Nutrient remediation rates in municipal wastewater and their effect on biochemical composition of the microalga *Scenedesmus* sp. AMDD

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**ABSTRACT**

Microalgae are very efficient at removing nutrients from municipal wastewater and may be a viable tertiary wastewater treatment while additionally improving the economics of microalgal cultivation for biofuel production. The relative quantities and productivities of fatty acids, carbohydrates and proteins were determined in the microalga *Scenedesmus* sp. AMDD grown in treated municipal wastewater in continuous chemostats under different dilution rates or hydraulic retention times. The dilution rate of the chemostat exerted a strong control over the biochemical composition of the cultivated biomass and clear differences in the patterns of accumulation of cellular constituents were detected. Maximum carbohydrate and protein productivities were estimated to be 130 and 120 mg L⁻¹ d⁻¹, respectively, at dilution rates of 0.5 d⁻¹ and 1.05 d⁻¹, respectively. Fatty acid productivity was fairly constant at about 20 mg L⁻¹ d⁻¹ across all tested dilution rates. Total fatty acid only accumulated when growth rates were very low or when a prolonged nutrient starvation regime was imposed by interrupting the supply of wastewater to the chemostat. Monounsaturated fatty acids increased by 250% whereas polyunsaturated fatty acids decreased by 60% and saturated fatty acids remained fairly constant, from the highest dilution rate of 1.05 d⁻¹ to nutrient starved cells. The rate of wastewater nutrient remediation therefore strongly controls the composition of the biomass, thereby controlling its commercial applicability.

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**1. Introduction**

The production of biofuels from microalgae faces several significant techno-economic challenges which must be resolved before a viable industry can develop. These include challenges associated with the high costs of mass cultivation and processing of algal biomass to fuel and non-fuel co-products [1]. Equally important in this regard are issues related to the long-term sustainability of this technology, particularly in relation to requirements for freshwater and nutrients. The environmental and economic benefits of coupling municipal wastewater bioremediation with biofuels production as an operational strategy to minimize potable water use and to reclaim and recycle nutrients for algal growth have regained considerable attention recently [2–5]. Municipal wastewater that has undergone traditional biological treatment has proven to be an ideal algal growth medium in certain algal strains as it provides an appropriately balanced quantity of macro- and micronutrients, and dissolved salts [6,7]. It has been shown that microalgae are very efficient at removing N and P from municipal wastewater as growth nutrients and may therefore be applied as a viable tertiary wastewater treatment strategy to mitigate the environmental effects of eutrophication [3,4,8,9].

There are multiple strategies for the production of a variety of fuels from microalgae [10]. Oleaginous microalgae can produce significant quantities of lipids in the form of triacylglycerides (TAGs) potentially suitable for biodiesel or biojet fuel production; in addition algal carbohydrates can be fermented to produce bioethanol [11,12]. Methane purified from biogas produced from anaerobic digestion of algal biomass can be compressed to a liquid fuel or used to generate electricity [11,12]. Anaerobic digestion has the added advantage of eliminating the high energy costs of harvesting and chemical extraction [13,14]. Alternatively, wastewater cultivated biomass can be processed into different fractions in order to produce a combination of fuels [15].

Most studies using wastewater as a medium for algal biofuels have been conducted in batch culture [9,16–19], and occasionally in unbalanced semi-continuous culture [3,20,21]. However, optimal operation of a microalgal wastewater remediation system requires careful consideration of the most efficient method of cultivation and the most suitable strain(s) of algae. Wastewater remediation rate, as well as algal biomass productivity, over a longer period of time may be enhanced by employing a continuous system [7]. A continuous treatment system in which the goal is to remove nutrients with the shortest possible hydraulic retention time (HRT) resembles, in principle, the operation of an algal chemostat [22]. In a chemostat, biomass productivity is controlled by a single, limiting nutrient and by the rate of culture dilution [22,23]. By varying the dilution rate, growth rates can be regulated...
and maintained for long periods of time, desired biochemical components can be more easily obtained and there is a higher degree of control than in batch or semi-continuous cultures [24]. At steady-state, the system assumes both a physiological and hydraulic equilibrium in which the rate of dilution is balanced by the growth rate: biomass productivity and culture volume are thereby held constant. Applied to a problem like wastewater treatment, an algal-based remediation system could be operated as a type of chemostat where the HRT is controlled by simply adjusting up or down the rate of dilution and growth.

Algal growth in a chemostat is controlled by a limiting nutrient, such as nitrogen or phosphorus, and this offers additional advantages for a coupled wastewater remediation with algal biofuels production system. For example, nutrient limitation is well known to cause an increase in lipid and TAG contents or starch content in algal biomass [10,25–30]. Nutrient-limited continuous systems may therefore help enhance the productivity of lipid and/or carbohydrate. In addition, since the limiting nutrient carried in the inflowing media is quickly assimilated by the algal cells it is usually undetectable in the culture and outflow [7]. If the N:P ratio of the wastewater is such that excess cellular uptake is not saturated, then the non-limiting nutrient, N or P, will be simultaneously removed [7,31,32]. The physiological range of N:P in Scenedesmus, for example, is from ~5 to ~100 (N or P) limitation to ~100 under P limitation [33,34]. The cellular N:P ratio of Scenedesmus can closely match the N:P ratio of the medium being supplied [35] and this stoichiometric flexibility is advantageous for wastewater treatment.

