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Improving Microalgae Feasibility Cultivation: Preliminary Results on Exhausted Medium Reuse Strategy

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Abstract: Although microalgae exploitation is very promising, process sustainability is undermined by biomass production and harvesting. Among the various bottlenecks of the production process, particular attention should be paid to the water footprint. Indeed, a huge volume of water is required in microalgae production. Water reuse can support both the water footprint and medium cost reduction, saving water and unconverted substrates. The present study reports preliminary results regarding the utilization of a water reuse strategy for two Chlorophyta microalgae under batch conditions. Growth parameters and chlorophyll content are monitored and the optimal amount of reused medium is assessed. The results show that 70% of the medium can be reused with no loss of specific growth rate and chlorophyll fraction for *Pseudococcomyxa simplex* in three consecutive batch cultivations. By contrast, a significant decline in *Chlorella vulgaris* growth was observed after the first cultivation in reused medium, across all tested conditions.

Keywords: microalgae; water footprint; substrate recovery; medium reuse

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1. Introduction

The increasing world population and the climate crisis present several pressing issues that need to be addressed [1,2]. The population growth will lead to such a high food demand that it could only be satisfied by more than one Earth [3]. A potential solution to this food shortage is the introduction of microalgae into both the human diet and animal feed [4]. Indeed, microalgae are a promising industrial source of edible and high-quality proteins [5]. Moreover, microalgal strains with high oil content are an efficient feedstock for producing carbon neutral biodiesel, supporting the transition to renewable energy sources [2]. A further advantage of microalgae is related to the wide spectrum of secondary metabolites characterized by impressive biological effects, including antioxidant, antimicrobial, anticancer, antiviral, and immunomodulatory activities. These metabolites—mostly peptides and phenolic compounds—are very attractive to pharmaceutical and nutraceutical industries [6].

Microalgae are photoautotrophic microorganisms that include both eukaryotes (microalgae) and prokaryotes (cyanobacteria) [7]. The photosynthetic system allows the conversion of energy-poor inorganic substances—such as CO_2, water and mineral salts—into energy-rich organic compounds necessary for cell growth.

Microalgae are typically cultivated at large industrial scale by supplying a microalgae suspension with a light and inorganic carbon source, usually CO_2 [8]. Cultivation processes are divided into open and closed systems, as detailed in the cultivation literature. Open systems are more affordable and easier to manage since they rely on environmental conditions for light and CO_2 supply. In contrast, closed systems offer better process control and minimize contamination risks. However, open systems have less control over growth and

suffer higher water loss due to evaporation, while closed systems incur higher costs [9,10]. The CO_2 supply can be increased in both systems by using a gas stream enriched with CO_2 at concentrations of up to 15% v/v [11]. Aeration is achieved through spargers at the reactor bottom, ensuring proper culture mixing, preventing cell adhesion, and reducing biofilm formation. Photoautotrophic cultivation has the advantages of fixing CO_2 without the need of an organic carbon source but it is characterized by an upper limit in cell concentration due to low photosynthetic efficiency [10]. The light source can be sunlight or artificial illumination [12]. The light is irradiated from the source and is gradually adsorbed through the suspension. As the cell concentration increases, the light penetration depth is reduced as a consequence of the shielding effect of the cells, and the photosynthetic efficiency is reduced [13]. This shielding effect at high concentration requires a large reactor working volume to fulfil high mass production. Consequently, the substantial consumption of water and nutrients emerges as a critical concern in industrial processes. In general, raw materials and wastewater management have a larger impact on the operating costs (OPEX) in microalgal cultivation [14].

Microalgal cultivation is a water-intensive process as water acts as a controlling tool for the temperature and as a medium for nutrient delivery. During the cultivation stage, water is mainly lost to evaporation and is involved in photosynthesis. A huge amount of water is lost in the stages of harvesting and drying. The management of the water used for microalgal cultivation requires particular attention to the following:

1. Reduce operating costs;
2. Reduce competition with human use of water;
3. Increase process sustainability [15].

The environmental impact of water for different processes is compared through the water footprint index (WF). The WF is an indicator of water use throughout the product value chain. Indeed, the WF considers the direct water use by the producer or the consumer and the indirect water use through the production of energy and raw materials necessary to construct and run the process [16].

