table 3), but growth rates and biomass yield measured in the current study were in line with maximum growth rates measured under natural conditions for an *Ulva* strain (SGR up to 6.2% day^{-1}, biomass yield between 20 and 30 g FW m^{-2} day^{-1}) collected from the exact location as the *Ulva* used in the current study (Jansen et al. 2022). Nevertheless, growth rates and biomass yield were low compared to other studies on *Ulva* spp. (Table 3; Revilla-Lovano et al. 2021). Given the high nutrient concentrations that prevailed in the current research prevented the *Ulva*'s growth limitation by the macronutrients), the role of trace elements and other limiting factors should be considered in future studies. Such studies are relevant for integrated RAS, where the main nutrient supply to the extractive species is not controlled but is determined by nutrients that the fed species do not retain.

Conclusion

Integrated aquaculture systems, where marine fish in RAS systems are combined with seaweed production are characterised by high nutrient concentrations, which is different to most studies that have previously examined nutrient uptake kinetics of *Ulva* species. Therefore, deliberately exposing *Ulva* spp. to high nutrient concentrations allowed us to answer the hypotheses that were put forward for the present study, as follows:

Hypothesis (I)—Ulva spp. performance is not influenced by stoichiometry in fish waste effluents under high nutrient concentrations: it was indeed confirmed that nutrient concentrations in the culture medium and tissue content were above the critical threshold for maximum growth, suggesting that other factors than the macronutrients limited seaweed growth. The unfavorable stoichiometry in fish wastes (N:P < < Atkinson ratio) typical for RAS systems does not limit seaweed growth.

Hypothesis (II)—Orthophosphate concentrations in RAS effluents are not toxic for Ulva spp.: The orthophosphate concentration of 0.9 mmol L^{-1} was probably toxic since it was associated with the degeneration of the seaweed. The exact details of phosphorus toxicity remain to be further elucidated, but our results suggest that the toxic concentration approaches 0.9 mmol L^{-1}. This hazard requires consideration in the design and operation of integrated RAS systems.

Table 3 A literature overview of *Ulva* performance (tissue content and growth) and nutrient removal rates. DM, dry matter; accl., acclimatization period; exp., experimental period; biomass & tissue content, nutrient removal rate determined based on biomass growth and initial and final tissue content; nutrient depletion, nutrient removal rate determined based on nutrient depletion in the media

Species	Tissue content (% DM)		Removal rate (µmol g^{-1} DM day^{-1})		Growth (% day^{-1})	Concentration culture medium (mmol L^{-1})		Experimental design		Ref
	N	P	DIN	DIP		DIN	DIP	Removal rate	Performance	
Ulva spp.	3.67–3.82	0.30–0.37	140–155	4–7	4.3–5.0	0.7–5 NO_3	0.01–0.1	Flow-through, 1 wk accl., 1 wk exp, biomass & tissue content	Flow-through, 1 wk accl., 1 wk exp,	This study
	5.18	0.27–0.29	191	3	3.6–4.0	0.5–0.6 TAN	0.01–0.1			
Ulva sp.	1.30–3.52				0.8–6.2				Flow-through, 5 months, frequently sampled	Jansen et al. 2022
Ulva fasciata	5.8	0.39	528	260	7.5	0.7 NO_3	0.07–0.08	Flow-through, 3 wk accl., 4 d exp., nutrient depletion	Flow-through, 3 wk accl., 4 d exp	Shahar et al. 2020
	7.1	0.17	2736	82	14.3	0.8 TAN	0.06–0.07			
Ulva lactuca		0.83	885	27	0.6–4.4	5 NO_3	0.001–0.05	Daily media replacement, 10 d exp., nutrient depletion	Daily media replacement, 10 d exp	Lubsch and Timmermans 2018
Ulva lactuca	4.41	0.26	126	10	1.5–2.8	2.9–4.3 NO_3	0.195–0.291	No media exchange, 6 d exp., nutrient depletion; biomass & tissue content	No media exchange, 6 d exp	Tremblay-Gratton et al. 2018
			49–67	2						
Ulva rigida Wild type (WT) Sterile mutant (MT)	4.43 (WT) 2.79 (SM)		613 (WT) 858 (SM)	22 (WT) 29 (SM)	3.76 (WT) 13.97 (SM)	0.5 NO_3	0.025	No media exchange, but daily replenishment of N and P based on uptake rates, 12 d exp., nutrient depletion	Daily media exchange, 18–27 d exp	Gao et al. 2017
Ulva lactuca				100			0.0005–0.01	No media exchange, 4 h exp., nutrient depletion	Flow-through, 2 yrs, frequently sampled	Pedersen et al. 2010
		0.14–0.39				0.001–0.032 NH_4NO_3	0.00006–0.002			
Ulva lactuca				52—112			0.02	No medium exchange, no accl., 18 min exp., nut. depl		Runcie et al. 2004
				600		0.5 NH_4NO_3	< 0.0005	Regular media exchange, 17–18 d accl., 12 min exp, nutrient depletion	No media exchange, 13–15 d exp	
				1588		0.5 NH_4NO_3	0.05			
		0.35–1.53				0.04 NH_4NO_3	0.001–0.03			