The freshwater chlorophyte Scenedesmus sp. AMDD was chosen for this study as it is one of the most widely studied microalgal genera for wastewater treatment due to its high efficiency of nutrient removal, rapid growth rate, high biomass productivity and its ability to tolerate fluctuations in municipal wastewater conditions [3,36,37]. Previous work with Scenedesmus sp. AMDD established continuous chemostat cultivation as a promising process for large-scale microalgae-based wastewater treatment [7].

Our primary objective with this study was to apply the theoretical principles of chemostat operation to the practical problem of tertiary nutrient removal from municipal wastewater in order to explore the efficiency of this cultivation approach. Also of interest was to explore the biochemical composition of the algae under conditions where the HRT was manipulated by adjusting the dilution rate. There are no other studies to our knowledge of continuous, balanced microalgal cultivation on wastewater and the effect of different dilution rates on biomass productivity, biochemical composition and nutrient removal efficiencies.

2. Materials and methods

2.1. Microalgae cultivation and analyses

Isolation of the freshwater chlorophyte Scenedesmus sp. AMDD was described elsewhere [38]. Cultivation of Scenedesmus sp. AMDD was conducted in treated wastewater obtained from a local secondary sewage treatment plant (Mill Cove WWTP, Bedford, Nova Scotia). Twenty liter aliquots of wastewater were grab sampled at the plant and autoclaved in the laboratory. The initial concentrations of nitrogen and phosphorus ranged from 20 to 21 mg L−1 and from 2.4 to 3.0 mg L−1 respectively, equivalent to a molar N:P ratio of 16:1. Previous work with Scenedesmus sp. AMDD established continuous chemostat cultivation as a promising process for large-scale microalgae-based wastewater treatment [7].

Our primary objective with this study was to apply the theoretical principles of chemostat operation to the practical problem of tertiary nutrient removal from municipal wastewater in order to explore the efficiency of this cultivation approach. Also of interest was to explore the biochemical composition of the algae under conditions where the HRT was manipulated by adjusting the dilution rate. There are no other studies to our knowledge of continuous, balanced microalgal cultivation on wastewater and the effect of different dilution rates on biomass productivity, biochemical composition and nutrient removal efficiencies.

2.2. Biomass analyses

Fluorescence analysis was determined daily using a fast-repetition rate fluorometer (FIRe, Sylvania Octron 3500 k, 32 W) continuously illuminated one side of the chemostats and provided an incident light level of 200 μmol photons m−2 s−1 (established by taking the mean of 10 transect points within the chemostats filled with water), using a Biospherical Instruments Inc. QSL-2100 (San Diego, CA) scalar PAR irradiance sensor. The temperature of the cultures was maintained at 20 °C by a circulating water bath.

Algal cell concentrations were determined daily using a particle sizer and enumerator (Multi-Sizer III, Beckman-Coulter). Cell-free samples of filtered culture were taken daily for the determination of residual nutrient concentrations. Fifteen milliliter samples were filtered through a 0.22 μm filter and stored frozen until analysis. Residual free dissolved ammonium and phosphorus in the cultures were determined using commercially available, colorimetric assay kits according to the manufacturer’s instructions using a DR 2800 portable spectrophotometer (TNT830 and TNT843; Hach Co., Loveland, CO).

Total protein was determined by the Lowry protein assay kit (Biorad DC protein assay, Bio-Rad Laboratories, Hercules, CA) after extracting the filters in 0.2 M NaOH (samples were extracted with 1 mL of NaOH and incubated at 100 °C for 10 min, three times to fully extract from the filter). Standard curves were carried out using bovine serum albumin standards (Bio-Rad Laboratories, Hercules, CA). Cellular carbohydrate content was determined using phenol and sulfuric acid as described by Dubois et al. [41] after first hydrolyzing with 2.5 M HCl for 2 h at 100 °C [42]. Standard curves were carried out using dextrose.

Pigments were extracted from the filters with 90% acetone, sonicated for 1 h in an ultrasonic bath with sand, kept at −20 °C overnight and centrifuged at 2000 rpm for 2 min. The concentrations of chlorophyll a and b and total carotenoids in the extracts were determined spectrophotometrically using the extinction coefficients of Jeffrey and Humphrey [43] and Strickland and Parsons [44]. Fatty acid methyl esters (FAMES) were extracted and quantified by direct acidic transesterification followed by GC analysis following the method described in McGinn et al. [7].
Biomass productivity (BP) was calculated according to Griffiths and Harrison [26] as the product of the steady-state biomass concentration and the specific growth rate:

\[
BP' (\text{mg dw L}^{-1} \text{ d}^{-1}) = B' (\text{mg L}^{-1}) \times \mu (\text{d}^{-1})
\]

