

# Co-cultivation of microalgae in aquaculture water: interactions, growth and nutrient removal efficiency at laboratory and pilot scale

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## Abstract

Microalgae biotechnology is of increasing importance and a central application concerns the treatment of wastewater. Here, its implementation in a recirculating aquaculture system (RAS) to lower the discharge of wastewater is studied. To better cope with external variations in culture conditions, a co-cultivation of two species of microalgae, *Chlorella vulgaris* and *Tetrademus obliquus*, was used to obtain a more reliable and robust culture and was compared to monocultures. This approach was tested using RAS water both under sterile and non-sterile conditions at laboratory scale and then compared to a co-culture at pilot-scale in an open thin-layer photobioreactor. Performance of cultures was tested in terms of microalgae growth and nutrient removal efficiency. Furthermore, to better understand the interaction between environmental variables and each microalgae species, their relative frequencies in co-cultures as well as the presence of protozoa and bacteria were monitored. All growth experiments were carried out successfully and, unlike in a previous study, no crashes were observed. However, shifts in species frequency in co-cultures indicated that the two species were differentially affected by cultivation conditions. Despite nutrient limitation,

the pilot-scale cultivation had a high productivity ( $13.3 \text{ g m}^{-2} \text{ d}^{-1}$ ) and final dry weight ( $11.1 \text{ g l}^{-1}$ ) after 29 days and demonstrated its suitability for RAS water treatment.

## **Keywords**

Aquaculture, Nutrients, Protozoa, Water treatment, *Chlorella vulgaris*, *Tetrademus obliquus*

## **1. Introduction**

The growing importance of microalgae biotechnology is clearly reflected by the increasing number of publications related to it [1], with a special emphasis on the use of microalgae for wastewater treatment [2]. A promising field in this sector is aquaculture, as microalgae-based technologies have been proposed as a way of treating its effluents [3–5]. In addition, the growing interest in the replacement of fish meal with algal protein for the production of fish feed [6,7] promises a further valorisation of microalgal biomass that could be produced on-site.

State-of-the-art aquaculture relies on recirculating aquaculture systems (RAS), which are more eco-friendly, due to their low water demand and reduced waste output [8]. Water in a RAS must be treated to be reused internally, and several studies have successfully demonstrated the suitability of microalgae for RAS water treatment at laboratory [9,10] and pilot scale [5,11,12]. In these studies, known strains of microalgae species or characterized natural inocula with a predominance of a known species were used. Additionally, studies have addressed the role of microalgal-bacterial consortia [3,4] for aquaculture wastewater treatment. Nevertheless, little is known about the use of microalgae consortia (polycultures) for wastewater treatment. While the use of microalgal consortia for wastewater treatment has been addressed repeatedly [13], it has rarely been done with water from a RAS [14]. In addition, studies involving microalgal consortia typically focus on the treatment efficiency

achieved or the overall biomass produced, and few document the interactions among the microalgae species involved [15].

Previous research has demonstrated that polycultures of microalgae are more robust than monocultures, because they are less likely to suffer from biomass loss when cultivated [16]. In general, genetic diversity reduces the vulnerability of ecosystems to disturbances [17]. Therefore, the co-cultivation of multiple species of microalgae promises to reduce the frequency and extent of culture crashes caused by viral, bacterial or fungal infections or predation by protozoa, all of which may target only specific species [18], yet not a complete polyculture. Further, different nutrient requirements may improve overall nutrient utilization and, thus, the efficiency of wastewater treatment [14].

Protozoa are well known pests in microalgae cultures [19] and prevention strategies are actively researched, e.g. the use of CO<sub>2</sub> [20] or chemicals [21]. However, such interventions may also affect the microalgae culture itself and less aggressive strategies are desirable. To this end, gaining a better understanding of the interactions between specific microalgae and protozoa will help to develop operational strategies that minimize crashes.

Species of the genera *Chlorella* and *Tetradismus* are common in freshwater ecosystems and are among the most used and best understood microalgae [22–24]. Here, specific strains of the species *Chlorella vulgaris* and *Tetradismus obliquus* were selected to conduct the experiments, which also allowed their differentiation by light microscopy. Previous research with these strains showed specific differences in performance depending on the presence of protozoa and possibly on the location in the RAS, where water has been sampled [9]. Additionally, the presence of *C. vulgaris* and *T. obliquus* was beneficial for the growth of Nile Tilapia in aquaculture that uses autotrophic biofloc technology [25]. Co-cultivation of both *C.*

*vulgaris* and *T. obliquus* has been carried out before using sterilized municipal wastewater [26], which showed that this is a promising consortium for nutrient removal.

Another paramount feature for the success of cultivation is the specific design of the photobioreactor (PBR) [23]. PBRs are mainly classified into two types: closed and open, each one with its own advantages and disadvantages [27]. In this study an open thin-layer photobioreactor was used [28]. In this type of PBR, photosynthetic efficiency is optimized thanks to the high turbulence and thin cultivation layer. CO<sub>2</sub> is injected in this PBR to enhance microalgae growth, and besides pure CO<sub>2</sub>, flue gas has been used successfully [29]. Additionally, the open design and the high surface area to volume ratio result in a high evaporation rate. This allows the continuous addition and treatment of RAS water [5].

The aim of this study was to analyse the performance of a co-culture of *C. vulgaris* and *T. obliquus* and test whether culture robustness can be improved. To achieve this, the performance of a co-culture of *C. vulgaris* and *T. obliquus* was tested at laboratory scale under controlled conditions in non-sterile and sterile aquaculture water, and at pilot scale in an open thin-layer PBR using untreated water from the fish tank of a RAS. Growth of microalgae and final dry weight achieved, variation of species proportion over time (relative amount of each species), presence of protozoa and bacteria and finally nutrient removal efficiency were monitored.

