

Communication

# High-Density Microalgae Cultivation in Open Thin-Layer Cascade Photobioreactors with Water Recycling

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**Featured Application:** Open microalgae mass culture with water recycling as a sustainable feedstock for feed, fuels or chemicals.

**Abstract:** (1) Background: Recycling of water and non-converted nutrients is considered to be a necessity for an economically viable production of microalgal biomass as a renewable feedstock. However, medium recycling might also have a negative impact on algal growth and productivity due to the accumulation of growth-inhibiting substances. (2) Methods: Consecutive batch processes with repeated water recycling after harvesting of algal biomass were performed with the saline microalga *Microchloropsis salina* in open thin-layer cascade photobioreactors operated at a physically simulated Mediterranean summer climate. The impact of water recycling on culture performance was studied and the composition of the recycled water was analyzed. (3) Results: Water recycling had no adverse effect on microalgal growth and biomass productivity (14.9–21.3 g m<sup>-2</sup> d<sup>-1</sup>) if all necessary nutrients were regularly replenished and KNO<sub>3</sub> was replaced by urea as the nitrogen source to prevent the accumulation of K<sup>+</sup> ions. Dissolved organic carbon accumulated in recycled water, probably promoting mixotrophic growth. (4) Conclusion: This study shows that repeated recycling of water is feasible even in high-density cultivation processes with *M. salina* of more than 30 g L<sup>-1</sup> cell dry weight, increasing culture performance while reducing nutrient consumption and circumventing wastewater production.

**Keywords:** microalgae; *Microchloropsis salina*; open photobioreactor; thin-layer cascade; water recycling

## 1. Introduction

Algal biomass is widely recognized as a key feedstock for a future biobased economy and listed as a main sector of biomass supply in the EU's bioeconomy strategy [1,2]. Due to their ability to sequester carbon dioxide, harvest light energy, and utilize municipal or agricultural wastewater, algae can become

an integral part of a low-carbon circular bioeconomy [3]. In contrast to conventional crop plants, algae and especially microalgae yield higher productivities and consume less freshwater without competing for agricultural land [4,5]. Nevertheless, feasible microalgal production processes have only been developed for few specialty chemicals, nutraceuticals or other high-value products [6,7].

Despite these advantages, the main barrier to commercialization of microalgal commodities is the high costs of biomass feedstock production [8]. Among others, these comprise costs and energy consumption for water pumping, dewatering of dilute cultures as well as use of fertilizers for nutrient supply [9]. Therefore, low-cost open photobioreactor production systems are required that achieve high productivities and product concentrations [10,11]. Additionally, the recycling of the used process water is necessary to recover residual nutrients, all while reducing costs for medium preparation and wastewater treatment [12,13].

While raceway ponds are the most commonly employed open photobioreactors, they suffer from low biomass concentrations of up to 0.3–1.0 g L<sup>-1</sup> dry weight due to mutual shading and low flow turbulence in the high fluid layer depth of 15–30 cm [5,14]. An alternate, though far less recognized, production system designed to overcome these drawbacks is the open thin-layer cascade (TLC) photobioreactor [11]. Originally developed in the 1960s in Czechoslovakia, this photobioreactor offers the advantage of a very high surface-to-volume ratio in a thin fluid layer of 5–8 mm, gravimetrically flowing down a sloped lane [15,16]. Recently, the TLC has been redesigned and investigated indoors under a realistic physical reproduction of a southern Spain summer climate. Thereby, a maximum cell dry weight (CDW) concentration of 50 g L<sup>-1</sup> was achieved with the saline microalga strain *Microchloropsis salina* (formerly *Nannochloropsis salina*) in a nutrient replete batch process [17], while *M. salina* biomass with a maximum lipid content of 46% (w/w) and up to 6.6 g L<sup>-1</sup> of total lipids were produced at nutrient-limited conditions [18].

Recycling of growth medium in microalgae processes has mostly been studied in dilute cultures (<6 g CDW L<sup>-1</sup>) or cost intensive closed photobioreactors. Reported impacts of water recycling on algal cell growth and productivity appear to be divergent and system-dependent. In that context, water recycling in semi-continuous outdoor raceway pond cultivations was suggested to result in increased *Tetraselmis* sp. biomass productivity due to mixotrophic growth on dissolved organic carbon (DOC) sources [19]. In contrast, no significant effect of water recycling on growth was observed with *Tisochrysis lutea* and *Nannochloropsis* sp. in a 220 L flat-panel photobioreactor [20], as well as with *M. salina* in shake flasks [21]. Moreover, a decrease in culture performance after water recycling and nutrient replenishment was reported with *Nannochloropsis* sp. in 120 L closed annular photobioreactors [22], as well as with *Scenedesmus* sp. in shake flasks [23]. This decrease was attributed to the accumulation of cell debris and auto-inhibitory substances. In this respect, Richmond and Zou [24] stated that growth of *Nannochloropsis* sp. in flat-plate photobioreactors declined at 6–7 g CDW L<sup>-1</sup> if the culture medium was not completely replaced with fresh water every 48 h. To the best of our knowledge, there are currently no data on water recycling studies with high-density cultivations >20 g CDW L<sup>-1</sup> in open photobioreactors, where an increased effect of growth inhibition due to cell debris and soluble substances like exopolysaccharides or small organic molecules, being secreted or released via cell lysis, can be expected [25,26].