Nutrients are a pressing item on the OPEX list. According to Yang et al. (2011) [17], to generate 1 kg biodiesel from microalgae, 3730 kg water, 0.33 kg nitrogen, and 0.71 kg phosphate are required. The investigation carried out by Yang et al. (2011) [17] has highlighted that a large fraction of the initial nutrients is still present in the medium after cultivation. In addition to the loss of money for the underexploited substrate, the presence of unconverted nutrients in the wastewater stream increases the cost of the wastewater treatment [17].

Potential solutions to reduce the pressure of water management and underexploited nutrients include wastewater recycling or reuse. The use of wastewater as a culture medium has the advantages of lowering overall production costs and utilizing the nutrients already present in it. The chemical and physical properties of aqueous waste used for microalgal growth vary based on its origin. Various types of domestic, agricultural, and industrial wastewater have already been tested for this purpose, yielding positive results [10]. Wastewater exploitation as culture media is a promising strategy to couple microalgae-based metabolite production with bioremediation purpose: due to their biosorption, bioaccumulation, and biodegradation capabilities, microalgae are a promising biological route for wastewater treatment, providing some high-value products too [18]. However, using wastes as water feedstock conflicts with regulations concerning when products should be delivered to pharma/food/feed markets.

The recycling of exhausted medium/water is a promising strategy when dealing with products devoted to pharma/food/feed markets. Reusing the medium offers the potential to reduce water consumption in upstream units and recover unconverted substrates, ultimately decreasing the water footprint (WF) and overall process costs. This technique can impact microalgal growth either positively or negatively. The effect on growth depends on the nature of the substances released by microalgae into the medium, making this impact strain-dependent. Furthermore, without medium purging, metabolites accumulate

with each reuse cycle, altering their growth effects [15]. Besides metabolites released by microalgae, growth in reused medium may also be influenced by other substances such as flocculating agents or contaminants' byproducts [18–20]. Therefore, the feasibility of water reuse needs to be investigated for each new strain studied. Several studies have shown the successful reuse of medium for known strains like *Chlorella* sp. and *Scenedesmus* sp. For instance, *Chlorella* spp. exhibited minimal decreases in growth rates after a single reuse cycle if harvested by centrifugation or filtration (harvesting methods that do not change the composition of the exhaust medium) [21–23]. Similarly, *Scenedesmus* spp. demonstrated comparable growth in reused medium to fresh medium after filtration, at both bench and pilot scales [24–26]. Batch tests involving several *Chlorophyta* and *Spirulina* genera are also available in the literature, but the results vary significantly from negative to unaffected or enhanced biomass productivity. This variance depends on the harvesting system, the treatment of the exhausted medium, and the strain [27]. However, most studies emphasize fresh inoculum in reused medium without assessing the stability of medium reuse strategies that avoid continuous inoculation. This creates ambiguities when scaling up to continuous or semi-continuous cultivation.

This paper provides preliminary results of the water reuse strategy applied to batch cultivation of two Chlorophyta microalgae: the industrially cultivated *C. vulgaris* and the relatively unknown *Pseudococcomyxa simplex*. The growth performances of these two strains have been tested in three consecutive batch cultivations using reused medium and always supplying a nitrogen source up to fresh medium concentration. The reuse strategy was optimized based on the fraction of exhausted medium purged and replaced with fresh medium. The results also included chlorophyll a content to assess the impact of the reuse strategy on the photosynthetic apparatus. The tests were conducted by inoculating cultured cells from the previous batch to evaluate the stability of the repeated batch cultivation strategy with partially or fully reused medium.

2. Materials and Methods

2.1. Strain Selection and Culture Medium

Two Chlorophyta microalgae were selected from the Algal Collection of University Federico II of Naples (ACUF): *Pseudococcomyxa simplex* (ACUF 127) and *Chlorella vulgaris* (ACUF 266). Both strains are mesophile aeroterrestrial microalgae and were cultivated in Bold Basal Medium using nitrate as a nitrogen source at an initial pH of 6.8. Medium recipe and strain details are reported in Table 1. *C. vulgaris* was chosen because it is already extensively cultivated on an industrial scale for both protein and high-value molecule production. Additionally, *C. vulgaris* has been used for wastewater bioremediation, demonstrating its resilience in suboptimal conditions [28]. *Pseudococcomyxa simplex* was selected as an alternative Chlorophyta strain with untapped potential, showing comparable biomass productivity to *C. vulgaris* in fresh medium batch cultivation (see Section 3). Moreover, *P. simplex* has proven resilient in harsh conditions, such as low pH and heavy metal presence, due to its protective algaenan layer [29].