## **2. Materials and Methods**

### **2.1. Recirculating aquaculture system**

RAS water was obtained from an aquaponic system situated in a greenhouse on the Grüental Campus of the Zurich University of Applied Sciences in Wädenswil, Switzerland. The aquaponic system consisted of two subunits: a recirculating aquaculture system (RAS) and a

hydroponic unit. Water was collected from the fish tank of the RAS and was used for both laboratory-scale and pilot-scale experiments. Average water characteristics are (in mg l<sup>-1</sup>): 152.8 NO<sub>3</sub>-N; 16.1 PO<sub>4</sub>-P; ≤0.1 NH<sub>4</sub><sup>+</sup>; 246.4 Na<sup>+</sup>; 168.9 Ca<sup>2+</sup>; 39.6 Mg<sup>2+</sup>; 195.1 K<sup>+</sup>; 0.3NO<sub>2</sub><sup>-</sup>; 147.3 Cl<sup>-</sup>; 420.6 SO<sub>4</sub><sup>2-</sup>; 38.4 COD; 4.2·10<sup>5</sup> cfu ml<sup>-1</sup> (bacteria) and pH 7.5. A complete description of the system and a characterization of the RAS water is given by [9].

## **2.2. Microalgae strains used and their cultivation**

Two commonly used microalgae were tested: *Chlorella vulgaris* CCAP 211/52, obtained from the Culture Collection of Autotrophic Organisms of the Institute of Botany of the Czech Academy of Sciences, and *Tetrademus obliquus* (syn. *Acutodesmus obliquus*, *Scenedesmus obliquus*) SAG 276-1, obtained from the Culture Collection of Algae at Göttingen University. Both microalgae are freshwater species. Stock cultures of both strains were kept under controlled conditions (as explained in section laboratory-scale experiments) in mineral medium [30].

### **2.2.1. Laboratory-scale experiments**

Microalgae cultivation was carried out in 100-ml Erlenmeyer flasks covered with a cotton stopper in an incubator (Multitron Pro, Infors HT, Bottmingen, Switzerland) at 25 °C, 2 % CO<sub>2</sub> atmosphere, 115 rpm, constant illumination (warm white LEDs, 3500 K, photosynthetic photon flux density 90 μmol s<sup>-1</sup> m<sup>-2</sup>). The setup of the incubator ensures an even distribution of light and flasks were distributed randomly to avoid positional effects. Water was collected from the fish tank and used either directly (referred to as non-sterile) or after filtration (0.22 μm, referred to as sterile) for cultivation at laboratory scale. *C. vulgaris* and *T. obliquus* were cultivated separately (monocultures) and combined (co-cultures starting with equal cell numbers of each species) in 60 ml of non-sterile and sterile RAS water. Initial cell

concentrations were always  $10^6$  cells per ml for every species (no coenobia were observed in *T. obliquus*). Treatments were replicated four-fold to obtain 3 (2 monocultures, 1 co-culture) x 2 (sterile/non-sterile RAS water) x 4 (replicates) = 24 independent cultures.

Growth experiments lasted 19 days, until a stationary phase was reached (no decay was observed). Microalgae growth was measured as cell density, optical density (750 nm) and dry weight, protozoa (e.g. rotifers, amoebae, ciliates, or flagellates) were counted under a light microscope, and bacteria were counted as colony forming units on agar plates. Cell density of microalgae was determined by light microscopy (phase contrast, 400-fold magnification) with haemocytometer (Neubauer-improved Marienfeld, Lauda-Königshofen, Germany). Cells in coenobia were counted individually. Protozoa were quantified likewise (a lower magnification was used to survey the whole chamber containing approximately 10  $\mu$ l). Optical density was measured at 750 nm with an automated plate reader (Infinite 200 Pro, Tecan). Dry weight was measured using 5-ml samples in pre-weighed tubes that were centrifuged for 5 min at 5000 *g*, decanted, and dried at 105 °C overnight. A 100- $\mu$ l sample was collected daily to measure absorbance and count microalgae, protozoa and bacteria. For all methods, kindly refer to [9]. In all cultures, absorbance was measured daily, cell density and protozoa every three days, bacteria at days 3, 7, 11 and 19 of cultivation, and dry weight at days 4, 9, 13 and 19, once per independent cultivation. At the last sampling date, the complete remaining volume was sampled. Nitrate and phosphate were measured at day 0 and at the end of the cultivation using photometric test kits (LCK 339 and LCK 349 respectively, Hach-Lange, Rheineck, Switzerland). Initial concentrations were 96.3 mg NO<sub>3</sub>-N l<sup>-1</sup> and 9.9 mg PO<sub>4</sub>-P l<sup>-1</sup>. pH was 6.9 and bacteria initial concentration in non-sterile RAS water was  $3 \cdot 10^4$  cfu ml<sup>-1</sup>.

### **2.2.2. Pilot-scale cultivation**

Co-cultivation of *C. vulgaris* and *T. obliquus* was carried out in August/September 2018 in an open thin-layer photobioreactor situated next to the RAS. The photobioreactor (Figure 1) was constructed by BCS Engineering S.A., Brno, Czech Republic [28] and has been used previously in a similar configuration [5].