In this study, we report on high-density microalgae processes with the marine microalga *Microchloropsis salina*, applying repeated recycling of process water after harvesting of the microalgae biomass. Pilot scale batch cultivations were carried out in open TLC photobioreactors, operated in the TUM-AlgaeTec Center (Technical University of Munich, Germany). Specifically, the TUM-AlgaeTec Center is designed to evaluate and scale photoautotrophic processes under controlled climate conditions by applying physical simulations of outdoor sunlight intensity, temperature and air humidity with dynamic day-and-night cycles [17].

## 2. Materials and Methods

### 2.1. Climate Simulation

TLC photobioreactors were located inside glass halls of the TUM-AlgaeTec Center illuminated by natural sunlight. An LED-based artificial sunlight (FutureLED, Berlin, Germany) supplemented the local irradiance in the 400–750 nm range according to a set dynamic target irradiance. Automatically controlled windows as well as an air conditioning system controlled the dynamic air temperature inside the glass halls. Irradiance and air temperature were reproduced daily according to climate data of 15 June 2012 in Almería, Spain, a typical summer's day with a 14:10 h light-dark cycle and a temperature range of 14–30 °C. Air temperature was measured in the shadow inside the climate simulation halls. The maximum photon irradiance in the photosynthetically active radiation (PAR) range (400–700 nm) was  $1823 \mu\text{mol m}^{-2} \text{s}^{-1}$ , measured at the mean height of the inclined reactor surface. Almería was chosen as a suitable location for microalgae mass production due to its warm and sunny climate, availability of seawater and its already large-scale greenhouse agriculture for fruit and vegetable export. A detailed description of the TUM-AlgaeTec Center, climate simulation, and its impact on water temperature in the TLC was given previously [17].

### 2.2. Microalgal Strain and Reaction Medium

The marine strain *Microchloropsis salina* (SAG 40.85), obtained from the Culture Collection of Algae at the University of Goettingen, Germany, was used in this study. A modified artificial seawater (ASW) [27] was used for growth of *M. salina*. ASW was composed of NaCl (27 g L<sup>-1</sup>), MgSO<sub>4</sub> · 7 H<sub>2</sub>O (6.6 g L<sup>-1</sup>), CaCl<sub>2</sub> · 2 H<sub>2</sub>O (1.5 g L<sup>-1</sup>), KNO<sub>3</sub> (1 g L<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (0.07 g L<sup>-1</sup>), Na<sub>2</sub>EDTA · 2 H<sub>2</sub>O (0.021 g L<sup>-1</sup>), FeCl<sub>3</sub> · 6 H<sub>2</sub>O (0.014 g L<sup>-1</sup>), and 1 mL L<sup>-1</sup> of a trace element solution of ZnCl<sub>2</sub> (0.04 g L<sup>-1</sup>), H<sub>3</sub>BO<sub>3</sub> (0.6 g L<sup>-1</sup>), CuCl<sub>2</sub> · 2 H<sub>2</sub>O (0.04 g L<sup>-1</sup>), MnCl<sub>2</sub> (0.4 g L<sup>-1</sup>), (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> · 4 H<sub>2</sub>O (0.37 g L<sup>-1</sup>). To prevent nutrient limitations in batch processes, ASW was additionally supplied with a concentrated feed medium, composed of the same components as ASW including the trace element solution but without NaCl, MgSO<sub>4</sub> and CaCl<sub>2</sub>. The production of a seed culture from laboratory to pilot-scale open TLC reactors was described previously [18].

### 2.3. Thin-Layer Cascade Photobioreactor Operation

Open TLC photobioreactors with an illuminated surface area of 8 m<sup>2</sup> consisted of two 4 m × 1 m polyethylene channels with an inclination of 1° in opposite direction, connected by a flow reversal module. A working volume of 55 L was circulated day and night at 2.4 L s<sup>-1</sup> by a centrifugal pump (MKPG, Ventaix, Monschau, Germany), resulting in a fluid layer depth of  $5.5 \pm 1.4$  mm. During the day, pH was maintained constant at pH 8.5 by addition of pure CO<sub>2</sub> via a mass flow controller (red-y smart, Voegtlin, Aesch, Switzerland) through perforated hoses (Solvocarab, Linde, Pullach, Germany) installed in the retention tank at the end of the lower channel. About 500 mL of tap water was automatically added via a magnetic valve to compensate for evaporation as soon as a binary level-sensor (LFFS, Baumer, Friedberg, Germany) registered a reduced volume in the retention tank. A detailed description of design, construction and computational fluid dynamics simulations of the TLC was given previously [17,28].

TLC reactors were inoculated with *M. salina* to achieve initially 0.3 g L<sup>-1</sup> CDW from a 4 m<sup>2</sup> pre-culture TLC reactor, identical in design to the 8 m<sup>2</sup> TLC but with two 2 m × 1 m channels. To prevent nutrient limitations and achieve a high biomass density in batch processes of 12 to 14 days, 2 times concentrated feed medium was added to the ASW medium at the start of the cultivation. Additional concentrated feed medium was added manually to keep the nitrate concentration at 1–3 g L<sup>-1</sup> or, in case urea was used as the nitrogen source, at 0.5–1.5 g L<sup>-1</sup> urea. On average, with fresh ASW, feed medium according to 0.80 g L<sup>-1</sup> KNO<sub>3</sub> or 0.24 g L<sup>-1</sup> urea was supplied per 1 g L<sup>-1</sup> CDW. In some processes, the sulfate concentration was also regularly controlled by manual addition of MgSO<sub>4</sub> · 7 H<sub>2</sub>O to keep sulfate at 0.8–2.6 g L<sup>-1</sup>. Since the TLC reactor is an open reaction system, microalgae cultures

were not axenic. Nevertheless, *M. salina* was the predominant species in all experiments, evaluated via light microscopy (Eclipse E200 LED MVRs, Nikon, Tokyo, Japan) and flow-cytometry (Cytotflex, Beckman-Coulter, Brea, USA).

