



Co-cultivation of mixotrophic non-green algae with the green alga *Haematococcus pluvialis* in aquaculture effluent

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Abstract

Microalgae cultivation in aquaculture effluents offers an opportunity for high-value biomass production coupled with bioremediation but can be suppressed by bacterial competition. We hypothesized that co-cultivation of *Haematococcus pluvialis*, known for the synthesis of the valuable carotenoid astaxanthin, with mixotrophic non-green algae could reduce bacterial pressure, leading to enhanced production of algal biomass and nutrient removal. The growth of algae in fourteen two-species co-cultures was screened on 24-well plates, and the best-performing combinations were tested in 650-mL culture bottles in a long-term experiment (28 days) for stability, biomass production, nutrient removal, and biomolecular composition. Contrary to our hypothesis, mixotrophs stimulated bacterial proliferation rather than suppressed it. Co-cultivation reached equal biomass production in comparison to the monoculture of *H. pluvialis* and frequently improved the nutritional quality of biomass in terms of higher contribution of proteins, total and essential fatty acids (EFAs). However, the highest content of proteins, fatty acids, and EFAs was detected in monocultures of mixotrophs. Particularly *Gymnodinium impatiens* was rich in EFAs and was successfully cultivated both in mono- and co-cultures for 28 days. Removal of phosphorus and nitrogen by co-cultures was equal to or lower than that by the monoculture of *H. pluvialis*. Overall, although non-green mixotrophic algae did not decrease bacterial abundance or increase biomass production or nutrient removal, they improved the quality of the produced algal biomass, producing lipid- and protein-rich biomass in aquaculture effluent.

Keywords Co-cultivation · Mixotrophy · Aquaculture effluent · *Haematococcus* · Non-green alga · Biomolecules

Introduction

Microalgae have demonstrated high growth rates in aquaculture effluents (Stevčić et al. 2019; Tejido-Núñez et al. 2019; Villar-Navarro et al. 2021; Jakhwal et al. 2024), making them a promising solution for mitigating environmental impacts associated with aquaculture, such as eutrophication caused by nutrient discharge (Verdegem 2013; Yuan et al. 2019; MacLeod et al. 2020). Simultaneously algal cultivation produces valuable biomass which can be further processed for use in biofuels, dietary supplements, and pharmaceutical products (Caporgno and Mathys 2018; Khavari et al. 2021; Wicker et al. 2024). However, bacteria compete with algae for available nutrients, reducing both the quantity

and quality of the produced biomass (Halfhide et al. 2014; Stemmler et al. 2016). Commonly proposed solutions to mitigate bacterial pressure in aquaculture include chemical disinfection, filtration, antibiotic treatment, and irradiation of the water; however, all of these approaches increase operational costs due to the need for additional water treatment (Moreno-Andrés et al. 2020; Calderini et al. 2021).

Because biomass production correlates positively with nutrient removal (Jakhwal et al. 2024), increased biomass production via decreased bacterial pressure could lead to improved nutrient removal from aquaculture effluents. Noteworthy, it has been shown that algal monocultures are often unable to remove all nutrients from the aquaculture effluents: a comparative study of six green algal species cultivated as monocultures showed that 4–43% of the nitrate-nitrogen (NO₃-N) in RAS wastewater remained in the water after the cultivation period, whereas phosphate-phosphorus (PO₄-P) was almost entirely removed (Stevčić et al. 2019). Similar results have also been reported elsewhere, suggesting that phosphorus availability may limit algal growth and,

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subsequently, nitrate uptake (Tejido-Núñez et al. 2019; Jakhwal et al. 2024; Timilsina et al. 2025). Since algal groups differ in their nutritional strategies (Crane and Grover 2010), co-cultivation of algal species could enable more efficient nutrient removal under conditions where one resource becomes limiting. Indeed, previous studies have shown successful co-cultivation of different green algal species with an equal or improved nutrient removal in wastewater-simulating media (Mandal et al. 2018), recirculating aquaculture system (RAS) effluent (Tejido-Núñez et al. 2020), and dairy wastewater (Gupta and Marchetti 2024). While most research has focused on combinations of green algae (Mandal et al. 2018; Tejido-Núñez et al. 2020; Gupta and Marchetti 2024) and co-cultures of heterotrophic microbes and algae (Han et al. 2016; Berthold et al. 2019; Esposito et al. 2025; Khalatbari et al. 2025), non-green mixotrophic algae have gained less attention in co-cultivation with green algae. In addition to distinct nutritional strategies, co-cultivation of a green algae with a non-green mixotrophic algae could have an additional benefit via reduced bacterial competition. Altogether, in comparison to one-species monocultures, nutrient uptake by the microalgal co-culture could result in increased production of valuable biomass and nutrient removal. Moreover, co-cultivation can have additional benefits by influencing the biochemical composition of the resulting biomass (Olofsson et al. 2019). Biomass produced through co-cultivation could exhibit a more diverse nutritional profile, making it a promising raw material for feed applications.

In our previous studies on microalgal cultivation in aquaculture effluents, we have detected bacteria to be particularly abundant in *Haematococcus pluvialis* cultures in comparison to other green algae, *Selenastrum* sp. and *Monoraphidium griffithii* (Calderini et al. 2021). *Haematococcus pluvialis*, which is known for its ability to produce super-antioxidant, the carotenoid astaxanthin (Shah et al. 2016; Mota et al. 2022), suffers from bacterial abundance particularly at the post-growth stage via microbial degradation of algal cells, which may reduce astaxanthin yields (Timilsina et al. 2025). Co-cultivation of *H. pluvialis* with mixotrophic non-green algae, which, in addition to performing photosynthesis, are capable of consuming bacteria as a nutrient source (Gerea et al. 2016), could alleviate competitive pressure from bacterial communities and promote more stable and productive algal cultures (Thingstad et al. 1996; Crane and Grover 2010; Bhatnagar et al. 2011).

We hypothesized that co-cultivation of *H. pluvialis* and mixotrophic algae would improve total algal biomass production in RAS effluent by reducing bacterial growth and its inhibitory effects on algal growth. Moreover, we hypothesized that co-cultivation would increase nutrient uptake from RAS effluent via increased biomass production. We also hypothesized that the biochemical composition of co-cultures would differ from monocultures. To test

our hypotheses, we first screened the suitability of 14 non-green algal strains for co-cultivation with the green alga *H. pluvialis* in effluent from a land-based RAS, in which high concentrations of nitrogen and phosphorus (up to 200 and 45 mg L⁻¹, respectively) accumulate in the effluent (Martins et al. 2010; van Bussel et al. 2013; Yogev and Gross 2019; Böpple et al. 2025). The aim was to identify the most promising algal strains for increased biomass production. We then selected four strains for the larger-scale experiment, where we quantified nitrogen and phosphorus uptake, algal growth, bacterial abundance, and the biochemical composition of the produced biomass.

Materials and methods

Algal cultures

Haematococcus pluvialis and 14 non-green microalgal strains were precultivated in modified Wright's Cryptophyte (MWC) medium (Guillard and Lorenzen 1972) at 22 °C under continuous (24 h per day) light with the intensity of 30–40 µmol photons m⁻² s⁻¹ using two fluorescent tubes (Philips TL-D 36W/830, the Netherlands). Non-green algal species included two strains of chrysophytes, four strains of dinoflagellates, and eight strains of cryptophytes (Table 1). The cell diameter (> 20 counts) of each strain was measured under a microscope with ZEN blue imaging software (ZEISS). Cell diameter was converted to biovolume by assuming spherical geometry for round cells and spheroidal geometry for elongated cells, with both major and minor diameters measured.