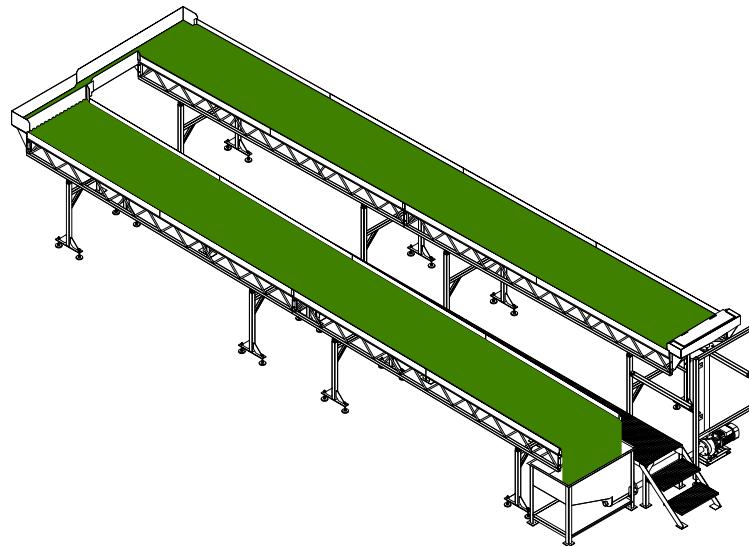


Figure 1 Scheme of the open thin-layer photobioreactor used in this study and in [5].

The reactor consisted of an inclined (1.7%) culture surface (18 m<sup>2</sup>) made of glass sheets in a steel frame on which the microalgal culture circulated. At the lower end of the surface, the culture was collected in a tank and then pumped up again with a centrifugal pump.

To control and maintain optimal cultivation conditions, several sensors were used to monitor parameters such as thickness of the culture suspension on the platform, partial pressure of oxygen and carbon dioxide, pH, temperature, photosynthetically active photon flux density, water volume and water added, and turbidity. The thickness of the suspension layer was kept at 8 mm by coupling an ultrasonic sensor to the pump *via* a proportional integral (PI) controller. During the day, pure CO<sub>2</sub> was injected into the suction pipe of the cultivation circulating pump. Dissolved CO<sub>2</sub> was measured just before the point of injection by means of a Severinghaus electrode (InPro<sup>®</sup>5000i, Mettler Toledo, Greifensee, Switzerland), which then

regulated the supply of CO<sub>2</sub> to a partial pressure of 10 mbar via a PI controller. At night, no CO<sub>2</sub> was supplied. Switching between day and night modes was based on local sunrise and sunset times. Oxygen partial pressure in the culture was measured with an optical sensor (InPro®6860i, Mettler Toledo). The pH of the algal suspension was measured with a pH electrode (InPro®3253i, Mettler Toledo), which also measured temperature. PAR (photosynthetically active radiation) was measured in terms of photosynthetically active photon flux density (PPFD,  $\mu\text{molm}^{-2} \text{s}^{-1}$ ) with two sensors (SKL2620, Skye Instruments Ltd., Powys, UK) placed above and below the glass platform. The number of photons absorbed was calculated as the difference between the measurements of both sensors. The volume of the circulating algal suspension was continuously monitored by means of a pressure sensor in the cultivation tank. Total volume in the reactor was 200 l and water loss by evaporation was balanced whenever the volume fell below 195 l. The volume of water that was added was measured by means of a water meter at the inlet pipe.

The cultivation was started with equal numbers of both *C. vulgaris* and *T. obliquus*, with an initial concentration  $10^6$  cells per ml (cells in coenobia were counted individually as in laboratory-scale experiments) and lasted 29 days. Water was supplied to the system from a storage tank (filled every two days with water from the RAS). After every refilling, concentrations of nitrate and phosphate, and salinity (conductivity, HQ40D, Hach Lange) were measured in the storage tank. Low concentrations of phosphate were consistently found, which is likely due to the fact that phosphate adsorbs to solids, which, in the RAS, are removed with a drum filter. It was therefore decided to supplement phosphate (as KH<sub>2</sub>PO<sub>4</sub>) to prevent phosphorous limitation of growth and, thus, to achieve a simultaneous depletion of both N and P in the cultivation. The supplied phosphate was dosed to achieve an N:P ratio close to an empirically derived optimal ratio for *C. vulgaris* [31]. A single 100-ml sample was taken



daily two hours after sunrise at the end of the platform. Biomass growth was monitored daily *via* optical density (absorbance at 750 nm, following the above-mentioned procedure), dry weight (25-ml sample, HB43-S moisture analyser, Mettler Toledo) and cell density (following above-mentioned procedures), and continuously *via* turbidity (SOLITAX sc, Hach Lange). Abundance of bacteria and protozoa during the experiment was measured daily (following above-mentioned procedures). Salinity (conductivity) was measured daily both in the circulating suspension and in the storage tank for RAS water. Biomass samples for CHN analysis were taken daily, beginning at day 5 and stored at -20 °C. After the experiment was stopped, biomass samples were thawed, dried at 105 °C for 24 h and grinded with a ball mill. 100 mg of dried biomass were combusted at 950 °C and analysed by means of infrared spectroscopy and thermal conductivity (TruSpec Macro Analyser, Leco Instruments Ltd., UK).

### 2.3. Data analyses

Data reported are average values and error bars are the standard error of the mean (SEM). In some cases, data points and error bars were shifted to improve visibility in the graphs. The differences in growth (measured as cell density, optical density, and dry weight) between non-sterile and sterile cultures were calculated as the ratio of mean values in non-sterile and sterile cultures minus 1. Thus, positive values indicate better growth of non-sterile cultures and *vice versa*.

$$NS/S = \frac{[\text{non - sterile data}]_{\text{mean}}}{[\text{sterile data}]_{\text{mean}}} - 1$$

Where required, values are expressed as mean  $\pm$  SEM, from a four-fold replication of each culture experiment. To robustly test for correlations and differences, non-parametric Spearman rank correlation and Welch's t-test assuming unequal variances were used. To test

for differences in bacterial numbers over the whole cultivation period analysis of variance was used (time and culture type as categorical factors).