#### 2.4. Cell Harvest and Water Recycling

At the end of each batch process, the algal biomass was harvested with an Evodos 10 spiral plate centrifuge (Evodos B.V., Raamsdonksveer, Netherlands) at  $3000\times g$  and a volume flow of approximately  $1.7\text{ L min}^{-1}$ . The supernatant was immediately used as recycled growth medium for the following cultivation. Ten liters of fresh ASW medium were added to replace the volume loss during the separation process. At a reaction volume of 55 L, this resulted in a water recycling rate of 82%. Subsequent batch processes were inoculated to  $0.3\text{ g CDW L}^{-1}$  from the pre-culture TLC reactor.

#### 2.5. Optical Density and Cell Dry Weight

Cell dry weight was determined via optical density measurement at 750 nm ( $OD_{750}$ ) in triplicate with a UV-Vis-Spectrophotometer (Genesys 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham, MA, USA). To obtain a linear correlation factor between CDW and  $OD_{750}$  for each experiment, CDW was also measured gravimetrically once per day by filtration on pre-dried and weighted glass-microfiber filters (GF/C, Whatman, GE Healthcare, Chicago, IL, USA) in triplicate. Loaded filters were washed with three times the sample volume of deionized water and dried at  $80\text{ }^{\circ}\text{C}$  for at least 48 hours before weighing. In case of the pre-culture TLC, CDW was measured gravimetrically once per week and estimated daily via  $OD_{750}$  measurement.

#### 2.6. Analysis of Growth Medium

##### 2.6.1. Nitrate, Urea, Sulfate and Salinity

Salinity and nitrate or urea, depending on the nitrogen source used, were analyzed daily in the supernatant of a centrifuged sample ( $14,500\times g$ , 4 min at room temperature, Espresso, Thermo Fisher, Waltham, MA, USA). The salinity was measured by a refractometer (Hanna Instruments, Voehringen, Germany). The urea concentration was measured photometrically at 340 nm via a urea/ammonia assay (r-biopharm, Darmstadt, Germany) based on a coupled enzymatic reaction with urease and glutamate dehydrogenase with stoichiometric conversion of NADH to  $\text{NAD}^+$ . For a quick estimation of nutrient availability on site, semi-quantitative test strips (Quantofix Nitrate/Nitrite, Macherey-Nagel, Düren, Germany) were used to estimate the nitrate concentration. In some processes, the sulfate concentration was also estimated twice per week with semi-quantitative test strips (Quantofix Sulfate, Macherey-Nagel, Düren, Germany).

##### 2.6.2. Salt Composition

Ion chromatography (Dionex IC25, Thermo Fisher Scientific, Waltham, MA, USA) with suppressed conductivity detection was used for quantitative determination of the anions  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$ . The anions were eluted with  $8\text{ mmol L}^{-1}\text{ Na}_2\text{CO}_3$  as the mobile phase at  $1\text{ mL min}^{-1}$  flow rate. The column was regenerated using  $2.5\text{ mmol L}^{-1}\text{ H}_2\text{SO}_4$ .

The monovalent cations  $\text{Na}^+$ , and  $\text{K}^+$  were determined by flame photometry (BWB Technologies, Berkshire, UK) after calibration with  $10\text{ mg L}^{-1}$  sodium and  $10\text{ mg L}^{-1}$  potassium. Complexometric titration with ethylenediaminetetraacetic acid (EDTA) as complexing agent was used for quantitative analysis of the divalent cations  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ .

##### 2.6.3. Dissolved Organic Carbon

Dissolved organic carbon concentrations were determined as non-purgeable carbon using a total carbon analyzer (TOC-L, Shimadzu, Japan) equipped with a combustion catalytic oxidation reactor ( $680\text{ }^{\circ}\text{C}$ ) and a non-dispersive infrared (NDIR) detector. The instrument was calibrated prior to sample

analysis by measuring standard solutions of potassium hydrogen phthalate. The linear dynamic range of the instrument was 1.7–25.0 mgC L<sup>-1</sup> with a limit of quantification of 1.7 mgC L<sup>-1</sup>. Measurements were performed after dilution (1:100 with milliQ water) and filtration of samples through 0.2 µm membrane filters (Whatman®cellulose filters).

#### 2.6.4. Xanthophyll and Chlorophyll a Concentration

High performance liquid chromatography coupled to a mass spectrometer (Exactive Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) was performed for quantitative analysis of chlorophyll a and xanthophyll in the supernatant of the recycled medium.

Prior to analysis, samples were diluted in a 1:10 ratio with the internal standard consisting of trans-β-Apo-8'-carotenal and zinc-phthalocyanine in acetonitrile/methanol (70/30 v/v). For complete removal of any flocculated substances, samples were filtered through a cellulose filter (0.2 µm pore size, Whatman®). Standard solutions were prepared by dilution of chlorophyll a, xanthophyll, zinc-phthalocyanine and trans-β-Apo-8'-carotenal, all purchased from Sigma Aldrich (Taufkirchen, Germany), with acetonitrile/methanol (70/30 v/v) covering the concentration range from 1 to 500 µg L<sup>-1</sup>. Calibration was done in triplicates.