Table 1. Culture medium recipe.

Compounds	Final Concentration (g/L)
$NaNO_3$	0.25
$CaCl_2$	0.0187
$MGSO_4*7H_2O$	0.075
K_2HPO_4	0.175
NaCl	0.025
KH_2PO_4	0.175
EDTA	0.05
H_3BO_3	0.01142
$FeSO_4*7H_2O$	0.0075

Table 1. *Cont.*

Compounds	Final Concentration (g/L)
$MnCl_2$*$4H_2O$	0.000025
$ZnCl_2$*$2H_2O$	0.00004
$CaCl_2$*$6H_2O$	0.000012
Na_2MoO_4*$2H_2O$	0.000024

2.2. Growth Test Operating Conditions

Growth tests were carried out in 50 mL operating volume flasks under continuous illumination (24/0 photoperiod, light/dark) at 80 μmol_{Ph} m^{-2} s^{-1} under constant and gentle mixing on an oscillatory plane (160 RPM). The temperature was set at 24 ± 2 °C. Growth occurred autotrophically and CO_2 from headspace air worked as a carbon source. CO_2 transfer and chemical absorption as carbonic acid were not controlled, and the absorption efficiency varied dynamically as pH increased due to nitrate depletion. No pH control system was used. All growth tests lasted until the first steady-state phase, and cultivation time was adjusted accordingly for each test. Strains were inoculated from solid phase to liquid medium (first growth), then stabilized in the media (fresh medium growth) before the start of the water reuse tests. All experiments reported in the manuscript were conducted in biological triplicate. The results presented in the figures and throughout the paper represent the mean values ± standard deviation. Every reagent has been purchased from Sigma-Aldrich® (St. Louis, MO, USA) culture grade.

2.3. Culture Monitoring

Each reaction system (triplicate) was monitored every day in terms of dry biomass concentration, chlorophyll content, liquid phase nitrate concentration, and pH. Biomass concentration was assessed by spectrophotometric (ONDA V10 spectrophotometer) measurement of optical density (OD) of the culture at 550 nm (the samples were diluted to fall within the calibration range of 0.1–0.8 OD); the dry mass concentration (X) has been reported on the basis of a relationship X = f(OD) previously determined for both strains. The suspension sample was centrifugated and soluble species were measured in the supernatant. Liquid-phase nitrate concentration was estimated using UV spectrophotometry at 220 and 280 nm according to Armstrong et al. (1963) [30]. Sodium nitrate (Sigma-Aldrich) was used for calibration. Chlorophyll content was estimated according to the assay proposed by Wellburn (1994) [31]:

- Solid–liquid methanol extraction in the dark at 36 °C for 24 h;
- Extracted samples were centrifuged;
- Chlorophyll concentration was measured in the liquid phase spectrophotometrically.

The specific biomass growth rate (μ_X), nitrate uptake yield (μ_N), and biomass–nitrate mass yield (Y_{X/NO_3^-}) were calculated according to Equations (1)–(3).

$$\mu_X = \frac{X(t) - X_0}{t - t_0} \quad (1)$$

$$\mu_N = \frac{N(t) - N_0}{t - t_0} \quad (2)$$

$$Y_{X/NO_3^-} = \frac{X(t) - X_0}{NO_3^-(t) - NO_{3\,0}^-} \quad (3)$$

where $X(t)$ and X_0 are the dry biomass concentration at inflection and the start of the exponential phase (respectively, t and t_0); $NO_3^-(t)$ and $NO_{3\,0}^-$ are the liquid phase nitrate concentration at inflection and the start of the exponential phase.

2.4. Medium Recycle and Purge Ratio

Figure 1 presents a synoptic diagram of the procedure followed for the tests. The initial cultures were carried out in fresh medium as the first step. At a pre-set time, 90% of the reaction volume was collected for biomass harvesting and supernatant storage. The remaining 10% of the suspension was used as inoculum for the subsequent growth cycle. The harvested biomass was extracted in methanol for chlorophyll assay. The stored supernatant was processed for the successive test, which aimed to assess the medium recycle. The purge ratio (PR) was assessed according to Equation (4):

$$\mathrm{PR} = \frac{V_{purged}}{V_f} \tag{4}$$

where V_{purged} is the volume of stored supernatant purged and replaced with fresh medium (BBM), and V_f is the final volume of the reaction system. The purge ratio ranged between 0% (no fresh medium) and 100% (only fresh medium). Tests were carried out at a PR equal to 0%, 10%, and 30% and were compared to the growth in fresh medium (PR = 100%). For tests carried out at PR < 100%, the nitrogen source was replenished in the culture broth up to the concentration set in fresh medium (150–180 mg/L of NO_3^-). The nitrogen source was depleted in each growth cycle. Other nutrients were integrated just by replacing purged supernatant with fresh medium.