Growth rates were calculated as the maximum first derivative of a smoothing spline that was fitted to the data on optical density (as not enough data on cell density and dry weight were available). Productivity in the photobioreactor was calculated as the maximum first derivative of a spline fitted to dry weight data.

### **3. Results and Discussion**

#### **3.1. Growth of microalgae**

Laboratory-scale cultivation confirmed that both microalgae, *C. vulgaris* and *T. obliquus*, were able to grow in RAS water (Figure 2 A and B) as has been previously demonstrated [25,32]. Upon closer inspection, all cultures showed a consistent pattern, where optical density was higher in non-sterile RAS water during the first days of cultivation, whereas optical density was higher in sterile RAS water in the second half of the experiment for both monocultures (Spearman's  $\rho = -0.71$  and  $-0.71$ ,  $p < 0.001$  for *C. vulgaris* and *T. obliquus*, respectively) and the co-culture (Spearman's  $\rho = -0.55$ ;  $p = 0.014$ ; Figure 2 C). However, the actual differences that caused this pattern were subtle (below 10%) and none of the non-sterile cultures showed a marked decay at any time during cultivation as has been observed in a previous study [9].

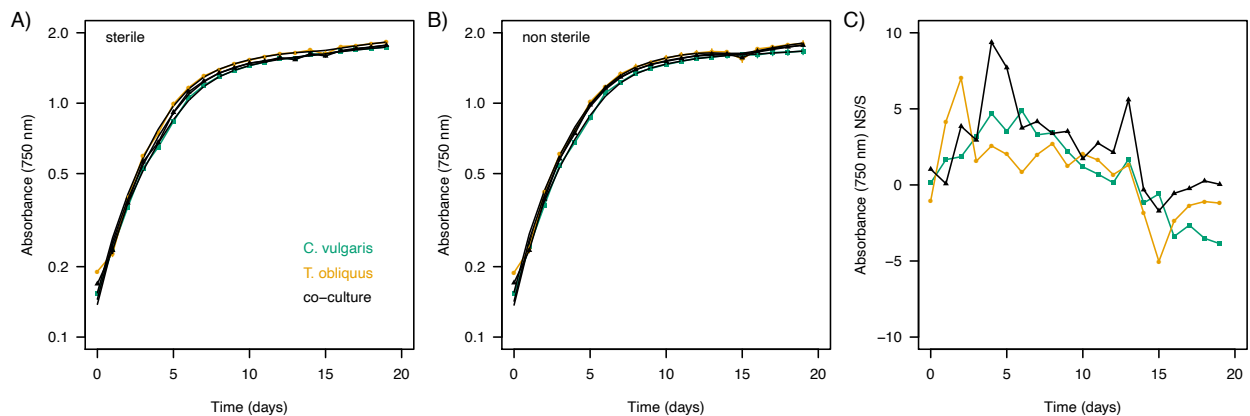


Figure 2 Growth curves based on optical density (absorbance at 750 nm) of sterile (A), and non-sterile (B) laboratory-scale cultures (average values  $\pm$  SEM,  $n = 4$ ) and percent differences between them (C).

Similar patterns were observed in cell density data (Figure 3 A and B). Both species and their co-culture, in both sterile and non-sterile RAS water, exhibited exponential growth during the first third of the experiment and then entered a plateau phase concurrently around day six. In co-culture, it was possible to follow growth of both species individually, which, again, revealed similar growth patterns, albeit at lower densities than in monocultures. When sterile and non-sterile cultivation experiments were compared (Figure 3 C), there was not a clear pattern as observed in Figure 2 C. *T. obliquus* showed the same trend (Spearman's  $\rho = -0.929$ ;  $p = 0.007$ ), whereas *C. vulgaris* in co-culture showed the opposite pattern (Spearman's  $\rho = 0.857$ ;  $p = 0.024$ ). All other trends were not significant.

When grown in co-culture, the proportion of cells of *C. vulgaris* increased during the first half of the experiment, followed by a stable ratio of approximately 60-70% cells of *C. vulgaris* and 30-40% cells of *T. obliquus* (Figure 4). This pattern occurred both in sterile and non-sterile RAS water and no significant trend was evident between the two treatments (Spearman's  $\rho = 0.5$ ;  $p = 0.27$ ). Taking into account the results obtained from a previous experiment [9], it was expected that in the presence of protozoa (grazers), *T. obliquus* becomes dominant in co-

cultivations with *C. vulgaris*. While this was unexpected, it is supported by the other results obtained herein, where both microalgae performed similarly.