Separation was performed using a C<sub>18</sub> column (Hypersil Gold, Thermo Fisher Scientific, Waltham, MA, USA) at 25 °C column temperature. The flow rate was set to 200 µL min<sup>-1</sup>. Twenty microliters of each sample were injected. The 13 min gradient elution profile was optimized employing acetonitrile/methanol (70/30 v/v), H<sub>2</sub>O and pure acetonitrile (see Table 1), resulting in good separation of all 4 components in the chromatogram. Atmospheric pressure chemical ionization (APCI) was operated in positive ion mode with a spray voltage of 5 kV, a sheath and auxiliary gas flow of 19 Lh<sup>-1</sup>, and a discharge current of 9 µA. The capillary temperature was set to 300 °C, the capillary voltage to 35 kV, the tube lens voltage to 110 kV, the skimmer voltage to 21 kV, and the vaporizer temperature to 500 °C. The components were identified by their specific retention time (RT) and the exact mass [M+H] with a mass accuracy of ± 7 ppm. RT and [M+H] of the components were as follows: trans-β-Apo-8'-carotenal, 4.50 min, 417.315; zinc-phthalocyanine, 5.77 min, 577.086; chlorophyll a, 11.04 min, 893.543. Note that xanthophyll is a collective term with the most prominent representatives of this group being lutein/zeaxanthin (2.60 min, 569.454), canthaxanthin (2.71 min, 565.404) and capsanthin (2.07 min, 585.430). Hence, for quantification of the xanthophyll content, the sum signal of all three components was used.

**Table 1.** Optimized elution profile for chlorophyll a, xanthophyll, zinc-phthalocyanine and trans-β-Apo-8'-carotenal on C<sub>18</sub> column at 25 °C.

Time, Min	H <sub>2</sub> O with 0.1% Formic Acid (FA), v%	ACN/MeOH 70/30 v/v with 0.1 v% FA, v%	ACN, v%
0	6	94	0
2	6	94	0
2.5	4	96	0
7.5	2	98	0
10	1.5	98.5	0
11	0	0	100
12	0	0	100
13	6	94	0

#### 2.7. Biomass Productivity and Specific Growth Rate

The volumetric and areal productivity P<sub>V</sub> and P<sub>A</sub> were calculated from the produced amount of cell dry weight per liter or per surface area, produced between time t and the starting time of the process t<sub>0</sub>:

$$P_V = (c_x(t) - c_x(t_0)) \cdot (t - t_0)^{-1} \quad (1)$$

$$PA = PV \cdot V \cdot A - 1 \quad (2)$$

with the cell dry weight concentration  $c_x$ , the reaction volume  $V$  and TLC reactor surface area  $A$ .

The biomass specific growth rate  $\mu$  in the exponential phase during the first five days of a batch cultivation was estimated based on the daily CDW concentration at 10:00 a.m. The nonlinear least squares fit and 95% confidence intervals were calculated with the Levenberg–Marquardt algorithm (Matlab R2017a, Mathworks, Natick, MA, USA) according to the function  $c_x(t) = c_x(t_0) \cdot e^{\mu t}$ .

### 2.8. Statistical Approach

Each series of eight consecutive batch processes with water recycling was performed once ( $n = 1$ ) in a respective timeframe of four months. Note that replication of consecutive processes may lead to a potential dependence of subsequent cultivations on previous cultivations. A 95% confidence interval level was used to evaluate differences in specific growth rates between cultivations in a single process series. A two-tailed paired t-test was calculated to evaluate growth rates between two series of consecutive batch processes. Overall CDW and productivity data of each process series were based on the mean and single standard deviation of CDW or productivity over time of all cultivations in this respective series. In this respect, linear interpolation between samples was used to calculate means when samples were taken at different time points in different cultivations.

## 3. Results and Discussion

### 3.1. Microalgae Processes with Water Recycling

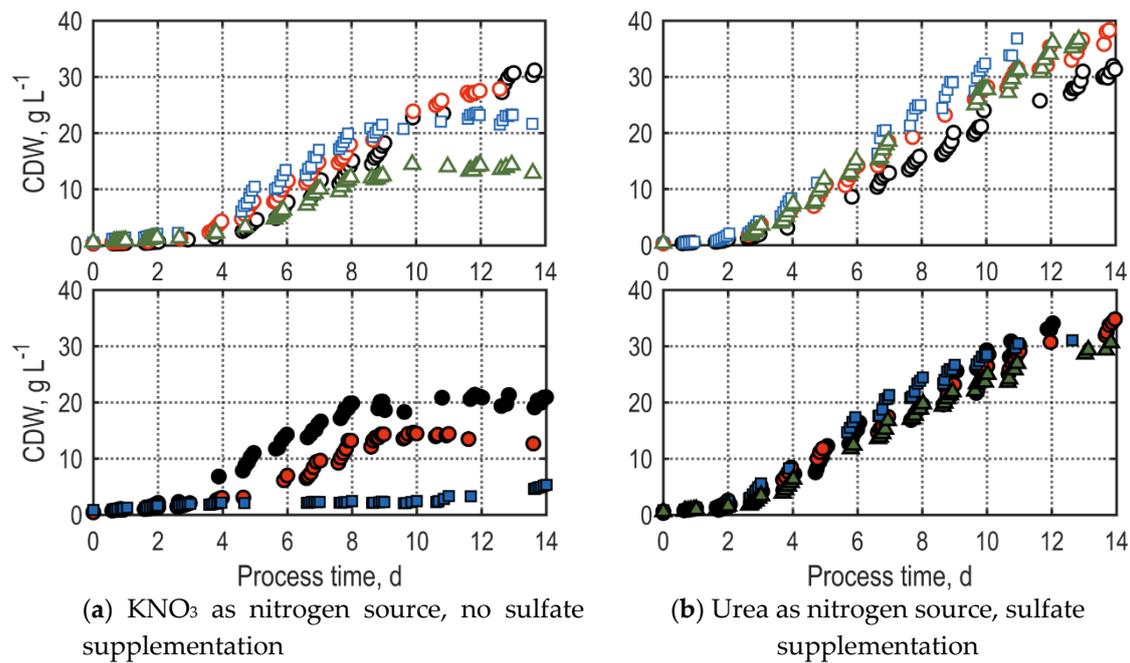
Batch processes with *Microchloropsis salina* were performed in 8 m<sup>2</sup> open TLC photobioreactors at a physically simulated Mediterranean summer climate. Cells were harvested by centrifugation at the end of each process after 12 to 14 days. The supernatant was supplemented with a concentrated feed medium solution and used as recycled growth medium for subsequent cultivations.