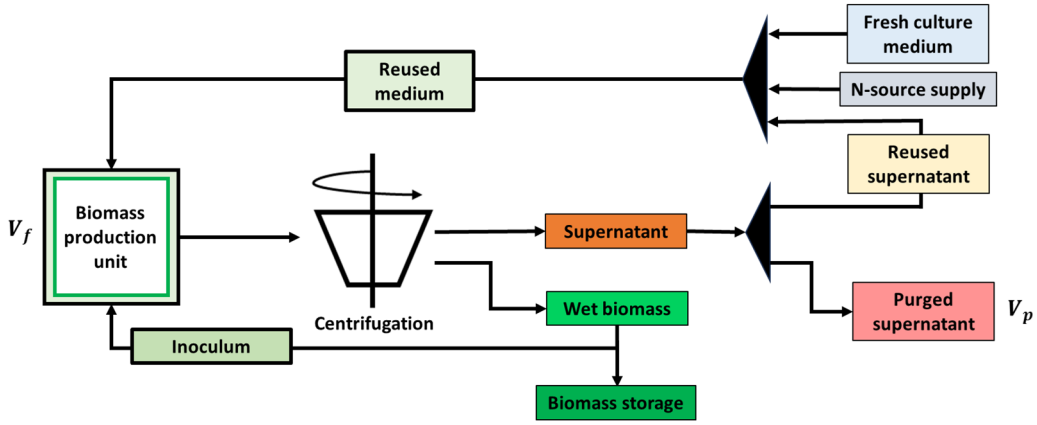

Figure 1. Synoptic diagram of the tests.

3. Results and Discussion

Figure 2 presents the results regarding the proposed medium reuse strategy (Figure 1) applied to the cultivation of the strains ACUF 127 and ACUF 266, specifically focusing on biomass growth rate. The biomass growth rate measured in each cycle (Figure 2a) was approximately equivalent to that in fresh medium (cycle 0) for *P. simplex* with a purge ratio (PR) of 30%. However, for *C. vulgaris*, none of the PRs tested yielded a growth rate comparable to fresh medium (Figure 2b), leading to a significant decline in specific biomass growth rate as the cycle number increases, even at a PR = 30%. For PRs below 30%, the biomass growth rate decreased with each reuse cycle, and this effect became more pronounced as the PR approached 0% for *P. simplex*, indicating the strain potential in this strategy. Notably, the impact of exhausted medium purging depends on the growth cycle. At PR = 10%, the biomass growth rate is similar to that of PR = 30% and fresh medium growth after two batches, but it drops to 0.06 g/L day in the third batch. Likewise, the biomass growth rate of *C. vulgaris* increases with PR when it rises from 10% to 30% in the third batch. Furthermore, in the first exhausted medium cultivation test, the biomass growth rate was similar across all PRs and for both strains, suggesting that the nitrogen supply was sufficient to support growth and that other substrates or accumulated byproducts did not limit the growth.

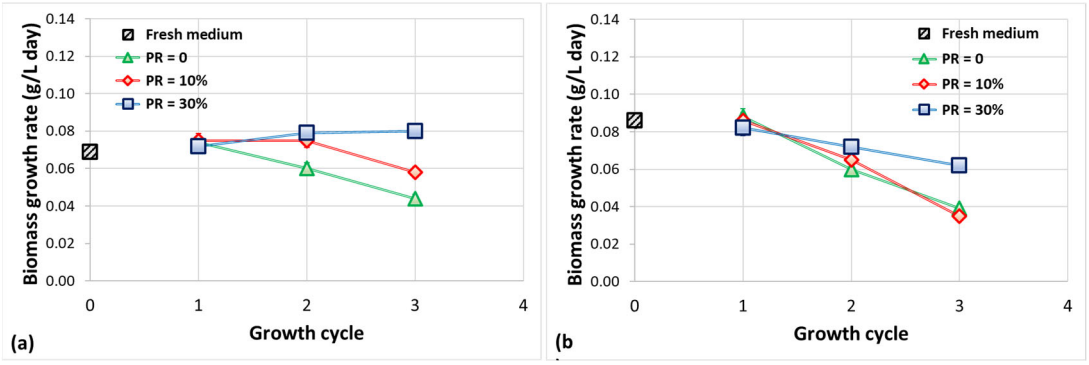

Figure 2. Results of exhausted medium reuse tests across three consecutive growth cycles at different purge ratios in terms of biomass growth rate: (**a**) *P. simplex* (ACUF 127); (**b**) *C. vulgaris* (ACUF 266). Data assessed for the fresh medium growth are also reported.