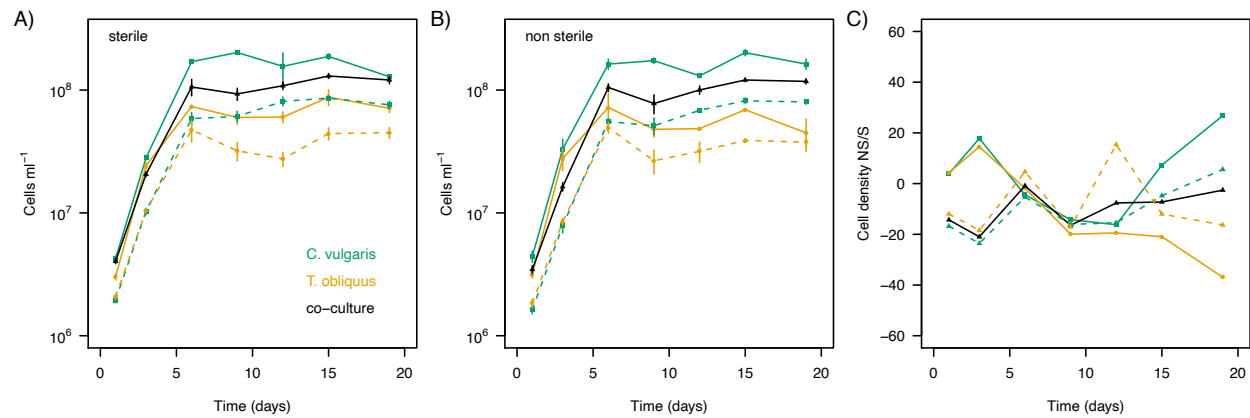


Figure 3 Cell density evolution during laboratory scale sterile cultivations (A), non-sterile (B) (average values  $\pm$  SEM, n = 4) and percent differences between them (C). Dashed lines represent cell counts of each microalgae species in the co-cultivation.

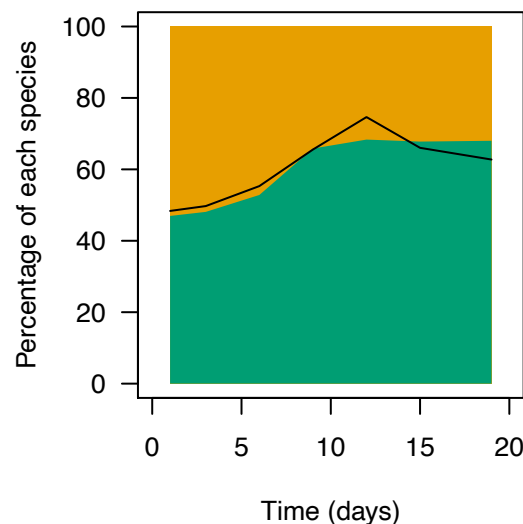


Figure 4 Relative amount of each species in non-sterile cultivations (coloured: green corresponds to *C. vulgaris*, orange to *T. obliquus*) and in sterile cultivations (black line, *T. obliquus* is represented by the area above the line and *C. vulgaris* below the line).

Taking into account dry weight results (see supplementary material Figure S1), highest final values were obtained in mono-cultures of *T. obliquus*, with yields being less variable and higher in sterile ( $7.52 \pm 0.11$  g l<sup>-1</sup>) than in non-sterile ( $6.38 \pm 0.67$  g l<sup>-1</sup>) RAS water, yet not significantly higher (Welch's two sample t-test:  $t_{3,2} = -1.7$ ,  $p = 0.18$ ). Slightly lower yields were

obtained in co-cultures (sterile:  $6.17 \pm 0.06 \text{ g l}^{-1}$ , non-sterile:  $6.12 \pm 0.07 \text{ g l}^{-1}$ , difference not significant:  $t_{5,9} = -0.62$ ,  $p = 0.56$ ), followed by *C. vulgaris* mono-cultures (sterile:  $5.09 \pm 0.08 \text{ g l}^{-1}$ , non-sterile:  $4.40 \pm 0.32 \text{ g l}^{-1}$ , difference not significant:  $t_{3,4} = -2.08$ ,  $p = 0.12$ ), which again showed more variation in non-sterile RAS water. The reverse results obtained with cell density (cells  $\text{ml}^{-1}$ ), where values for *C. vulgaris* were higher (Figure 3), can be explained with the cell size of these species [33,34], where the larger species, *T. obliquus*, achieves a higher dry weight despite a lower cell count. These values are comparatively high [10,29,35]. Moreover, the dry weight yields in both sterile and non-sterile cultivations reached those obtained in mineral medium in a previous study ( $4.34 \text{ g l}^{-1}$  with *C. vulgaris* at day 13 and  $6.71 \text{ g l}^{-1}$  with *T. obliquus* at day 18; [9]), which confirms the suitability of aquaculture water for microalgae cultivation along with nutrient removal and further valorisation.

Maximum growth rates (estimated from data on optical density) were similar (rate and timepoint) between all cultures grown both under sterile and non-sterile conditions (*C. vulgaris*:  $0.182 \text{ d}^{-1}$  at  $t = 5.1 \text{ d}$ ,  $0.189 \text{ d}^{-1}$  at  $t = 5.1 \text{ d}$ ; *T. obliquus*:  $0.201 \text{ d}^{-1}$  at  $t = 4.3 \text{ d}$ ,  $0.201 \text{ d}^{-1}$  at  $t = 4.4 \text{ d}$ ; co-culture:  $0.189 \text{ d}^{-1}$  at  $t = 4.7 \text{ d}$ ,  $0.201 \text{ d}^{-1}$  at  $t = 4.3 \text{ d}$ ).

A successful co-cultivation was carried out at pilot scale. Cultivation lasted 29 days, a total of 1771 l of RAS water was treated, a final dry weight of  $11.1 \text{ g l}^{-1}$  was reached, and no decay was observed (see supplementary material Figure S2). Design and configuration of the photobioreactor allow high densities up to  $40 \text{ g l}^{-1}$  using mineral medium [30]. The lower final dry weight obtained here could be due to a rapid decline of nitrate content in the cultivation in the first 5 days (supplementary material Figure S3), which may have caused an insufficient supply of nitrogen as will be explained in the section "Nutrient removal". Nevertheless, compared to other studies at pilot scale that used aquaculture water [4], the same microalgae

species [23], or autotrophic cultivation [36], final dry weight achieved in the present study was higher. Another parameter of interest when upscaling microalgae-based water treatment technologies is the productivity achieved by the photobioreactor [37]. High productivities are desired, especially if biomass is to be valorised. The highest productivity achieved was 13.3 g m<sup>-2</sup> d<sup>-1</sup>. Productivity peaked at day five (supplementary material Figure S4) and decreased afterwards until the end of the experiment. The culture was likely nutrient-limited after day five (supplementary material S3), and, thus, growth was not optimal. A similar effect was observed in [5], where it was concluded that typical nutrient levels in RAS water were below the demand of the microalgae culture. Therefore, while RAS water can be fully treated to be reused, lower productivities are reached when compared to non-limiting conditions (i.e. [30,38,39]).