In the first process series, with a total of seven consecutive batch processes with medium recycling, KNO<sub>3</sub> was employed as the standard nitrogen source. Using fresh ASW medium, an overall biomass productivity of 15.6 g m<sup>-2</sup> d<sup>-1</sup> (2.3 g L<sup>-1</sup> d<sup>-1</sup>) was achieved after 14 days at a CDW of 31.2 g L<sup>-1</sup> (Figure 1). While single reuse of growth medium had no negative effect on growth rate and process performance, the second and third recycling resulted in a stationary CDW of 22.8 ± 0.7 and 13.8 ± 0.5 g L<sup>-1</sup>, respectively, thereby reducing the productivity at the end of each process to 10.7 and 6.2 g m<sup>-2</sup> d<sup>-1</sup>, respectively. This observation was additionally emphasized by a decrease in the specific growth rates during the first 5 days of each cultivation from 0.63 ± 0.15 d<sup>-1</sup> with fresh ASW to 0.38 ± 0.06 d<sup>-1</sup> with three times recycled water (Figure 2a). The batch process with four times reused water showed a slight recovery to a productivity of 10.0 g m<sup>-2</sup> d<sup>-1</sup>; due to an increased volume loss during the preceding cell harvest, the addition of 15 L of fresh ASW was required instead of the usual 10 L ASW. A sixth reuse of water, however, followed the negative trend in microalgal growth with increasing number of medium recycling steps, reaching only 5.3 g L<sup>-1</sup> CDW and a productivity of 2.2 g m<sup>-2</sup> d<sup>-1</sup> after 14 days.

The analysis of salt concentrations showed an accumulation of potassium ions from 0.4 g L<sup>-1</sup> in fresh ASW to 9.9 g L<sup>-1</sup> at the end of the sixth water recycling process, which could be attributed to the regular addition of KNO<sub>3</sub> as nitrogen source in the concentrated feed medium. Though potassium facilitates important physiological functions in ion and especially phosphate transport, osmosis and enzyme activity [29,30], this increase in K<sup>+</sup> concentration by a factor of nearly 25 might cause ion imbalances and possibly has a detrimental effect on algal growth [31].

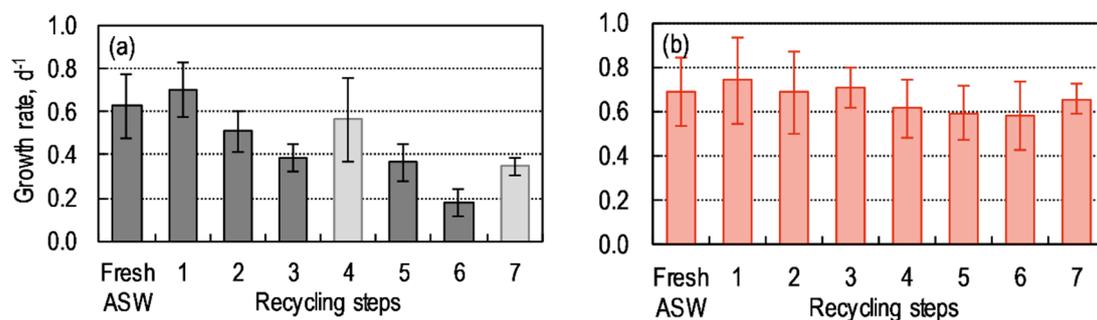
Furthermore, a sulfate depletion was observed, which may also decrease algae growth efficiency since a low sulfate availability—being an ubiquitous element of amino acids, vitamins and cofactors—is generally associated with a decreased photosynthetic activity and protein synthesis [32]. In that context, sulfate was only partially replenished by the addition of 10 L fresh ASW at the start of each batch process, as it was not included in the feed solution. Supplementing 2.6 g L<sup>-1</sup> MgSO<sub>4</sub> · 7 H<sub>2</sub>O after the seventh water recycling, however, resulted in a partial recovery of CDW concentration and productivity

to 19.2 g L<sup>-1</sup> and 9.5 g m<sup>-2</sup> d<sup>-1</sup>, respectively (Figure A1), indicating that a sulfate limitation was one but not the only factor limiting algal growth and productivity in recycled water.



**Figure 1.** Consecutive batch processes applying water recycling after harvesting of *M. salina* in thin-layer cascade reactors at physically simulated climate conditions. Left side: Water recycling without sulfate supplementation and with KNO<sub>3</sub> as nitrogen source. Right side: Water recycling with sulfate supplementation and with urea as nitrogen source. Cultivation with fresh artificial seawater (ASW) (○), 1st recycling (○), 2nd recycling (□), 3rd recycling (△), 4th recycling (●), 5th recycling (◐), 6th recycling (■), and 7th recycling (▲).