Effluent sampling and characteristics

RAS effluent for experiments was collected after the drum filtration from the fish farm of Natural Resources Institute Finland (LUKE) in Laukaa, Finland. A part of the collected RAS effluent was filtered through a 3 µm pore size cellulose nitrate filter (Whatman GF/C, Cytiva, UK) to remove protozoa but to allow bacteria to pass through. The filtered effluent was later used for experiment I (setups are described below in "Experimental setup"). The effluent for experiment II was filtered using a tangential filtration device (Millipore, Durapore cassette, pore size 0.22 µm), which is more suitable for high water volumes. The pH was measured with a pH-meter (SevenEasy, Mettler Toledo, Switzerland). The concentration of dissolved organic carbon (DIC) was measured from a 5 mL subsample of the filtered effluent, which was then acidified with 200 µL of 85% H₃PO₄ to release DIC from water into gaseous CO₂, after which the partial pressure of CO₂ was quantified by an Agilent 7890B gas chromatograph (Agilent Technologies, USA) (Taipale and

Table 1 Groups, species, strains, and cell biovolumes of microalgae used in the study

Group	Species	Strain	Cell biovolume (μm^3)
Chrysophyta	<i>Uroglena sp.</i>	CPCC 276	318
	<i>Synura petersenii</i>	NORCCA K-1875	420
Dinoflagellata	<i>Gymnodinium impatiens</i>	CCAC 0025	6120
	<i>Peridinium cinctum</i>	CCAC 0102 B	23,720
	<i>Peridinium volzii</i>	SCCAP K-1611	30,525
	<i>Woloszynskia sp.</i>	CCAC 6779 B	15,667
Cryptophyta	<i>Chroomonas sp.</i>	CCAC 0060	150
	<i>Cryptomonas ozolinii</i>	unknown	259
	<i>Cryptomonas ovata</i>	CCAC 0064	5129
	<i>Cryptomonas pyrenoidifera</i>	unknown	1628
	<i>Cryptomonas sp.</i>	CPCC 336	159
	<i>Cryptomonas sp.</i>	NORCCA K-1876	501
	<i>Rhodomonas lacustris</i>	NIVA 8/82	174
	<i>Rhodomonas sp.</i>	CCAC 0194 B	506
Chlorophyta	<i>Haematococcus pluvialis</i>	NORCCA K-0084	4633 (Exp I); 3089 (Exp II)

Sonninen 2009; Vesamäki et al. 2024). Dissolved organic carbon (DOC) and total nitrogen (TN) were measured from the filtered (Sartorius 0.45 μm pore size) and acidified (80 μL of 2 M HCl to 20 mL of sample) effluent by the Shimadzu TOC-V cph total organic carbon analyzer (Sharp et al. 2004; Wang et al. 2012). The concentrations of $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, and $\text{PO}_4\text{-P}$ were measured using LCK339, LCK341, and LCK349 kits (Hach Lange, Germany), respectively, and a spectrometer (Dr. Lange LASA 100, Hach Lange), which are often used in nutrient bioremediation and algal cultivation studies (e.g., Stevčić et al. 2019; Tejido-Núñez et al. 2020; Jakhwal et al. 2024). The molar ratio of nitrogen and phosphorus (N:P) was defined as the ratio of TN and $\text{PO}_4\text{-P}$. Removal efficiencies of TN and $\text{PO}_4\text{-P}$ were quantified as a percentage decrease of nutrient concentration relative to the start at the end of experiment II. The bacterial density in the effluent was quantified by the plate-count method. Sample water was diluted with MWC medium, after which 500 μL of the diluted sample was inoculated on the R2A agar plate (Reasoner and Geldreich 1985; Massa et al. 1998). Plates were incubated at 30 °C, and the colony-forming units (CFUs) were counted every 24 h for 5 days, enabling the detection of slowly growing bacteria. Effluent characteristics are summarized in Table 2.

Experimental setup

To identify which mixotrophic algae are most appropriate for co-cultivation with *H. pluvialis* to reduce bacterial competition, enhance algal growth, and nutrient removal, two experiments were conducted, referred to as experiment I and experiment II. Experiment I was conducted to screen the best combinations for maximum biomass production. It consisted of *H. pluvialis* and 14 strains of non-green mixotrophic

Table 2 Characteristics (mean $\pm$ standard deviation) of RAS effluent used in experiment I (filtered through 3 μm) and II (filtered through 0.22 μm ; detailed setup descriptions in “2.3. Experimental setup”)

	Experiment I	Experiment II
pH	6.84	7.17 $\pm$ 0.03
DIC (mg L ⁻¹)	2.24 $\pm$ 0.06	9.80 $\pm$ 0.41
DOC (mg L ⁻¹)	12.63 $\pm$ 0.06	9.51 $\pm$ 0.46
TN (mg L ⁻¹)	48.32 $\pm$ 0.27	13.46 $\pm$ 0.14
$\text{NO}_3\text{-N}$ (mg L ⁻¹)	47.70 $\pm$ 0.42	12.54 $\pm$ 4.19
$\text{NO}_2\text{-N}$ (mg L ⁻¹)	0.39 $\pm$ 0.02	0.26 $\pm$ 0.00
$\text{PO}_4\text{-P}$ (mg L ⁻¹)	1.63 $\pm$ 0.01	0.69 $\pm$ 0.01
TP (mg L ⁻¹)	2.07 $\pm$ 0.04	n.a
N:P	65.62 $\pm$ 0.61	43.22 $\pm$ 0.99
Number of CFUs mL ⁻¹	2.2 $\times$ 10 ⁶ $\pm$ 1.3 $\times$ 10 ⁵	2.4 $\times$ 10 ⁵ $\pm$ 3.1 $\times$ 10 ³

DIC dissolved inorganic carbon; DOC dissolved organic carbon; TN total nitrogen; TP total phosphorus; CFU colony-forming units. The molar ratio of nitrogen and phosphorus (N:P) has been calculated based on TN: $\text{PO}_4\text{-P}$. n.a. = not assessed

algae that were grown pairwise as two-species co-cultures (ratio 1:1 by biovolume) and separately as monocultures on fifteen 24-well plates (Fig. S1). Each plate contained two two-species co-cultures, two monocultures (five wells each), and four wells filled with RAS effluent without any algal inoculum as controls to monitor contamination. Altogether, each treatment had ten replicates. To assess the biomass development during the cultivation period, biovolume was used as a proxy for biomass. The inoculated biovolume of each alga in monocultures and co-cultures was 15.43 mm³ L⁻¹ in experiment I. Wells were filled up to 2.5 mL with RAS effluent. Cultures were incubated at 23.0 $\pm$ 0.5 °C until algal population growth reached a stationary phase (16 days) under identical light conditions to precultivation.

Selection of strains for the upscaled experiment was based on two criteria: 1) the total biovolume was statistically higher than or equal to the monoculture of *H. pluvialis*, and 2) the biomass composed of two algal strains; thus, strains that were outcompeted were not selected for further testing. Based on these criteria, four strains with the highest average biovolumes and a sustainable ratio of the two algae were selected for nutrient uptake efficiency and biomass production in a larger-scale experiment II, addressing bacterial mitigation, nutrient removal, and biomass quantity and quality in co-cultures versus monocultures. Selected strains were *Cryptomonas pyrenoidifera*, *Gymnodinium impatiens*, *Peridinium cinctum*, and *P. volzii*. Experiment II was conducted in 650-mL polypropylene bottles with a total effluent volume of 400 mL ($n=4$; Fig. S2). Total initial biovolume was $60.72 \text{ mm}^3 \text{ L}^{-1}$ to ensure sufficient initial biomass. Selected strains were grown as two-species co-cultures with *H. pluvialis* and as monocultures. Controls had no algal inoculum. Cultures were continuously aerated with ambient room air via air pumps (EHEIM Air400, Germany) and maintained at $23.0 \pm 0.5 \text{ }^\circ\text{C}$ for 28 days under light conditions identical to precultivation.