Figure 3 presents a detailed overview of the PR effect on *P. simplex* growth in terms of pH shift, maximum biomass and chlorophyll a concentration, nitrate uptake rate, and biomass–nitrate yield. Tests carried out at a PR smaller than 30% showed the following issues (Figure 3a–d):

- The maximum biomass concentration decreases slightly in the batch growth cycle, ranging around 1.28 ± 0.27 g/L at PR = 0% and 1.45 ± 0.11 g/L at PR = 20%. Notably, at PR = 20%, maximum biomass concentration is comparable to that measured in the fresh medium test (1.65 ± 0.12 g/L), although it is achieved later, resulting in a lower biomass growth rate, as indicated in Figure 2a;
- Nitrate is fully consumed during each growth cycle and within the same time frame, except for the third batch at PR = 0%, which indicates that cells can still absorb substrates in reused media;
- The biomass–nitrate yield decreases with each successive cycle because the achievable biomass concentration decreases, although the nitrate absorbed remains consistent in each test;
- The maximum chlorophyll concentration significantly declines with each cycle except at PR = 30%, where the chlorophyll levels are similar to those in fresh medium cultivation;
- A negligible pH shift (difference between final and initial pH) occurs at all PRs and growth cycles, indicating no significant changes in the electrochemistry of the liquid phase or impact on CO_2 absorption. This consistent pH shift is due to the complete depletion of nitrate and the consistent CO_2 supply system used in each test.

Tests carried out at PR = 30% (Figure 3e,f) were characterized by the absence of fluctuation of performance indicators—maximum biomass concentration, biomass–nitrate mass yield, maximum chlorophyll concentration, pH shift—with the cycle number. This reported observation would suggest that the limiting factor in biomass growth rate may be related to photosystem inefficiency. Indeed, the photosystem efficiency may be undermined by magnesium and trace metal depletion. Therefore, the replacement of 30% of supernatant with fresh medium is sufficient to provide enough metals to support photosystem activity. The negligible pH shift measured at any PR and growth cycle would suggest that the growth rate reduction is not due to an alteration of electrochemical equilibria of reaction media that can negatively affect both cell ion exchange and the chemical absorption of CO_2.