The mean CDW concentration and overall biomass productivity at the end of eight consecutive batch processes were 19.0 ± 8.5 g L<sup>-1</sup> and 9.1 ± 4.2 g m<sup>-2</sup> d<sup>-1</sup>, respectively (Figure 3). The specific growth rate during the first 5 days of each process decreased from 0.63 ± 0.15 d<sup>-1</sup> with fresh ASW to a minimum of 0.18 ± 0.07 d<sup>-1</sup> after the sixth water recycling (Figure 2).



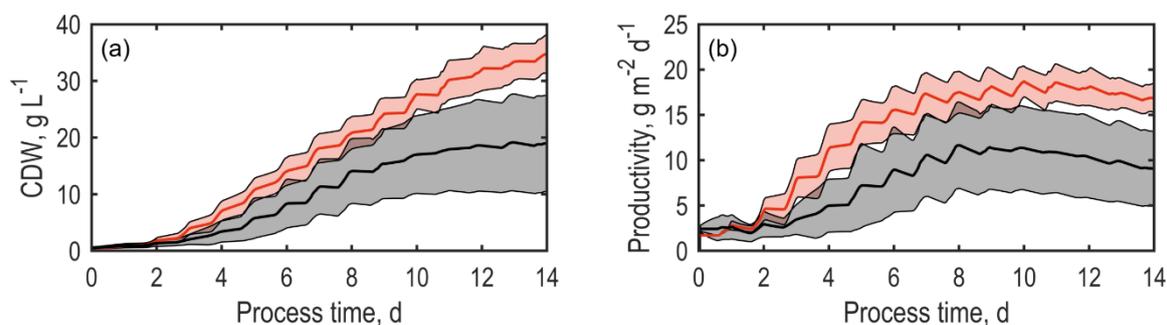
**Figure 2.** Specific growth rates of *M. salina* during the first 5 days of each batch process in thin-layer cascade photobioreactors applying water recycling. (a) Eight consecutive batch processes with water recycling were performed with KNO<sub>3</sub> as nitrogen source and without sulfur supplementation in most of the batch processes (dark grey). At the 4th recycling step, additional growth medium had to be supplemented to compensate a water loss during the preceding cell harvest. At the beginning of the 7th recycling step, sulfate was supplemented to mitigate a possible sulfate limitation; (b) 8 consecutive batch processes were performed with regular sulfate supplementation and urea as nitrogen source. Error bars represent 95% confidence intervals.

### 3.2. Water Recycling with Urea and Sulfate Supplementation

A second series of eight batch processes with *M. salina* in a TLC photobioreactor with repeated water recycling was performed at the same simulated Mediterranean summer climate. To circumvent algal growth inhibition due to the accumulation of potassium ions,  $\text{KNO}_3$  as a nitrogen source was replaced with urea in ASW and in the concentrated feed medium. In this case,  $0.3 \text{ g L}^{-1}$  of urea was used per  $1 \text{ g L}^{-1}$  of  $\text{KNO}_3$ , such that the molar nitrogen concentration remained constant. Additionally, the sulfate concentration in the growth medium was regularly measured and manually supplemented with  $4.5 \text{ g L}^{-1} \text{ MgSO}_4 \cdot 7 \text{ H}_2\text{O}$  when the sulfate concentration dropped below  $0.8 \text{ g L}^{-1}$  in order to restore the original sulfate concentration in the ASW medium.

Analogous to the initial batch process series, a CDW concentration of  $31.3 \text{ g L}^{-1}$  was achieved after 14 days with fresh ASW. However, the changes in nitrogen supply and sulfate supplementation surprisingly instigated an overall productivity increase by 37% from  $15.6 \text{ g m}^{-2} \text{ d}^{-1}$  ( $2.3 \text{ g L}^{-1} \text{ d}^{-1}$ ) with fresh ASW to a maximum of  $21.3 \text{ g m}^{-2} \text{ d}^{-1}$  ( $3.1 \text{ g L}^{-1} \text{ d}^{-1}$ ) with twice recycled water. Thereafter, the productivity remained stable with three- and four-times recycled water at  $19.4$  and  $19.3 \text{ g m}^{-2} \text{ d}^{-1}$ , respectively. In contrast to the initial batch process series, the productivity with five- to seven-times reused water decreased only slightly, from  $16.8$  to  $14.9 \text{ g m}^{-2} \text{ d}^{-1}$ . With urea and sulfate supplementation, specific growth rates during the exponential phase in the first 5 days of each process remained stable at  $0.58$ – $0.74 \text{ d}^{-1}$  with no significant difference at the 95% confidence interval level (Figure 2b). However, growth rates with urea and sulfate supplementation were significantly higher ( $p = 0.0052$ ) than growth rates in the first series of batch processes with water recycling (Figure 2a), in which  $\text{KNO}_3$  was used and sulfate was not regularly supplemented.

In summary, the mean CDW at the end of eight serial batch processes with water recycling was  $34.7 \pm 3.5 \text{ g L}^{-1}$ , thereby increasing the mean overall productivity by 86% to  $16.8 \pm 1.7 \text{ g m}^{-2} \text{ d}^{-1}$  compared to water recycling without sulfur supplementation and  $\text{KNO}_3$  replacement with urea (Figure 3). Thus, the decline in microalgal growth and productivity over eight serial batch processes with sequential water recycling was effectively mitigated by regular nutrient supplementation and replacement of  $\text{KNO}_3$  with urea to prevent the accumulation of potassium ions.