Biomass quantitation, biovolume monitoring, and algal ratios

In experiment I, algal growth was monitored daily by Spark multimode microplate reader (Tecan, Switzerland) targeting autofluorescence of chlorophyll *a* at the excitation wavelength of 430 nm and emission 680 nm. Additionally, at the end of experiment I, biovolume and the ratios of the two algal strains in co-cultures were determined using a Bürker cell counting chamber (Paul Marienfeld GmbH & Co.KG, Germany). Cell counts were converted to biovolume by using a predetermined single-cell biovolume as a conversion factor.

In experiment II, cell counting was conducted every second day to monitor biomass development and the ratios of algal strains. Algal dry weight (DW) per volume unit was measured by filtering 20–50 mL of sample through pre-washed (filtered with 100 mL of distilled water), precombusted (overnight $450 \text{ }^\circ\text{C}$) and preweighed filters (Whatman, GF/F, Cytiva, U K) at the end of the experiment. Filters were dried by keeping them at $60 \text{ }^\circ\text{C}$ overnight, and reweighed. The total dry biomass was calculated by subtracting the difference between the reweighed and preweighed filter papers.

Fatty acid analysis

For cultures with low biomass (i.e., control and monocultures of *Peridinium* sp.), samples for fatty acid analysis were collected by filtering 100–250 mL of culture through preweighed GF/C-filters (pore size $3 \text{ }\mu\text{m}$). For cultures

with high biomass, 200 mL of the culture was centrifuged at 1500 rpm for 15 min. Filters and pellets were frozen at $-80 \text{ }^\circ\text{C}$ and lyophilized overnight, after which lipids were extracted by applying the Folch's method (Folch et al. 1957). Filters laden with biomass or 3–5 mg of algal biomass were weighed and placed in 3 mL of chloroform–methanol (2:1 by volume), after which 0.75 mL distilled water and 16 μg internal standard (nonadecanoic acid; Larodan, Sweden) were added, and algal cells were sonicated for 10 min. The phases were separated by centrifugation (3000 rpm, 4 min), and the lower phase was transferred to a new tube. To maximize the lipid recovery, the phase separation was done twice by adding 2 mL chloroform to the samples after the first extraction and repeating the sonication and centrifugation. The extracted lipid pool was evaporated under N_2 stream and methylated by the acid-catalyzed transmethylation, which was conducted by adding 1 mL of n-hexane and 2 mL of 1% H_2SO_4 in methanol and incubating samples on heat blocks at $90 \text{ }^\circ\text{C}$ for 90 min. The produced fatty acid methyl esters (FAMES) were then separated by adding 2 mL of 2% KHCO_3 and n-hexane to the samples and centrifuging (2000 rpm, 2 min). Upper phases were then transferred to new tubes. To maximize the FAME recovery, 2 mL of n-hexane was added to the original sample, centrifuged, and combined with the first FAME extract. The solvent was then evaporated, FAMES redissolved with n-hexane, and transferred into GC-V-vials. The final run volume of samples was 1 mL for all samples and blanks (included to check there is no chemical contamination), except for the control treatment, whose running volume was set to 100 μL .

FAMES were analyzed with a gas chromatograph (Shimadzu Ultra, Japan) equipped with a mass detector (GC–MS) using an Agilent DB-Fast FAME column (length 30 m, diameter 0.25 mm, film thickness 0.25 μm). The splitless mode with a temperature of $260 \text{ }^\circ\text{C}$ was used for the injection. The interface and ion source temperatures were 250 and $220 \text{ }^\circ\text{C}$, respectively. Helium was used as the carrier gas (linear velocity = 36.3 cm s^{-1}). The following temperature program was used: $60 \text{ }^\circ\text{C}$ was held for 1 min, then the temperature was increased to $165 \text{ }^\circ\text{C}$ at a $40 \text{ }^\circ\text{C min}^{-1}$ rate, followed by a $4 \text{ }^\circ\text{C min}^{-1}$ rate increase to $230 \text{ }^\circ\text{C}$, where it was held for 4.5 min. The total run time of one sample was 25.38 min.

The FAMES were identified by using specific target ions and their retention times. Individual FAME was quantified with GC–MS solution software (v4.42, Shimadzu, Japan) by creating a four-point calibration curve (10, 50, 100, and 250 $\text{ng } \mu\text{L}^{-1}$) based on a known standard solution of a FAME standard mixture (GLC standard mixture 566c, Nu-Chek Prep, USA). Recovery of FAMES was corrected based on the internal standard, and the quantity of fatty acids in a sample was calculated as $\mu\text{g mg}^{-1}$.

FTIR

From high biomass cultures (all except control and monocultures of *Peridinium* sp.), 200 mL was collected and centrifuged at 1500 rpm for 15 min. The resulting pellet was frozen at -80°C and lyophilized overnight. Dried biomass was placed on the diamond crystal of the FTIR equipment (Thermo Scientific Nicolet iS50 FT-IR Spectrometer), and spectra were recorded in ATR mode in the range of $2000\text{--}600\text{ cm}^{-1}$ with 32 scans and a spectral resolution of 4 cm^{-1} . Spectra were corrected for background and baseline using OMNIC software (Fig. S3). Spectra were normalized by the minimum and maximum signals as follows:

$$Abs_{normalized} = (Abs - Min)/(Max - Min),$$

where $Abs_{normalized}$ is the normalized absorbance, Abs is the detected absorbance on a given wavenumber, Min is the lowest absorbance detected in the sample, and Max is the highest absorbance detected in the sample. To examine biochemical composition of algal biomass, the highest wavenumber at the range $1750\text{--}1730\text{ cm}^{-1}$ was used to assess lipid content, and $1160\text{--}1140\text{ cm}^{-1}$ was used for carbohydrates (Wagner et al. 2010). Protein content was assessed as an average of amide 1 and amide 2 signals at the ranges of $1680\text{--}1640\text{ cm}^{-1}$ and $1550\text{--}1540\text{ cm}^{-1}$, respectively (Giordano et al. 2001; Stehfest et al. 2005; Wagner et al. 2010). The proportion of each biomolecule class was then calculated as:

$$Biomolecule - \% = Abs_{Biomolecule}/Abs_{Sum} * 100,$$

where $Biomolecule - \%$ is the percentual proportion of lipids, proteins, or carbohydrates from biomass, $Abs_{Biomolecule}$ is the highest absorbance value detected in the defined biomolecule-specific wavenumber range, and Abs_{Sum} is the sum of three normalized absorbance values of lipids, proteins, and carbohydrates.

Bacterial growth

The bacterial density in mono- and co-cultures was quantified by the plate-count method at the end of the experiments and performed as described previously in “Effluent sampling and characteristics”.

The effect of carbon addition on bacterial growth in RAS effluent was tested by conducting an in vitro experiment with three different carbon sources: D-glucose, cellulose, or dead *H. pluvialis* cells. *Haematococcus pluvialis* cells were killed by heating at 40°C for 24 h (Nakajima et al. 2020), after which the biomass was freeze-dried overnight. Stock solutions of 100 mg C L^{-1} were prepared for all three substrates by adding each substrate into 100 mL of RAS effluent that was filtered through $0.45\text{ }\mu\text{m}$ pore size filters (Sartorius) to

remove bacteriovores and other than carbon except DOC. Then we created a gradient of 0 mg L^{-1} , 20 mg L^{-1} , and 75 mg L^{-1} added carbon (additional to the natural carbon content of RAS effluent, i.e., 12 mg L^{-1}). Then, 2.4 mL of RAS effluent with added carbon was pipetted into a 24-well plate and incubated at $23.0 \pm 0.5^{\circ}\text{C}$ in darkness. Four replicates were made for each carbon concentration. Bacterial growth was measured by the plate-count method after 7 days of incubation.