Growth patterns observed varied depending on the measure used (optical density, cell density, dry weight, turbidity). While total cell density appeared to reach a plateau after approximately 10 days, biomass increase (dry weight, turbidity, Supplementary Material S2) continued to the end of the experiment (albeit at a reduced rate after day 10). This is also reflected in the data of the C:N ratio of the biomass, where a sharp increase occurs until day 10, followed by a slow decline (Figure 8), yet still remaining at elevated levels [5]. Thus, day 10 possibly marks the occurrence of strong nutrient limitation (nitrate in the circulating water was depleted at day 5) that affected growth. Throughout the experiment, the proportion of *C. vulgaris* in the co-culture declined, reaching approximately 10% after 29 days (Figure 5). This shift was not expected based on the results of the laboratory-scale experiment.

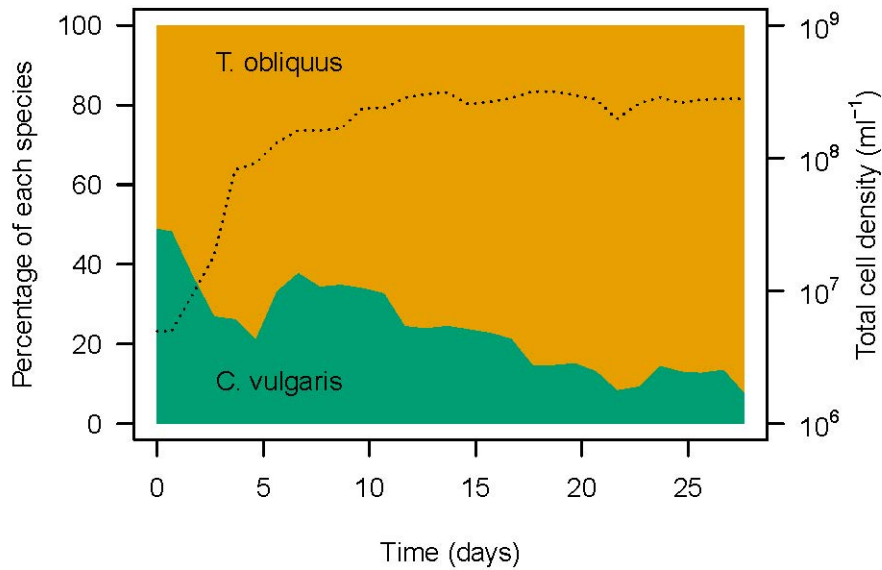


Figure 5 Relative amount of each species (coloured areas) and total number of microalgae cells from both species per ml (dotted line) in the pilot-scale cultivation.

It has been shown previously that biomass productivity depends on sunlight if the PBR is operated with RAS water [5]: Sunlight correlates with water temperature in the PBR and, thus, influences evaporation. This, in turn, determines directly the amount of RAS water that is added to the PBR and therefore also the nutrients that are supplied. Because biomass growth is nutrient-limited if nutrients are only supplied with RAS water, said dependency occurs. This was also observed here, as PAR correlated with temperature of the culture (Spearman rank correlation,  $p < 0.001$ ) and temperature with evaporation (Spearman rank correlation  $p < 0.001$ , Supplementary Material Figure S5).. Temporal pH shifts reflected the pattern of CO<sub>2</sub> addition (only during daytime) and daily fluctuations of oxygen concentration in the culture indicated photosynthetic activity. Both measurements confirmed that cultivation conditions were favourable throughout the experiment (Supplementary Material, Figure S6).

### 3.2. Presence of protozoa and bacteria

Protozoa in laboratory-scale cultures were detected in non-sterile RAS water conditions, as expected. The number of protozoa in mono- and co-cultures increased during the course of

the experiment and reached similar values in both (Figure 6). The numbers of protozoa that were observed here are markedly (up to 32-fold) lower than in a previous study [9]. This suggests that their number was not high enough to cause the strong effects on *C. vulgaris* growth performance as observed previously [9]. This is an important insight, as it is not the sterilization *per se* that is the main factor that determines which microalgae species performs better, but the actual presence of protozoa. If their number is not sufficiently high and if they do not contain specific species [19], their effect will differ. If the presence of protozoa in wastewater has no effect on microalgae growth or can be controlled [40] to remain below damaging levels, there is no need to sterilize the wastewater. These results obtained here and in a previous study [9], both of which used water from the same RAS system, demonstrate that presence and identity of protozoa likely varies through time and, thus, cultivation success also. The term protozoa encompasses a wide range of organisms that are well known to vary in their preferred prey, with many targeting bacteria instead of microalgae [41]. If bacteria compete with microalgae for nutrients, the effect of protozoa on microalgae might even be beneficial.