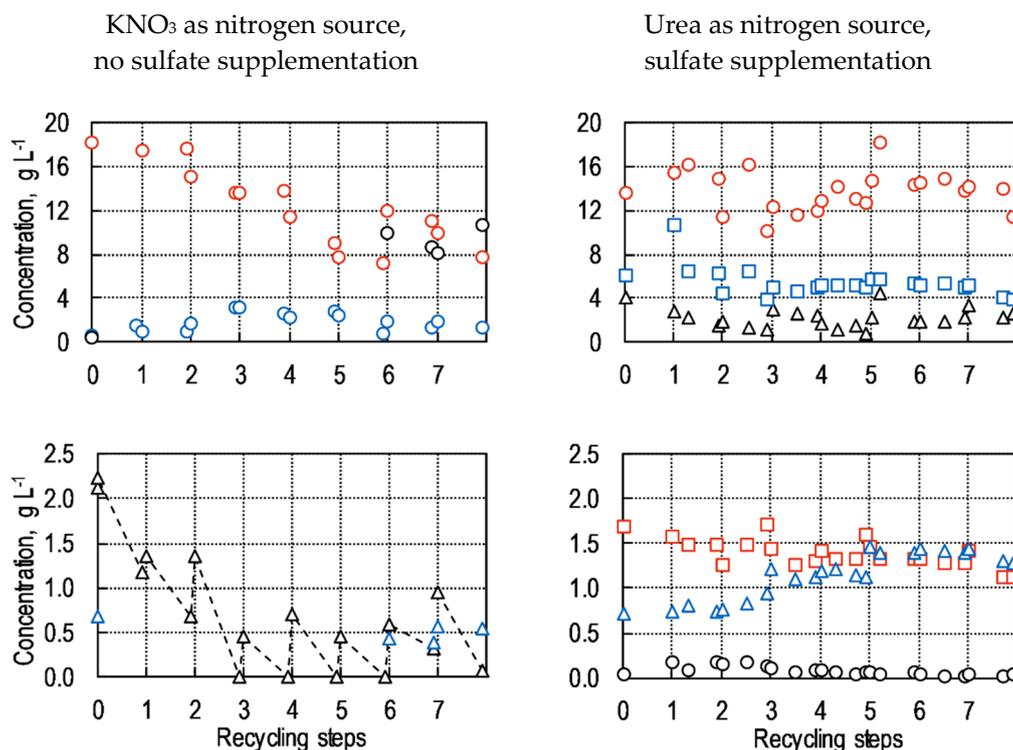
**Figure 3.** Mean cell dry weight (CDW) concentration (a) and areal biomass productivity (b) with water recycling in 8 consecutive batch processes with *M. salina* in thin-layer cascade photobioreactors. Water recycling was performed without regular sulfur supplementation and with  $\text{KNO}_3$  as nitrogen source (black), or with sulfur supplementation and urea as nitrogen source (red). Shaded areas represent  $\pm 1$  standard deviation of the respective consecutive processes.

### 3.3. Impact of Recycling on Water Composition

To investigate the effect of the improved water recycling strategy—applying sulfate supplementation and using urea as the nitrogen source—on the water composition, a detailed analysis of ion concentrations, dissolved organic carbon and carotenoid concentrations was performed. Because no sulfate was supplemented in the first six recycling steps of the process series using  $\text{KNO}_3$ , apart from the ASW additions that were needed to compensate water losses during cell harvest, sulfate concentration steeply declined until being completely consumed after three cultivations. Additionally,

the accumulation of  $K^+$  led to an increase in salinity which had to be mitigated by a reduction in NaCl in the fresh ASW for water compensation. This, in turn, decreased  $Cl^-$  (and probably  $Na^+$ ) concentration over time from 18.2 to  $7.7\text{ g L}^{-1}$  after the seventh water recycling.

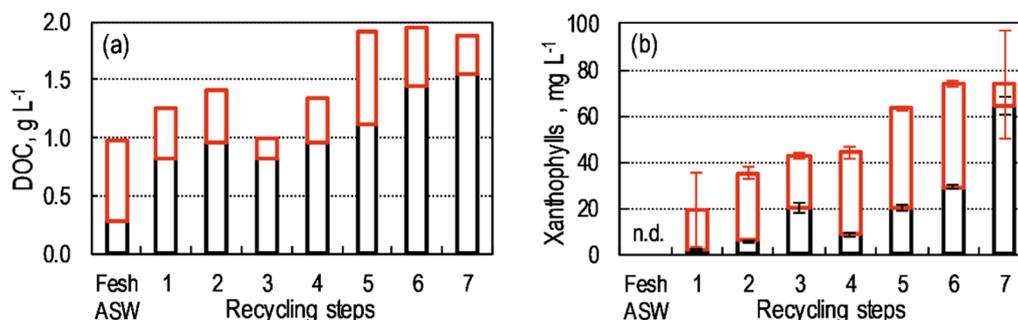
However, the water analysis of the improved process series with urea and sulfate supplementation confirmed that none of the measured salt species ( $Na^+$ ,  $Cl^-$ ,  $SO_4^{2-}$ ,  $Ca^{2+}$ ,  $Mg^{2+}$  and  $K^+$ ) was depleted over the series of eight batch processes while  $Mg^{2+}$  was the only component showing a slight accumulation from  $0.72\text{ g L}^{-1}$  in fresh ASW to a maximum of  $1.46\text{ g L}^{-1}$  after five recycling steps (Figure 4, Table A1). Since reference seawater has a similar  $Mg^{2+}$  concentration of  $1.31\text{ g L}^{-1}$  [33], it is unlikely that this increase would be adverse. The accumulation of  $K^+$  ions up to  $9.9\text{ g L}^{-1}$ , which was seen in the first recycling series, was effectively prevented by the replacement of  $KNO_3$  with urea as nitrogen source.