Statistical testing

Pairwise permutational multivariate analyses of variance (PERMANOVA) were performed on fatty acid profiles and biomolecular composition of algal dry mass (Bray–Curtis similarity matrix, number of permutations = 999, R package vegan). Additionally, SIMPER analysis was conducted for the biomolecular composition dataset to evaluate the contribution of each measured factor to differences between treatments.

Since the normal distribution was not often met, pairwise permutation-based Wilcoxon tests (one-way, 9999 resamples; implemented in the R package coin) were conducted for univariate datasets (biovolume, nutrient removal, algal biomass, protein contribution to drymass, total FA content, essential fatty acid (EFA) content, and number of CFUs) to assess differences between treatments (Holt and Sullivan 2023). Permutation tests have an additional benefit in comparison to Mann–Whitney’s U-test since p-values include the effect size estimation and thus, the strength of the p-value reflects the magnitude of the difference between treatments. A simple linear regression analysis with F-test was performed to examine the relationship between DOC and the number of CFUs in monocultures at the end of experiment II. The normality of residuals was tested by the Shapiro–Wilk test and the independence of observations by the Durbin–Watson test. All statistical tests were performed using RStudio v2023.12.1. Statistical difference was considered significant if $p < 0.05$.

Results

Strain screening

In experiment I, algae were cultivated until they reached the stationary phase (Fig. S4). Most of the tested co-cultures had a slightly but not statistically significantly lower total biovolume than the monoculture of *H. pluvialis* at the end of the experiment ($p > 0.05$; Table S1). *Cryptomonas ozolinii* ($p = 0.030$), *Chroomonas* sp. ($p = 0.007$), *Rhodomonas* sp. ($p = 0.002$), *R. lacustris* ($p = 0.021$), and *C. ovata* ($p = 0.013$) showed significantly lower biovolumes in comparison to the

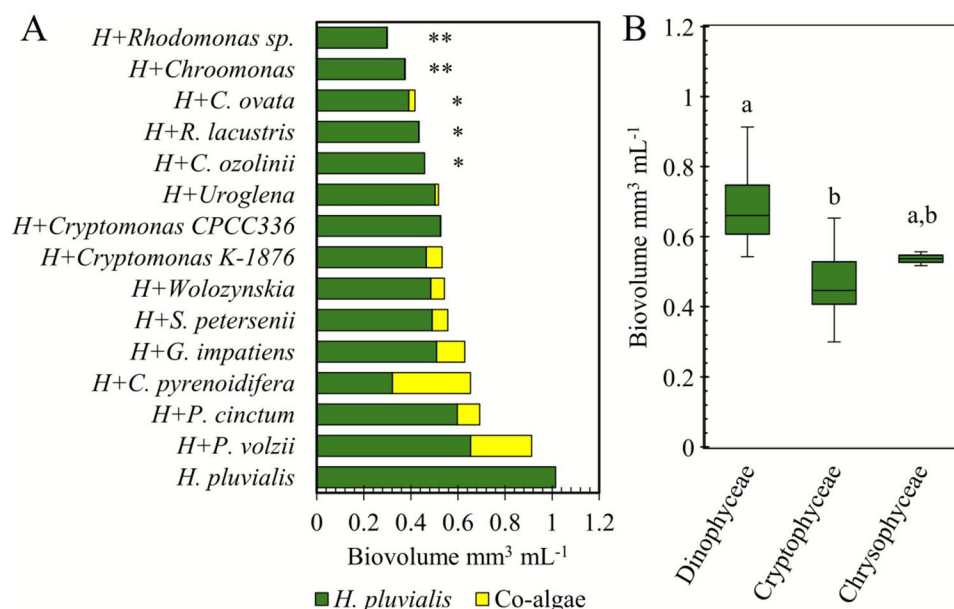


Fig. 1 Screening of the best combination of *Haematococcus pluvialis* (H) and non-green algae (experiment I). **A**) Total biovolumes and ratios of the two algae in co-cultures after 16 days (n = 10). Asterisks indicate the statistical difference in comparison to the monoculture of *H. pluvialis*: * $p < 0.05$, ** $p < 0.01$. **B**) Biovolumes of non-green algal strains (Dinophyceae n = 4, Cryptophyceae n = 8, and Chrysophyceae n = 2) belonging to different families in co-cultures. These bio-

volumes were calculated from the mean biovolume non-green algal partner in each co-culture (n = 10). The highest final biomass was observed when *H. pluvialis* was co-cultured with algae belonging to the order Dinophyceae. Letters indicate the statistical difference: if the groups share the same letter, there is no statistical difference

monoculture of *H. pluvialis* (Fig. 1A). Overall, dinoflagellates were the most promising group for co-cultivation since they were associated with the highest total biomass in co-cultures, with a significant difference in comparison to cryptophytes (Fig. 1B; $p = 0.012$). Based on the biomass production and coexistence with *H. pluvialis* in the co-cultures, *C. pyrenoidifera*, *G. impatiens*, *P. cinctum*, and *P. volzii* were selected for experiment II.

Biomass quantity and quality

In experiment II, co-cultivation of the green alga *H. pluvialis* and any non-green algae yielded equal biomass in comparison to the monoculture of *H. pluvialis* ($p > 0.05$, Fig. 2A). However, *C. pyrenoidifera* and both strains of *Peridinium* sp. grew poorly and/or decreased over time in co-cultures, indicating that they were outcompeted by *H. pluvialis*. In contrast, *G. impatiens* was not outcompeted by *H. pluvialis* during the four weeks of incubation, indicating their capacity to coexist in the same culture (Fig. S5). Among monocultures, *H. pluvialis* had significantly higher total biomass than any other algae (*C. pyrenoidifera*; $p = 0.027$, *G. impatiens*; $p = 0.027$, *P. cinctum*; $p = 0.031$, *P. volzii*; $p = 0.030$; Table S2).

The relative biochemical composition (protein, lipid, and carbohydrate normalized to 100%) differed between all combinations except between the monoculture of *H.*

pluvialis and the co-culture of *P. cinctum* and *H. pluvialis* (PERMANOVA, Table S3). Overall, the composition of biomass was dominated by proteins (58–80%), followed by carbohydrates (8–27%) and lipids (8–22%; Fig. 2B). SIMPER analysis showed that differences between *H. pluvialis* monoculture and each co-culture were explained by distinct contributions of lipids (*G. impatiens* + *H. pluvialis*), proteins (*C. pyrenoidifera* + *H. pluvialis* and *P. cinctum* + *H. pluvialis*), and carbohydrates (*P. volzii* + *H. pluvialis*; Table S4).

The highest contribution of proteins was measured for *C. pyrenoidifera* ($80.53 \pm 2.62\%$), which was significantly greater than in *H. pluvialis* ($62.50 \pm 1.65\%$; $p = 0.030$) and their co-culture ($70.13 \pm 1.42\%$; $p = 0.029$). Furthermore, the combination of *C. pyrenoidifera* and *H. pluvialis* exhibited the highest protein content among all co-cultures tested. In contrast, no significant differences were observed in protein contribution between *G. impatiens*, *H. pluvialis*, or their co-cultures (Table S5).

Haematococcus pluvialis had the highest carbohydrate content ($27.00 \pm 1.60\%$) among the monocultures, resulting in an elevated contribution of carbohydrates to biomass also in co-cultures with *P. volzii* ($31.24 \pm 1.53\%$) and *C. pyrenoidifera* ($19.71 \pm 3.23\%$; Fig. 2B). *Gymnodinium impatiens* had the lowest measured carbohydrate fraction ($19.05 \pm 0.56\%$). In the co-culture of *G. impatiens* and *H. pluvialis*, the contribution of carbohydrate reached $25.15 \pm 1.16\%$.