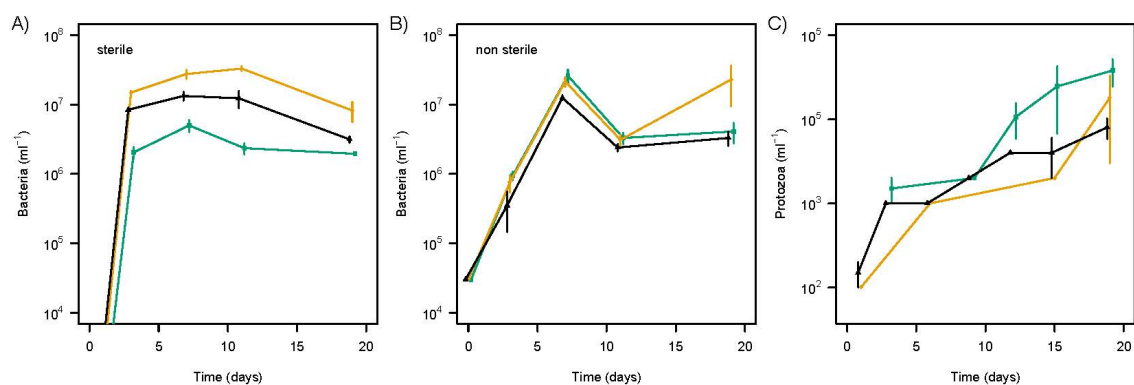


Figure 6 Change in bacterial numbers in sterile (A) and non-sterile (B) laboratory-scale cultures and change in protozoa abundance in non-sterile laboratory-scale cultures (C). *C. vulgaris* is represented in green, *T. obliquus* in orange and co-cultures in black. Data points are means  $\pm$  SEM, n = 4.



As expected, initial bacterial concentrations in sterile cultures were below the detection limit. However, bacteria density quickly increased and reached levels comparable to non-sterile cultivations (Figure 6). Interestingly, after the first day, bacterial density in initially sterile cultivations depended on the type of cultivation applied, with cultures of *C. vulgaris* containing more bacteria than cultures of *T. obliquus*, and co-cultures in between ( $F_{2,45} = 127.4$ ,  $p < 0.001$ ; Figure 6 A). Non-sterile cultures contained bacteria from the beginning, yet differences between cultures were much less pronounced and even declined intermittently ( $F_{2,45} = 5.24$ ,  $p = 0.009$ ; Figure 6 B). This indicates that the presence/absence of bacterivorous protozoa has a stronger effect on bacteria than the identity of microalgae. While the former can even decrease the concentration of bacteria, the latter may influence bacterial growth e.g. by exudates that either benefit or harm bacteria. A similar antagonistic effect between protozoa and bacteria has been observed previously [9]. It should be noted that presence of bacteria in cultivations was accepted because when scaling up microalgae treatments, biological contamination is an important issue [18] and maintaining axenic cultures in open systems is impossible.

In pilot-scale cultivation, abundance of protozoa and bacteria also increased during the experiment (Figure 7). Dynamics of protozoa encompassed three stages: abundance was low from day 0 to 12 (more than 400-times lower than previous results [9]); reached similar values to laboratory-scale experiments from days 13 to 20; and finally, values similar to [9] from day 14 to 28. Comparing these results to the growth patterns observed for each microalgae species, it can be hypothesised that the observed decrease in *C. vulgaris* abundance during the experiment is caused by protozoa, because only after day 12 the number of protozoa was sufficiently high to have a measurable effect on *C. vulgaris* growth. Note, however, that the same number of protozoa that was reached in the PBR at day 12 did not affect growth of *C.*

*vulgaris* in the laboratory-scale cultivations. Open pond systems are prone to contamination by zooplankton [40] but thin layer cascade systems are less vulnerable than other types of systems thanks to higher operational biomass concentrations and higher volumetric productivity [42]. While keeping grazers at low levels is important for successful microalgae cultivation [18], their management is easy in a thin-layer cascade system because the complete culture can be harvested at regular intervals. CO<sub>2</sub> addition may additionally be used to cause zooplankton asphyxiation [40]. Finally, repeated cultivations throughout the year would allow to observe the dynamics of both microalgae species and protozoa under varying seasonal conditions. This would give insight into the respective advantages of both species depending on environmental factors.

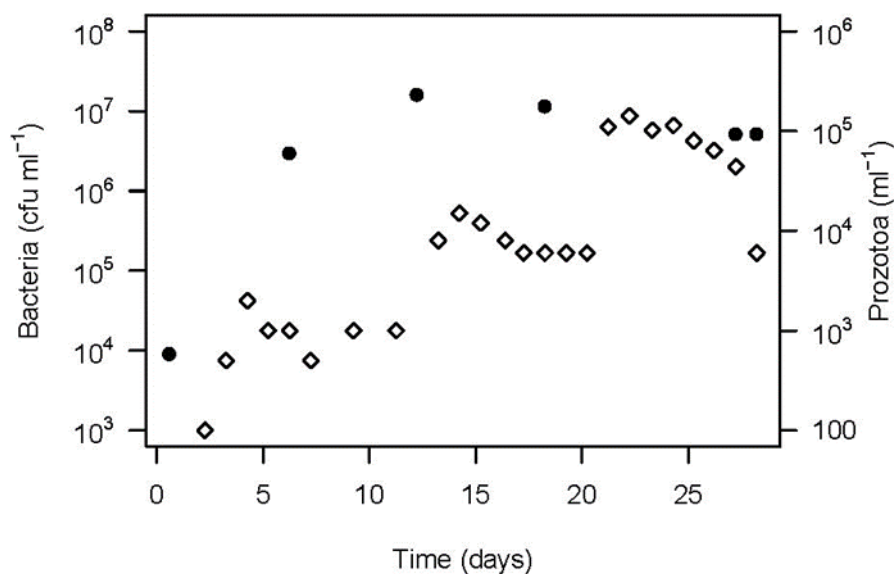


Figure 7 Number of protozoa (open diamonds) and titer of bacteria (closed points) in the pilot-scale cultivation.