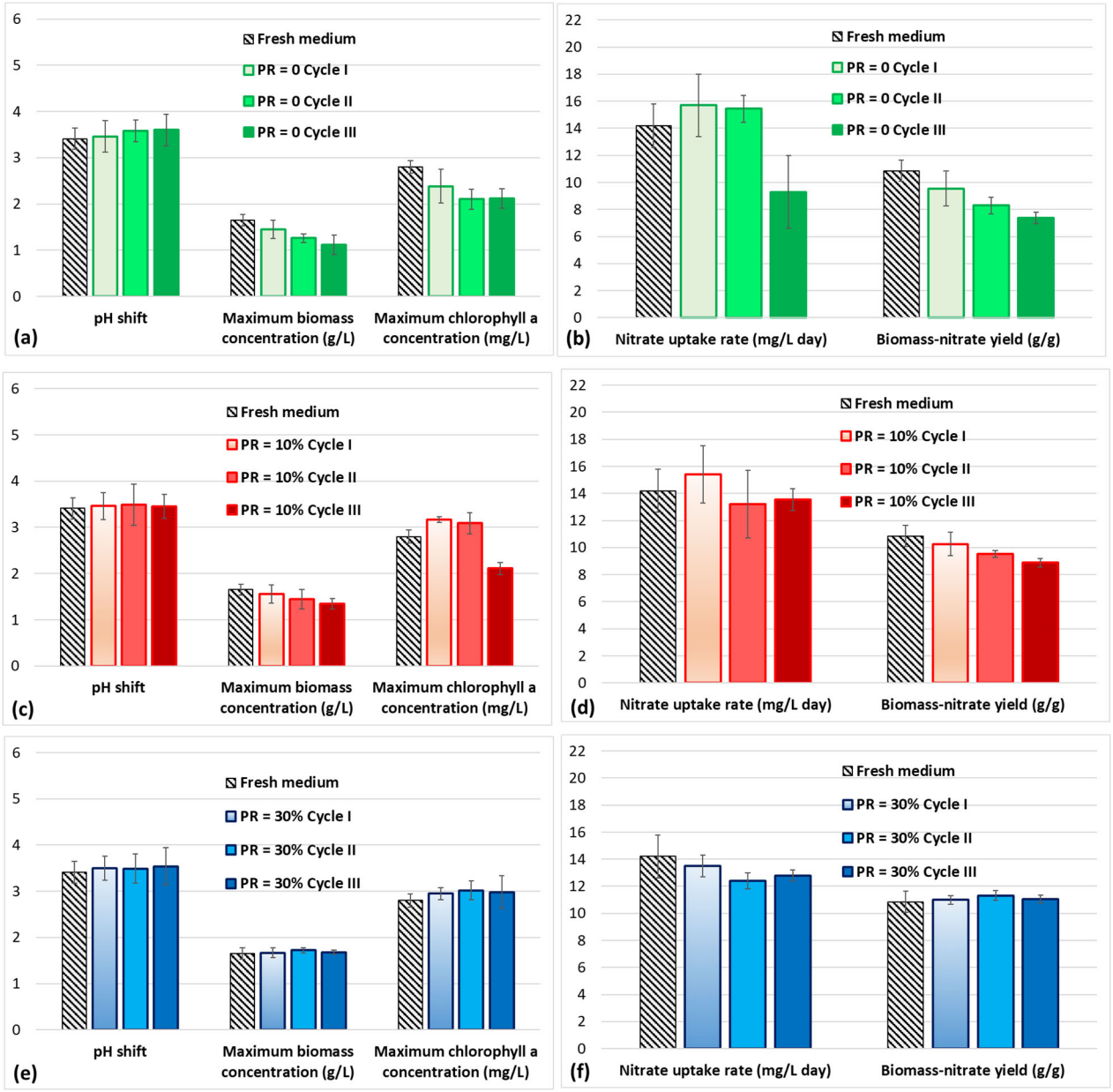

Figure 3. Effect of purge ratio on pH shift, maximum biomass and chlorophyll a concentration, and nitrate uptake rate and biomass–nitrate yield in cultivation tests of *P. simplex* (ACUF 127) across three consecutive growth cycles using exhausted medium reuse strategy. PR = 0 (**a**,**b**), 10% (**c**,**d**) and 30% (**e**,**f**). Data assessed for the fresh medium growth are also reported.

Figure 4 presents the results regarding the proposed medium reuse strategy (Figure 1) applied to the cultivation of the strain ACUF 266. The behavior observed for ACUF 127 concerning pH shift, chlorophyll content, and biomass–nitrate yield was also observed for ACUF 266, and to a greater extent for PRs below 30%, as reported in Figure 4. It is worth noting the following:

- The decline in chlorophyll content is significantly more pronounced, even at PR = 30%, and the average chlorophyll content of *C. vulgaris* (4.72 ± 0.12 mg/L) is higher than that of *P. simplex* (2.8 ± 0.14 mg/L) in fresh medium cultivation, as shown in Figure 3;
- The decrease in the maximum biomass concentration with the cycle number is coupled with a sharp decrease in biomass–nitrate mass yield;

- Similarly to *P. simplex*, the complete depletion of nitrate results in a consistent pH shift in each test, indicating that the reduction in biomass–nitrate yield is solely due to the lower achievable biomass concentration;
- The nitrate uptake rate is affected by both PR and growth cycle: at PR = 0%, after the second batch, it significantly drops down to 6.2 ± 1.8 mg/L day;
- Even purging 30% of medium before recycling, *C. vulgaris* can only be cultivated in partly exhausted medium for one additional batch.