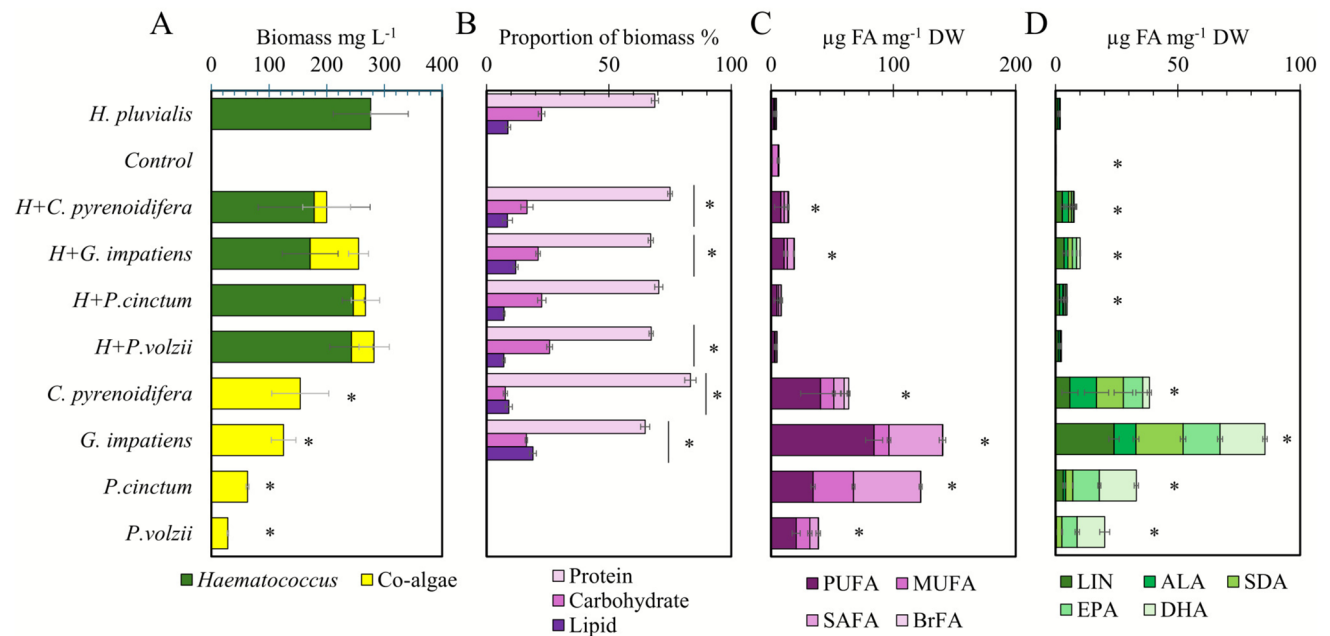


Fig. 2 Quantity and quality of algal biomass in experiment II (n=4). **A**) biomass, **B**) proximate composition of biomass, **C**) proportion of polyunsaturated (PUFA), monounsaturated (MUFA), saturated (SAFA) and branched-chain fatty acids (BrFA) to fatty acid profiles of the cultures, and **D**) quantity of essential fatty acids (LIN=linoleic acid, ALA=α-linoleic acid, SDA=stearidonic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid) per mg of dry weight

(DW) in the co-cultures and single species cultures after 28 days of cultivation. Asterisks indicate statistical difference: $*p < 0.05$. Lines in Fig. 2B indicate that the whole profiles were compared between *H. pluvialis* monoculture and other mono- and co-cultures. For clarity, only statistical differences in comparison to the monoculture of *H. pluvialis* have been shown. Other comparisons are presented in the supplementary materials (Tables S2 and S3)

The total FA content was significantly higher in all monocultures of non-green algae compared to the *H. pluvialis* monoculture (Fig. 2C, Table S2). Moreover, co-cultures of *H. pluvialis* with *G. impatiens* and *C. pyrenoidifera* exhibited higher total FA levels than the *H. pluvialis* monoculture. The total amount of EFAs was highest in the monoculture of *G. impatiens*. The monoculture of *G. impatiens* had 85-fold higher EFA content than the monoculture of *H. pluvialis* ($p = 0.029$). In comparison to *H. pluvialis*, *C. pyrenoidifera* had 21-fold ($p = 0.027$), *P. cinctum* 18-fold ($p = 0.031$), and *P. volzii* 11-fold ($p = 0.031$) higher EFA content. Overall, the co-cultivation of non-green algae with *H. pluvialis* increased the amount of EFAs in all co-cultures in comparison to the monoculture of *H. pluvialis* (Fig. 2D, Table S2).

In addition to differences in total quantity, PERMANOVA analysis revealed distinct FA profile compositions among all monocultures and between the *H. pluvialis* monoculture and its co-cultures with *G. impatiens* and *P. volzii* ($p = 0.036$ and $p = 0.022$, respectively; Table S6). FA profile in the co-culture of *H. pluvialis* and *C. pyrenoidifera* differed significantly from both monocultures of *H. pluvialis* and *C. pyrenoidifera*. Similarly, the FA profiles of the *H. pluvialis* and *G. impatiens* co-culture differed from *H. pluvialis* and *G. impatiens* monocultures, and the co-culture of *P. volzii* differed from monocultures of *H. pluvialis* and *P. volzii*. In

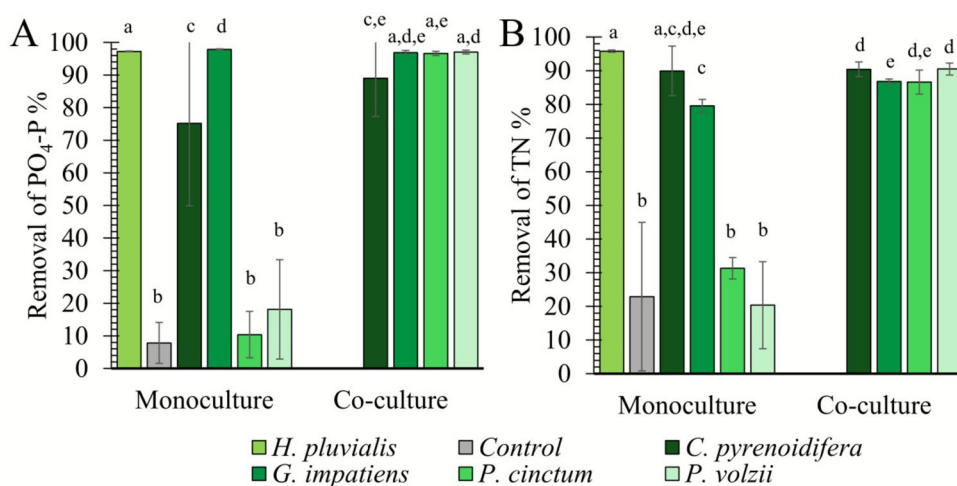
contrast, the co-culture of *P. cinctum* and *H. pluvialis* did not differ from the monoculture of *H. pluvialis*, confirming growth monitoring results, which indicated the extinction of *P. cinctum* in co-cultures.

FA profiles were dominated by polyunsaturated fatty acids (PUFAs) in all treatments, except for the control and the monoculture of *P. cinctum* (Fig. 2C), but SIMPER analysis showed that differences were explained mainly by distinct contributions of MUFAs in most of the comparisons (Table S7). Exceptions included *P. cinctum* monoculture versus co-culture, *C. pyrenoidifera* monoculture versus co-culture, *H. pluvialis* monoculture versus the monoculture and co-culture of *C. pyrenoidifera*, and *H. pluvialis* monoculture versus the co-culture with *P. cinctum*, which all differed mainly by the PUFA content (Table S8).