### 3.3. Nutrient removal

All laboratory cultures surpassed 98 % removal efficiency of nitrate and 99 % removal efficiency of phosphate, which confirms that RAS water has a nutrient composition that is suitable for treatment with microalgae. Average nutrient removal efficiencies were

98.73±0.06 % and 99.46±0.04 % for nitrate and phosphate respectively. Nutrient removal efficiency obtained in the present study was in line with previous results that also used aquaculture water [10,29,35,43], and even exceeded results obtained by us [9]. This could be related to the fact that protozoa presence did not affect microalgae performance.

Pilot-scale cultivation showed that the rapid decline of nitrate in the cultures reduced overall productivity and yielded a lower final dry weight compared to other studies, as explained in the section 'Growth of microalgae'. This has been reported previously [5], where a cultivation of *T. obliquus* reached 18.7 g l<sup>-1</sup> after 21 days at a similar time of the year and it was suggested this was due to an insufficient nutrient supply, rather than an inhibitory effect by the RAS water. Here, the course of the C:N ratio during the experiment (Figure 8) shows a rapid increase up to day 10, followed by slow decline that remains at elevated levels (compared to unlimited conditions reported in [5]). This could indicate a nitrate limitation caused by a supply rate if RAS water that does not meet the demand of the growing culture. Consequently, a comparatively high average nitrate removal efficiency of 98.6 ± 0.001 % was obtained and a total amount of 201.09 g of NO<sub>3</sub>-N were removed during the experiment (0.387 g day<sup>-1</sup> m<sup>-2</sup>). Nitrate concentrations lower than 3 mg NO<sub>3</sub>-N l<sup>-1</sup> were reached in the cultivation (Supplementary Material Figure S3), which makes water suitable for reuse in a RAS after microalgae separation [44,45] and also fulfils nitrogen requirements for water discharge, as ammonium nitrogen is practically negligible [45,46]. We note that the results obtained do not allow inferences about the limitation by other nutrients or their removal efficiency. However, at least for phosphate we expect that is removed as well, as its uptake is typically very efficient (our own results from the laboratory-scale experiments and a previous study [9]). Finally, comparing with other studies [4,23], removal efficiency obtained was in line with them, even

higher taking into account that this study was carried out with real water from the fish tank of a RAS.

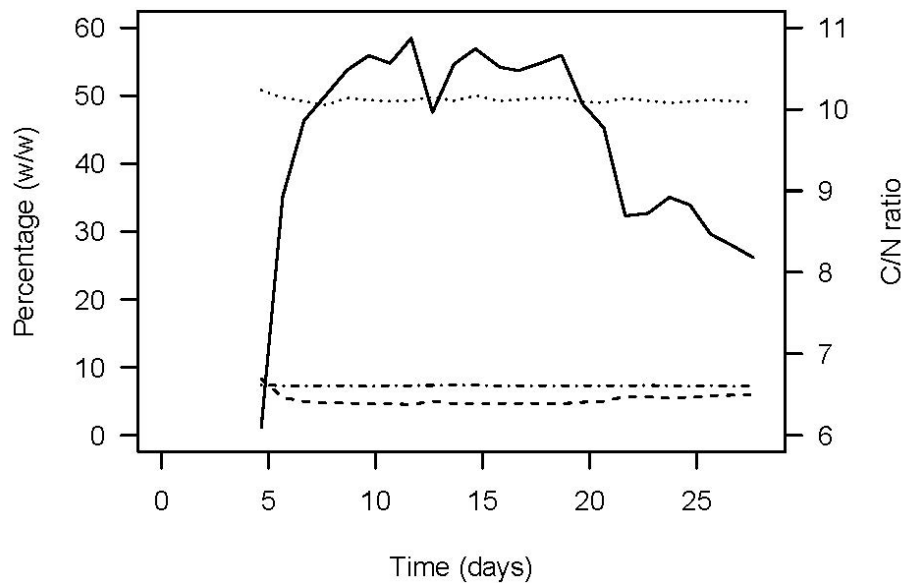


Figure 8 C:N ratio (solid line) of the microalgae biomass during the pilot-scale experiment. Percentages of elements (w/w) are shown for C (dotted line), H (dot-dashed line) and N (dashed line).

#### 4. Conclusions

Co-cultivation experiments were carried out successfully, as both microalgae, *C. vulgaris* and *T. obliquus*, were able to grow robustly and perform satisfactorily both in terms of biomass productivity and nutrient removal during prolonged periods of time without suffering crashes. None of the species was completely replaced, which suggests that they can be kept in co-culture over prolonged periods and may be a feasible approach to buffer species-specific crashes. Although protozoa were detected both in laboratory- and pilot-scale cultures, their effect was scale-dependent. The results suggest that the number and identity of protozoa in a culture determine to what degree they affect algal growth and that this effect is species specific. The nutrient removal that was achieved is sufficient to recirculate water to the RAS with a previous separation step, however as a nutrient limitation occurs, higher biomass productivities could be achieved if more evaporation occurs.

Further research will have to address the dynamics of microalgae species during longer periods (including changes of season) and the interactions between more microalgae species that could be useful for aquaculture.

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### **Conflict of interest**

No potential financial or other interests that could influence the outcomes of the research are perceived.

### **Author contributions**

**Yaiza Tejido-Núñez** conceived, designed and conducted the experiments, analysed the data, drafted and revised the article. Yaiza Tejido-Nuñez (ytejido@ceit.es) is the corresponding author and takes responsibility for the integrity of the work as a whole, from inception to finished article.

**Enrique Aymerich** planned the study and revised the article.

**Luis Sancho** planned the study and revised the article.

**Dominik Refardt** conceived and designed the experiments, analysed the data and revised the article.

### **Informed consent, human/animal rights**

No conflicts, informed consent, human or animal rights applicable.

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