**Figure 4.** Ion concentrations in the growth medium over 8 serial batch processes with *M. salina* in thin-layer cascade photobioreactors. Left side: Batch processes with water recycling without regular sulfate supplementation and with  $KNO_3$  as nitrogen source.  $K^+$  and  $Mg^{2+}$  were only determined in the 6th and 7th recycling step.  $Na^+$  and  $Ca^{2+}$  were not determined. Sulfate measurements were connected linearly to improve clarity. At the start of the 4th and 7th recycling step, additional sulfate was added (see Section 3.1). Right side: Batch processes with water recycling with sulfate supplementation and with urea as nitrogen source.  $Cl^-$  (○),  $NO_3^-$  (○),  $K^+$  (○),  $SO_4^{2-}$  (△),  $Mg^{2+}$  (△),  $Na^+$  (□), and  $Ca^{2+}$  (□) concentration.

Since water recycling leads to an accumulation of organic matter due to algal cell lysis and secretion of organic substances, e.g., exopolysaccharides or small organic molecules [25,26], the concentration of dissolved organic carbon was measured after inoculation and before harvest of each cultivation (Figure 5a). Though DOC was partially removed with each cell harvest, it increased from  $0.28\text{ g L}^{-1}$  at the start of the first process with fresh ASW to a maximum of  $1.94\text{ g L}^{-1}$  at the end of a cultivation with six times recycled water. Therefore, DOC species might have served as a heterotrophic substrate for algal growth in recycled water, leading to an increased biomass productivity. This hypothesis is supported by a 34% increase in the yield coefficient for urea (as an indicator for overall nutrient consumption) from  $4.2\text{ g CDW g}^{-1}$  urea in fresh ASW to a maximum of  $6.4\text{ g CDW g}^{-1}$  urea in three

times recycled water. Similar observations of enhanced growth in recycled water due to heterotrophic substrates were made with *Nannochloropsis oceanica* [34], *Chlorella vulgaris* [35] and *Tetraselmis* sp. [19]. In this respect, mixotrophic growth of *M. salina* was found to increase with the availability of a nitrogen source [36].



**Figure 5.** (a) Concentration of dissolved organic carbon (DOC) and (b) sum of xanthophylls lutein, canthaxanthin and capsanthin in 8 serial batch processes with *M. salina* in thin-layer cascade photobioreactors for each water recycling step with sulfur supplementation and urea as nitrogen source. Black bars represent the concentration at the beginning of each cultivation, red bars show the concentration before harvest.

This DOC accumulation over the batch process series was accompanied by an intensifying coloration of the recycled water, ranging from colorless to the yellow-orange spectrum. This coloration was caused by an accumulation of xanthophylls (lutein, canthaxanthin and capsanthin, Figure 5b) reaching up to  $74 \pm 1.4 \text{ mg L}^{-1}$ . These photosynthetic pigments are likely released into the water due to algae cell lysis occurring over the extended cultivation period. In contrast, chlorophyll a only accumulated in the supernatant in each respective cultivation, but was removed during cell harvest; hence, no accumulation over multiple processes was observed (data not shown).

#### 4. Conclusions

Water recycling studies with high-density *M. salina* cultures were performed in open thin-layer cascade photobioreactors. Using urea instead of  $\text{KNO}_3$  as the nitrogen source and regular supplementation of all necessary nutrients (N, P, Fe, S and trace elements) allowed for repeated water recycling without adversely affecting microalgal growth and productivity in eight consecutive batch processes. Accumulating dissolved organic carbon species, secreted or released via cell lysis, probably served as heterotrophic substrates in recycled water, leading up to a 37% increased biomass productivity, while at the same time reducing nutrient consumption for biomass growth by up to 34%. These results demonstrate that multiple water recycling in phototrophic *M. salina* biomass production processes is a viable strategy, even in high-density cultures of more than  $30 \text{ g L}^{-1}$  cell dry weight, leading to a reduced nutrient consumption and circumventing wastewater production. Applied to different microalgae species, this technique can contribute to the development of efficient bioprocesses for the production of feed, fuels or chemicals.

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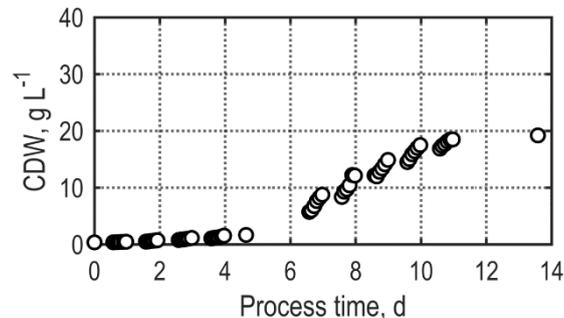
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## Appendix A



**Figure A1.** Batch processes with 7 times recycled water with  $\text{KNO}_3$  as nitrogen source in thin-layer cascade reactors at physically simulated climate conditions. Supplementation of  $2.6 \text{ g L}^{-1} \text{ MgSO}_4 \cdot 7 \text{ H}_2\text{O}$  at the start of the process led to a partial recovery of *M. salina* growth and productivity.

**Table A1.** Average concentration changes of ions in cultivation water per batch process with urea and sulfate supplementation. Error intervals represent 95% confidence intervals of the linear regression fit.

Ion	Average Concentration Change per Batch, $\text{g L}^{-1}$
$\text{Na}^+$	$-0.313 \pm 0.235$
$\text{Ca}^{2+}$	$-0.047 \pm 0.024$
$\text{Cl}^-$	$-0.022 \pm 0.379$
$\text{K}^+$	$-0.015 \pm 0.008$
$\text{Mg}^{2+}$	$0.103 \pm 0.025$
$\text{SO}_4^{2-}$	$-0.017 \pm 0.186$

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