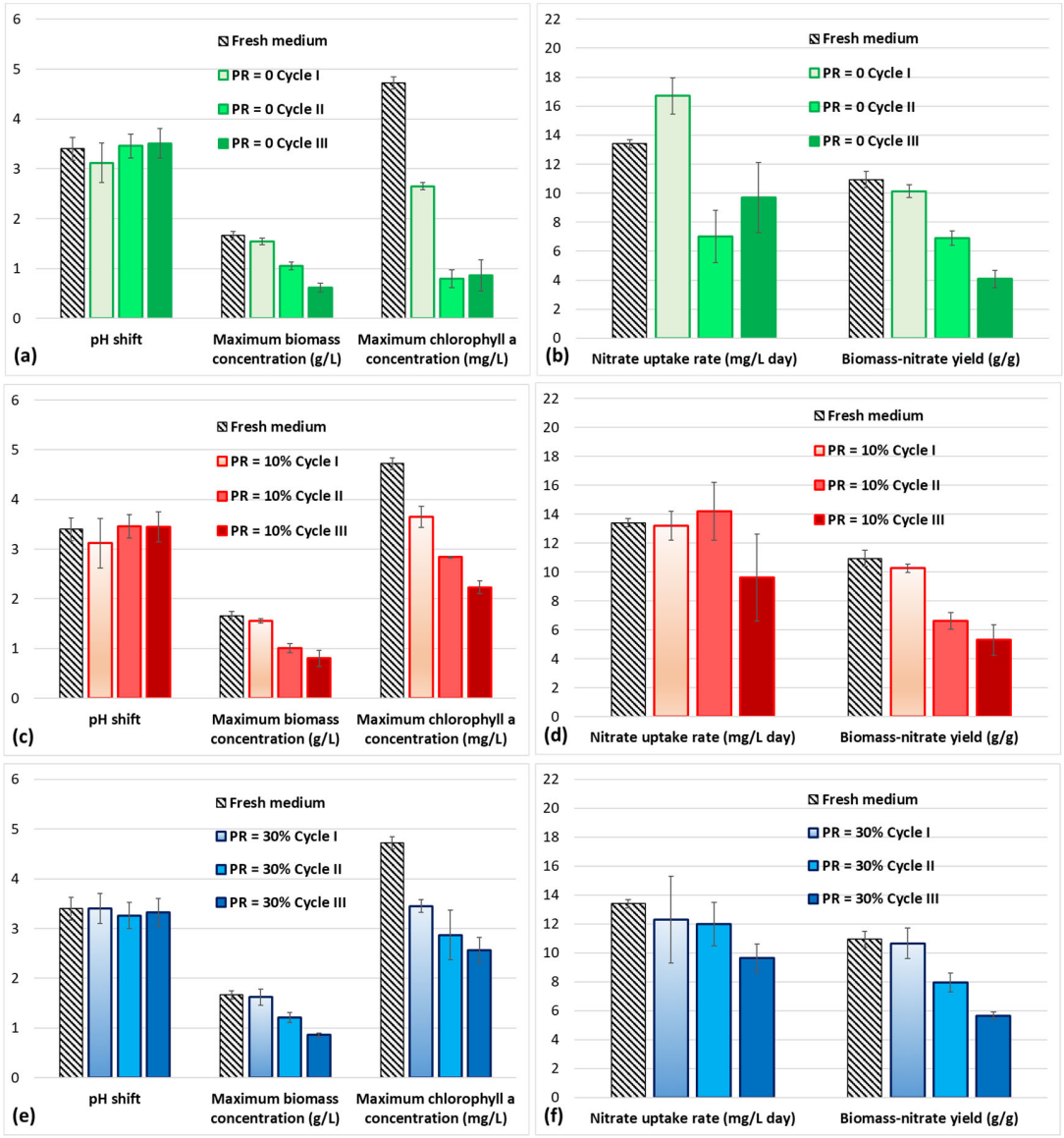

Figure 4. Effect of purge ratio on pH shift, maximum biomass and chlorophyll a concentration, and nitrate uptake rate and biomass–nitrate yield in cultivation test of *C. vulgaris* (ACUF 266) across three consecutive growth cycles using exhausted medium reuse strategy. PR = 0 (**a**,**b**), 10% (**c**,**d**), and 30% (**e**,**f**). Data assessed for the fresh medium growth are also reported.