Nutrient removal

Only the co-culture of *H. pluvialis* and *C. pyrenoidifera* had significantly lower $\text{PO}_4\text{-P}$ removal than the monoculture of *H. pluvialis* ($p = 0.028$; Fig. 3A), whereas other co-cultures did not differ from each other ($p > 0.05$; Table S9). Comparison between monocultures showed that *H. pluvialis* had higher $\text{PO}_4\text{-P}$ removal than other algae, with the removal of $97.9 \pm 0.2\%$, followed by *G.*

Fig. 3 Removal of **A)** $\text{PO}_4\text{-P}$ and **B)** total nitrogen (TN) from aquaculture effluent in monocultures, co-cultures with *H. pluvialis*, and the control (no algal inoculum) in 28 days ($n=4$). Letters indicate the statistical difference: if the groups share the same letter, there is no statistical difference



impatiens with $\text{PO}_4\text{-P}$ removal of $95.8 \pm 0.3\%$ ($p=0.031$), *C. pyrenoidifera* ($75.2 \pm 25.3\%$, $p=0.030$), *P. volzii* ($18.1 \pm 15.2\%$, $p=0.031$), and *P. cinctum* ($10.4 \pm 7.1\%$, $p=0.029$). *Gymnodinium impatiens* removed $\text{PO}_4\text{-P}$ more than *C. pyrenoidifera* ($p=0.031$), *P. volzii* ($p=0.029$), and *P. cinctum* ($p=0.030$). Notably, the removal of $\text{PO}_4\text{-P}$ by *P. cinctum* and *P. volzii* did not differ significantly from the removal in the control ($7.8 \pm 6.3\%$).

The monoculture of *H. pluvialis* removed significantly more nitrogen than any of the tested co-cultures (Fig. 3B; Table S9). The removal of TN by *H. pluvialis* reached $95.8 \pm 0.3\%$ and was followed by the co-cultures of *H. pluvialis* with *P. volzii* ($90.5 \pm 1.8\%$, $p=0.029$), *C. pyrenoidifera* ($90.4 \pm 2.2\%$), *G. impatiens* ($86.8 \pm 0.7\%$), and *P. cinctum* ($86.7 \pm 3.6\%$). Among monocultures, *H. pluvialis* had equal removal of TN than *C. pyrenoidifera* ($89.9 \pm 7.3\%$) but removed significantly more TN than *G. impatiens* ($79.6 \pm 1.9\%$), *P. cinctum* ($31.3 \pm 3.2\%$), or *P. volzii* ($20.3 \pm 12.9\%$). The removal of TN by *P. cinctum* and *P. volzii* did not differ from the control.

Effects of mixotrophic algae and carbon on bacterial growth

The presence of mixotrophic algae in co-cultures did not reduce the bacterial load. In experiment I, the number of CFUs in the monoculture of *H. pluvialis* was equal ($p>0.05$) to the control without algal inocula and co-cultures with *Chroomonas* sp. *C. ovata*, *P. volzii*, and both strains of *Rhodomonas* sp., whereas other co-cultures had significantly higher numbers of CFUs than the monoculture of *H. pluvialis* (Fig. 4A, Table S10). Despite the filtration of effluent through $0.22 \mu\text{m}$, which effectively removes bacteria, the same trend was observed in experiment II: in comparison to the monoculture of *H. pluvialis*, the number of CFUs was equal ($p>0.05$) in all co-cultures

except the co-culture of *H. pluvialis* and *P. volzii*, where significantly more CFUs were observed ($p=0.030$; Fig. 4B; Table S11) and no decrease in bacterial load was detected. However, the deviation in the number of CFUs was high in co-cultures, particularly in the one with *C. pyrenoidifera*, reflecting differences in algal succession between bottles.

Algal presence did not systematically lead to a higher number of CFUs: the control group without any algal addition had a similar number of CFUs to monocultures of *H. pluvialis*, *P. cinctum*, and *P. volzii*. In contrast, monocultures of *C. pyrenoidifera* and *G. impatiens* showed a higher number of CFUs in comparison to the control ($p=0.029$ and $p=0.026$, respectively). However, a statistically significant ($F=31.16$, $\text{df}=1$ and 22 , $p<0.001$, $R^2=0.59$) linear regression within monocultures at the end of experiment II suggested a positive relationship between DOC concentration and the number of CFUs (Fig. 4C). Therefore, we conducted an additional experiment to test how different carbon sources affect bacterial density.

The stock solutions of glucose dissolved completely, whereas stock solutions of cellulose and algal necromass contained 89.1% and 83.6% particulate organic carbon, respectively (Fig. 4D). After the 7-day incubation period, the number of CFUs indicated that glucose did not significantly increase bacterial growth with 20 mg L^{-1} nor 75 mg L^{-1} of added carbon (Fig. 4E) even though the number of CFUs doubled in 75 compared to 20 mg L^{-1} of added carbon. Furthermore, the addition of 20 mg L^{-1} of algal necromass-derived carbon increased bacterial growth ($p=0.029$), whereas cellulose did not affect the number of CFUs at the lower concentration of added carbon. In treatments with 75 mg L^{-1} of added cellulose and algal necromass carbon, bacterial abundance was significantly higher in comparison to the control group ($p=0.028$ for both; Table S12).

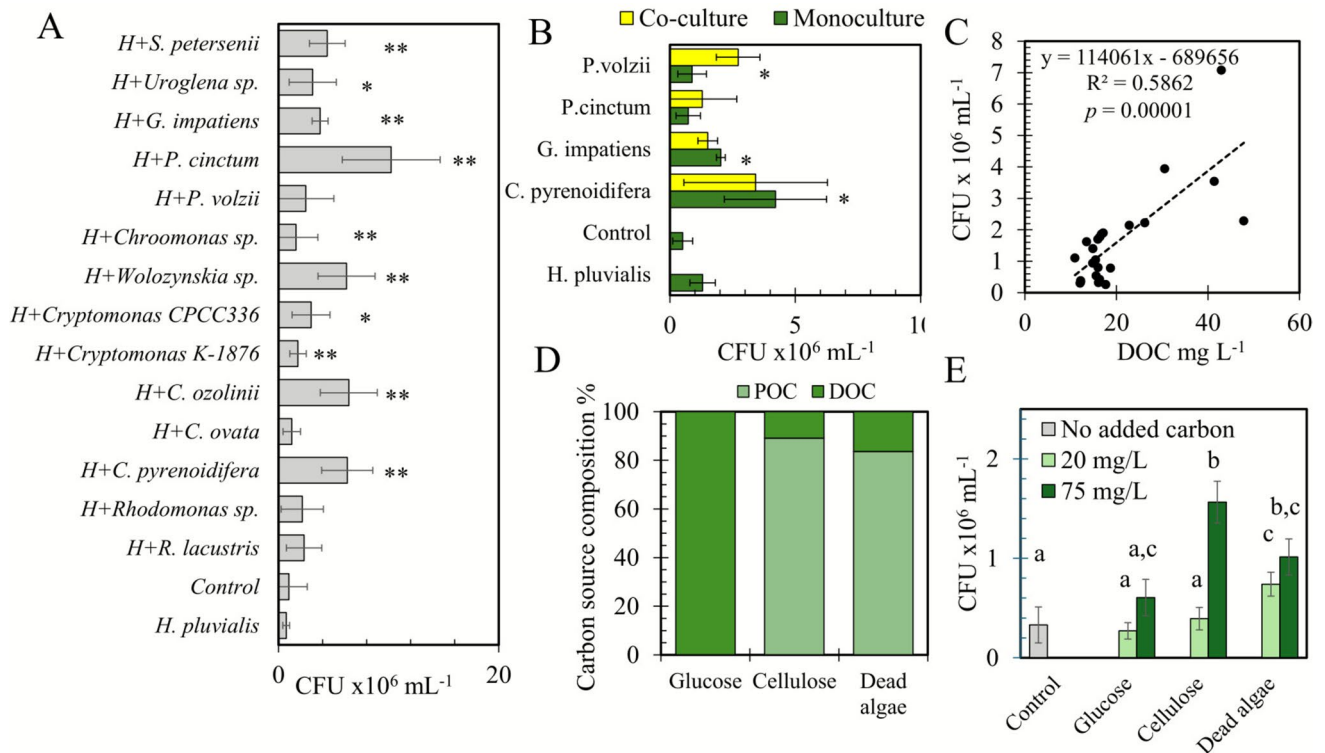


Fig. 4 Bacterial abundance expressed as mean and standard deviations of numbers of colony-forming units (CFU) at the end of **A**) experiment I (the monoculture of *Haematococcus pluvialis*, control, and co-cultures, $n=5$) and **B**) experiment II ($n=4$). **C**) In experiment II, a regression between the concentration of dissolved organic carbon (DOC) and the number of CFUs in co-cultures was detected among