Table 3 (continued)

Species	Tissue content (% DM)		Removal rate (µmol g^{-1} DM day^{-1})		Growth (% day^{-1})	Concentration culture medium (mmol L^{-1})		Experimental design		Ref
	N	P	DIN	DIP		DIN	DIP	Removal rate	Performance	
Ulva rotundata			2136	69		0.03–0.06 TAN	Up to 0.005	No media exchange, 7 h exp., nutrient depletion		Martínez-Aragón et al. 2002; Hernández et al. 2002
	1.57–2.62	0.07–0.10	50–90	1.5–5		0.02–0.06 TAN	Up to 0.005	Flow-through, 6 d accl. (starved), 1 wk exp., nut. depl		
	1.35–2.32	0.05–0.09	50–150	0–1		0.02–0.06 TAN	Up to 0.005	Flow-through, 6 d accl. (non-starved), 1 wk exp., nut. depl		
Ulva lactuca			1728			0.0035–0.085 TAN		No media exchange, 4–6 h		Pedersen and Borum 1997
			480			0.0035–0.045 NO_3		exp., nutrient depletion		
Ulva lactuca					7–11	0.010–0.014 TAN			Flow-through, 3 wk exp	Neori et al. 1991
					8–17	0.027–0.048 TAN				
					15—18	0.071–0.078 TAN				
Ulva lactuca	1.15		3312		5.3	0.04 TAN		No medium exchange, 1-2 h exp., nutrient depletion	Medium exchange every other day, 19d exp	Fujita 1985
	3.59		3312		9.3	0.2 TAN				

Hypothesis (III)—High nitrate concentrations in RAS effluents limit the phosphorus uptake: The toxic TAN excreted by fish in RAS systems is bacterially transformed into nitrate, which accumulates in the recycled water. In contrast to suggestions in the literature, *Ulva* V_s and V_m phosphorus uptake were not reduced in the presence of high nitrate concentrations (up to 5 mmol L^{-1}). *Hypothesis (IV)—High ammonium concentrations improve the performance of Ulva spp. compared to comparably high nitrate concentrations: Ulva* growth rate was similar with high concentrations of both N-forms, but the nitrogen content in tissue increased significantly in the ammonium-based treatments. Thus, at the tested high concentrations, the advantage of ammonium nutrition was not in growth but much higher rates of uptake and the accumulation of tissue nitrogen.

Growth and nitrogen removal rates did not differ between high and moderate nitrate concentrations, nor between high versus low N:P ratios, suggesting that maximum growth and nitrogen removal rates were achieved under all these conditions. These results contribute to a better understanding of the application of *Ulva* spp. as an extractive component in closed IMTA systems, where a continuous state of moderate to high nutrient concentrations prevails.

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Data Availability The datasets belonging to the current study are available from the corresponding author upon reasonable request.

Declarations

Conflicts of interest/Competing interests The authors have no conflict of interest or competing interests to declare relevant to the content of this manuscript.

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