The comparison of results obtained from the two tests series highlights several issues. Nitrate uptake itself is not impacted by the medium reuse strategy, but its rate is influenced by both the PR and the batch number. However, the metabolization of the nitrogen source is constrained both kinetically, resulting in a drop in biomass growth rate, and stoichiometrically, leading to a decrease in the maximum biomass concentration. It is worth noting that the specific needs of metals/substrates strongly depend on strain physiology;

thus, each strain should be carefully investigated. The maximum chlorophyll content of *C. vulgaris* is about twice that of *P. simplex* when grown in fresh medium. However, this difference diminishes when medium reuse is considered. A comparable biomass growth rate (0.08 g/L day) was measured for both *C. vulgaris* and *P. simplex* even though the amounts of chlorophyll a were 4 mg/L and 2 mg/L, respectively, and the biomass concentration was approximately 2 g/L (fresh medium results in Figures 3 and 4). The difference in required chlorophyll for the tested strains, resulting in a difference in magnesium stoichiometric requirement, can explain why a 30% PR is not feasible for *C. vulgaris* growth in partly reused media, suggesting that a detailed substrate mass yield quantification is mandatory to optimize the exhausted medium reuse strategy. However, it should be noted that both strains maintained their growth performance during the first batch cultivation even without purging the exhausted medium, but only *P. simplex* exhibited stability at PR = 30%. This difference in behavior may be attributed to the algaenan layer, which increases *P. simplex* resistance to unabsorbed metals and ions accumulating in the exhausted medium, or to its lower magnesium requirement for synthesizing the necessary chlorophyll a content. In future studies, it would be valuable to further explore the optimization of reuse strategies by establishing a precise mass balance of substrates and metals. This would involve accounting for unconverted substrates and the accumulation of metals, thereby enhancing the understanding of efficient resource utilization.

Results Comparison and Validation

The results of the present study were compared to the existing literature. To the best of the authors' knowledge, there are few data available on the cultivation of *P. simplex* in (partially) reused exhausted medium. Therefore, a data comparison was conducted with other Chlorophyta microalgae, particularly focusing on the *Chlorella* genus. However, the literature review yielded varying results regarding the effects of medium reuse as a cultivation strategy. Some studies have reported inhibitory, neutral, and stimulant effects, even among strains of the same species. For instance, Yang et al. (2016) observed an increase in biomass growth rate ranging from 5 to 11% compared to fresh medium control for three different *Chlorella* sp. when biomass was harvested through pH-induced flocculation [18]. Conversely, Richter et al. (2018) suggested that biomass productivity enhancement was influenced by bacteria metabolites released during previous growth cycles if not removed or inactivated during media sterilization [19]. Farooq et al. (2015) found that employing $FeCl_3$-supported flocculation for *C. vulgaris*, followed by reusing the supernatant, led to increased biomass and lipid productivity. This suggests that the choice of harvesting system can significantly influence the efficiency of exhausted medium reuse strategies [20]. However, Lee et al. (2013) reported an 80% decrease in biomass yield for a *Chlorella* sp. even when using flocculation [32], similar to the findings of Zhu et al. (2013) using *Chlorella zoofingeris* harvested by centrifugation [21]. When centrifugation and filtration methods were employed, a gradual decrease in biomass concentration was observed for *C. vulgaris* by Discart et al. (2014) [22], and an up to 30% decrease was reported by Boggess (2014) using a *Chlorella* sp. [23]. Several authors have not found significant differences in the cultivation of *Chlorella* genus in reused medium [32–34].

The results of the present study align with the neutral effect reported in the literature for the first growth cultivation in reused medium. It is noteworthy that many studies have focused on single-batch cultivation after fresh medium growth, often using a new inoculum. In contrast, in the present study, cells were inoculated from the previous growth cycle to evaluate their capacity to duplicate when introduced into recycled medium. This approach may explain the absence of biomass productivity enhancement observed in some studies. Furthermore, the accumulation of inhibitory/stimulating compounds, including cell debris, extracellular metabolites, and molecules potentially released by bacteria, was controlled through medium purging, optimized with the PR in this study. This introduces a useful variable to evaluate the stability of repeated batch cultivation, which can be extended to continuous cultivation where a fresh inoculum is generally not supplied.

4. Conclusions

Two unicellular Chlorophyta strains—*C. vulgaris* and *P. simplex*—characterized by about the same specific growth rates in fresh medium were investigated for their potential exploitation in medium (and water) reuse strategies. This study focused on kinetic performance, biomass–nitrate mass yield, and chlorophyll content. The key variable in the water reuse strategy was the purge ratio (PR), the fraction of supernatant volume to be purged with respect to the total volume. The PR was tuned. A PR set at 30% is sufficient to provide biomass productivity comparable to fresh medium for *P. simplex*. *C. vulgaris* exhibited a critical drop in specific growth rate.

The results suggest that reducing the water footprint and promoting substrate recovery from exhausted media could be a promising step towards achieving techno-economic feasibility in microalgae production.

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