monocultures. **D**) Form of organic carbon (particulate/dissolved) in the stock solutions (average, $n=3$). **E**) Bacterial abundance expressed as the number of CFUs per volume unit under different carbon sources and concentrations after 7 days. * $p<0.05$, ** $p<0.01$. Letters indicate the statistical difference: if the groups share the same letter, there is no statistical difference

Performance of co-cultures

Overall, none of the co-cultures outperformed the monoculture of *H. pluvialis* in terms of biomass production, nutrient removal, or mitigation of bacterial abundance. However, the nutritional quality improved in co-cultures with *C. pyrenoidifera* and *G. impatiens* in terms of elevated contributions of proteins and lipids, respectively (Table 3).

Table 3 Performance of four co-cultures in comparison to the monoculture of *Haematococcus pluvialis* in terms of nutrient removal, biochemical composition, and bacterial load

	<i>Cryptomonas pyrenoidifera</i> + <i>H. pluvialis</i>	<i>Gymnodinium impatiens</i> + <i>H. pluvialis</i>	<i>Peridinium cinctum</i> + <i>H. pluvialis</i>	<i>Peridinium volzii</i> + <i>H. pluvialis</i>
Biomass	Equal	Equal	Equal	Equal
Protein-%	1.1 × Higher	Equal	Equal	Equal
Total fatty acids	3.5 × Higher	4.3 × Higher	Equal	Equal
EFA	4.1 × Higher	5.5 × Higher	2.6 × Higher	Equal
TN removal	Lower	Lower	Lower	Lower
PO ₄ -P removal	Lower	Equal	Equal	Equal
Bacterial load	Equal	Equal	Equal	2.1 × Higher

Discussion

Mixotrophy and competition in co-cultures

We have previously detected high bacterial abundance in association with *H. pluvialis* cultured in aquaculture effluent (Calderini et al. 2021; Timilsina et al. 2025), with the suggestion that algal biomass production is hindered by

bacteria. The present study tested the co-cultivation of mixotrophic non-green algae with the green alga *H. pluvialis* to enhance biomass quantity and quality in aquaculture effluent. We hypothesized that mixotrophic algae co-cultivated with *H. pluvialis* would lead to diminished bacterial competition pressure and consequently increase the production of algal biomass and nutrient removal efficiency. However, bacterial abundance remained equal or increased in all co-cultures in comparison to the monoculture of *H. pluvialis*. This suggests that mixotrophic algae in our experiments favored autotrophic metabolism rather than heterotrophy (i.e., consuming bacteria). Subsequently, instead of mutualistic interaction, there was competition between *H. pluvialis* and the mixotrophic non-green algae. Among the co-cultures tested, the dinoflagellate *G. impatiens* was the only strain able to coexist with *H. pluvialis* over a 28-day growth period, demonstrating its compatibility for co-cultivation with *H. pluvialis* in aquaculture effluent. The other two dinoflagellates *P. cinctum* and *P. volzii* were outcompeted by *H. pluvialis*. The growth of *C. pyrenoidifera* in the co-culture was unstable, and the population either collapsed in the beginning or was slowly outcompeted by *H. pluvialis*.

According to the competitive exclusion principle, two species within the same niche cannot coexist without one outcompeting the other (Hardin 1960). Although nutritional strategies of mixotrophic algae partly overlap with those of relying purely on autotrophy, their niches are hypothesized to be sufficiently distinct for coexistence (Crane and Grover 2010). Mixotrophs rely primarily on photosynthesis but acquire additional resources, such as growth-limiting nutrients, by feeding on bacteria, which then support photosynthesis (Caron et al. 1993; Crane and Grover 2010; Rottberger et al. 2013). Indeed, 75% of mixotrophic algal biomass carbon is taken up via photosynthesis, whereas 25% originates from organic matter and bacteria (Caron et al. 1993). Therefore, organic carbon limitation typical to RAS effluent (Rojas-Tirado et al. 2019) can promote autotrophy over heterotrophy in mixotrophic algae (Rottberger et al. 2013). Mixotrophic algae then rely completely on autotrophy, leading to resource competition with the solely autotrophic green algae in the co-culture, as was seen in the present study. Furthermore, under continuous light that enhances the growth rate of mixotrophic algae in comparison to light cycles with a dark period (Patel et al. 2019), the mixotrophic mode is likely induced by decreased nutrient concentration and the increased availability of organic carbon sources and bacteria. In nutrient-rich RAS effluent with adequate light, mixotrophic algae likely favor photosynthesis over heterotrophy and start to use mixotrophic metabolism only when nutrient availability becomes a growth-limiting factor or the utilization of organic carbon sources provides a significant competitive advantage. In theory, this would offer mixotrophic algae a competitive advantage over co-cultivated,

solely autotrophic green algae at late-exponential or stationary growth stages of the algal culture. Because *H. pluvialis* encysts in nutrient-limited conditions typical of these growth stages (Shah et al. 2016), astaxanthin-rich aplanospores of *H. pluvialis* would potentially be protected against competition from mixotrophic algae. If so, the biomass and astaxanthin content of *H. pluvialis* would remain stable, while simultaneously the mixotrophic algal strain would increase its biomass and, subsequently, total biomass of the algal culture, by utilizing organic carbon and nutrient sources.

Biochemical composition of algal biomass

The biochemical composition of low lipid and high protein content indicate that the vegetative cells of *H. pluvialis* were at the late growth stage (Abomohra et al. 2013). The lipid contribution (10% of the biomolecular content) was 5–15% lower than previously reported for the vegetative growth phase (Damiani et al. 2010; Shah et al. 2016). High protein content (63% of the biomolecular content) also supported the suggestion of the late exponential growth stage. Typical protein content of *H. pluvialis* ranges from 18 to 63% in the green stage, and changes to 21–23% in the red stage (Ba et al. 2016; Shah et al. 2016; Zhu et al. 2019; Marinho et al. 2021). Thus, the results of biochemical composition reflect on the vegetative cells of *H. pluvialis* at the late growth stage.

The growth medium can affect the protein content of algae, and particularly nitrogen concentration correlates positively with protein content (Zarrinmehr et al. 2020; Marinho et al. 2021). Our results indicated that proteins contributed 63% to biomolecular composition (the sum of lipids, carbohydrates, proteins) in the monoculture of *H. pluvialis* at the late exponential phase. Nitrogen-rich RAS effluent thus seems to provide a suitable growth medium for protein production by *H. pluvialis*. However, the monoculture of *C. pyrenoidifera* had the highest observed relative protein content, indicating that it would be particularly suitable for the production of the high-protein algal biomass. The co-cultivation of *C. pyrenoidifera* and *H. pluvialis* yielded more protein-rich biomass than the monoculture of *H. pluvialis*. Since *H. pluvialis* is a rich source of proteins and contains essential amino acids (Zhu et al. 2019), co-culture of *H. pluvialis* and *C. pyrenoidifera* could provide a rich source of algal amino acids in terms of quality and quantity. Moreover, co-cultivation could compensate for the decreasing protein content of *H. pluvialis* in the culture during the encystment.

Despite the competitive pressure, the co-cultures of *G. impatiens* and *C. pyrenoidifera* with *H. pluvialis* produced an equal amount of biomass with a higher contribution of total and essential fatty acids (EPA, DHA, LIN, ALA, SDA) than the monoculture of *H. pluvialis*. Although the production of EFAs in the co-culture of *H. pluvialis* and *G.*

impatiens was 5.5-fold higher than in the monoculture of *H. pluvialis*, it was 8.5-fold lower than in the monoculture of *G. impatiens*. Similarly, the monoculture of *C. pyrenoidifera* yielded 5.0-fold more EFAs than the co-culture with *H. pluvialis*, and the co-culture yielded 4.1-fold more EFAs than the monoculture of *H. pluvialis*. Co-cultures can thus provide more EFAs than the monoculture of *H. pluvialis*, however, due to changing ratios of two algal strains in a co-culture that affect the nutritional quality of total biomass, monocultures of non-green algae are likely more suitable for stable production of the desired fatty acids on a larger scale.

Nutrient removal by co-cultures

None of the co-cultures outperformed the *H. pluvialis* monoculture in overall nutrient removal. Supporting previous studies (Crane and Grover 2010; Stevčić et al. 2019), however, non-green algae used phosphorus and nitrogen in different ratios than *H. pluvialis*, indicating diverging nutrient demands. For instance, the *G. impatiens* monoculture exhibited a lower nitrogen removal efficiency than *H. pluvialis*. Moreover, the average removal of TN by the monoculture of *C. pyrenoidifera* was higher than the removal of $\text{PO}_4\text{-P}$. *Cryptomonas pyrenoidifera* exhibited low $\text{PO}_4\text{-P}$ removal, which reflected the removal % of $\text{PO}_4\text{-P}$ in the co-culture of *H. pluvialis* and *C. pyrenoidifera*. This co-culture had the lowest removal efficiency of $\text{PO}_4\text{-P}$, which was in line with the lowest average biomass among co-cultures. In other co-cultures, $\text{PO}_4\text{-P}$ removal was equal to that in *H. pluvialis* monoculture and at the same level as previously reported for other green algal strains (Stevčić et al. 2019; Tejido-Núñez et al. 2020; Gupta and Marchetti 2024). In contrast, while nitrogen was also efficiently removed, its removal efficiency was reduced in all co-cultures compared to the *H. pluvialis* monoculture, likely due to lower average biomass.

Although phosphorus has been reported to limit nitrogen removal by green algae in aquaculture effluent (Tejido-Núñez et al. 2020; Jakhwal et al. 2024; Timilsina et al. 2025), our results demonstrated efficient removal of both nitrogen and phosphorus in the monoculture of *H. pluvialis*, indicating that the phosphorus did not limit nitrogen uptake. The *H. pluvialis* monoculture achieved an average nitrogen removal efficiency of 95.8%, which is approximately 30% higher than previously reported values (Stevčić et al. 2019; Timilsina et al. 2025). This enhanced efficiency is likely explained by the long (28 days) cultivation period used in the present study: a previous study with 6 days of cultivation showed lower TN removal (Stevčić et al. 2019), whereas another study (Tejido-Núñez et al. 2020) with 29 days of cultivation reached similar nutrient removal levels as the present study. It seems likely that the longer cultivation time decreases the effect of luxury uptake of phosphorus on total nutrient removal. In microalgal luxury uptake, i.e.,

rapid consumption, phosphorus is stored in algal biomass as polyphosphates (Powell et al. 2009; Brown and Shilton 2014). Therefore, the low concentration of phosphorus in the effluent at the end of the algal cultivation period does not necessarily indicate phosphorus limitation. When all phosphorus is assimilated by algae, nitrogen uptake and growth may continue, since algae can use polyphosphates to complete their needs for phosphorus.

Bacteria benefit from algal POM

Contrary to our expectations, we did not detect bacterial growth mitigation by mixotrophic algae but instead found that algal inoculation boosted bacterial growth in RAS effluent. However, differences in bacterial abundance between algal co-cultures were seen, and the monoculture of *H. pluvialis* had the lowest mean number of CFUs in experiment I. This suggests that either other algal inocula consisted of greater initial bacterial abundances than *H. pluvialis*, or, contrary to our earlier findings (Calderini et al. 2021; Timilsina et al. 2025), *H. pluvialis* efficiently competed with bacteria suppressing their growth, or *H. pluvialis* secreted antimicrobial compounds (Darwesh et al. 2022) that inhibited bacterial growth despite the increased availability of algal POM.

Although a link between DOC concentration and bacterial abundance was observed in co-cultures, further examination suggested that POM is a more important factor than DOC for bacterial growth. The role of the physical form of the carbon source was seen in high-concentration treatments: completely dissolved glucose did not boost microbial growth, whereas cellulose, which contains the same elements as glucose but was only 11% soluble, boosted microbial growth more strongly. Although cellulose is one of the main components of the cell wall of *H. pluvialis* (Damiani et al. 2006; Kim et al. 2016) and the observed POC:DOC -ratios of cellulose and algal necromass were at the same level, algal necromass boosted microbial growth at a lower concentration (20 mg C L^{-1}) than cellulose, suggesting that carbon is not the only element affecting microbial growth even in nutrient-rich RAS effluent. Overall, results support the current view that microbial growth in aquatic environments is affected not only by the concentration of the carbon but also by the physical form and the molecular heterogeneity of the organic matter (Fischer et al. 2002; Eiler et al. 2003; Wurzbacher et al. 2016; Vesamäki et al. 2022, 2024).

Conclusions

We hypothesized that co-cultivation of *H. pluvialis* and mixotrophic algae in recirculating aquaculture effluent would reduce bacterial abundance and lead to enhanced

biomass production, nutrient removal, and biomass valorization. Against our hypotheses, mixotrophic non-green algae did not reduce bacterial load in co-cultures. On the contrary, mixotrophic algae favored autotrophic metabolism, supporting bacterial growth and leading to competition between *H. pluvialis* and non-green algae in co-cultures. Despite competition, co-cultures had equal biomass to the monocultures with an improved nutritional quality in comparison to the monoculture of *H. pluvialis*. However, monocultures of *G. impatiens* and *C. pyrenoidifera* outperformed the co-cultures of *H. pluvialis* and non-green algae in the production of EFAs, suggesting that monocultures of non-green algae are more efficient for the production of EFAs and/or proteins than the co-cultures. *C. pyrenoidifera* was particularly rich in proteins, and alone or together with *H. pluvialis* could provide a rich source of amino acids. From the point of view of nutrient bioremediation, co-cultures removed less nitrogen than the monoculture of *H. pluvialis*, but showed improved or equal nitrogen removal in comparison to the monocultures of non-green algae. Thus, although mixotrophic non-green algae did not reduce bacterial abundance in *H. pluvialis* culture in aquaculture effluent, the nutritional quality of produced green algal biomass may be elevated by the co-cultivation with non-green algae while maintaining equal biomass production, but with a trade-off of decreased nitrogen removal.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10811-026-03789-w>.

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Author's contributions J.S.V.: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. H.T.: Writing – review & editing, Investigation. M.H.: Writing – review & editing, Investigation, Conceptualization. J.P.: Writing – review & editing, Resources, Funding acquisition, Conceptualization. K.P.: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Data availability The data used in this study is freely available via Zenodo repository with URL: <https://zenodo.org/records/17571248>.

Declarations

Competing interest The authors declare no competing interests.

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