(1)

where, in a chemostat at steady-state, the specific growth rate is equivalent to the dilution rate (D) of the culture:

\[
\mu (\text{d}^{-1}) = D (\text{d}^{-1})
\]

(2)

and where the dilution rate (D) is equal to the daily inflow rate of the wastewater (F) divided by the volume of the culture vessel (V):

\[
D (\text{d}^{-1}) = \frac{\text{F (L d}^{-1})}{\text{V(L)}}
\]

(3)

The hydraulic retention time (HRT) is the inverse of D:

\[
\text{HRT (d)} = \frac{\text{V(L)}}{\text{F (L d}^{-1})}
\]

(4)

The cellular productivities of protein, carbohydrate and fatty acids were calculated as the product of BP and the fractional content (w/w) of each macromolecular pool in the biomass:

\[
\text{ProteinProd} (\text{mg Prot. L}^{-1} \text{ d}^{-1}) = \text{BP} \times \text{protein/biomass (w/w)}
\]

(5)

\[
\text{CarbohydrateProd} (\text{mg Carb. L}^{-1} \text{ d}^{-1}) = \text{BP} \times \text{carbohydrate/biomass (w/w)}
\]

(6)

\[
\text{FattyAcidProd} (\text{mg FA L}^{-1} \text{ d}^{-1}) = \text{BP} \times \text{fatty acid/biomass (w/w)}
\]

(7)

2.3. Statistical analyses

One-factorial ANOVAs were applied for statistical analyses. Tukey's honestly significant difference (HSD) multiple comparison tests were used to assess differences among treatments. Statistical analyses were performed using the OriginPro8 software.

3. Results

3.1. Biomass productivity and composition in relation to steady-state growth

Scenedesmus sp. AMDD had an average maximum specific growth rate (\(\mu_{\text{max}}\)) of 1.65 d\(^{-1}\) when cultures were initially grown in batch, before the pumps were switched on for continuous cultivation (data not shown). The highest continuous dilution rate employed in this study (1.05 d\(^{-1}\)) was set deliberately below \(\mu_{\text{max}}\) to avoid wash-out, which often occurs when the rate of cell division cannot keep pace with the dilution rate, typically resulting in a fairly rapid ‘crash’ of the population towards zero [22]. Nutrient-limited continuous chemostats were grown in triplicate at five different dilution rates in municipal wastewater. At each dilution rate, steady-state was confirmed by the observation that biomass concentrations varied at most by only 6.9% for at least 3 consecutive days of cultivation. Dry weights and cell counts were highly correlated with an R\(^2\) of 0.88. There was an inverse relationship between the growth rate and the biomass concentration in municipal wastewater at steady-state (Fig. 1A). As the dilution rate was decreased from 1.05 to 0.7 d\(^{-1}\), the mean biomass concentration increased from 290 to 422 mg dw L\(^{-1}\), an increase of 45%. Further reduction in the dilution rate by 3.5-fold resulted in an increase in biomass concentration to about 640 mg dw L\(^{-1}\). After sampling was conducted under the lowest growth rate, dilution was stopped and the cultures switched back to batch mode for three additional days, thereby imposing nutrient starvation. Under these conditions, biomass accumulated in the culture vessels to slightly in excess of 1000 mg dw L\(^{-1}\), on average, by which time growth had ceased. Compositional analyses of culture samples taken at different dilution rates were conducted to quantify the relative amounts of protein, carbohydrates and fatty acids in the biomass (Fig. 1A). As the steady-state dilution rate was decreased step-wise from 1.05 d\(^{-1}\) to 0.25 d\(^{-1}\), the cellular protein content decreased in parallel from approximately 40% (w/w) to about 20% while the carbohydrate content increased from approximately 30% to almost 60%. Across the same range of dilution rates, fatty acid content remained at about 6% and increased to 12% at the 0.25 d\(^{-1}\) rate. Under nutrient starvation, protein declined further to approximately 15%, while fatty acids accumulated to nearly 28% (Fig. 1A). Carbohydrates did not accumulate further in batch mode beyond 60%. Total pigments (chlorophylls and carotenoids) decreased slightly from highest to lowest dilution rates and decreased further under nutrient starvation (Fig. 1A). A significant component of residual biomass unaccounted for in the protein, carbohydrate, fatty acid and pigment pools remained which decreased from roughly 20% (w/w) at the highest dilution rates to about 10% at the lowest. After 3 days of nutrient starvation all the biomass could be accounted for in the four macromolecular pools assayed (Fig. 1A). The relative quantities of the four pools were also expressed in terms of elemental carbon using the average C-content values reported in Geider and LaRoche [33] (Fig. 1B). The overall trends were similar to the macromolecular level analyses shown in Fig. 1A with some differences. Carbon accounted for roughly 50±2% (w/w) of all biomass samples assayed. The proportion of C in protein (C\(_{\text{protein}}\)) essentially mirrored the proportion of protein in biomass. Due to the higher C content of fatty acids compared to carbohydrates (0.70 g C·g lipid vs. 0.40 g C·g carbohydrate [33]), the proportions of C in fatty acids (C\(_{\text{fatty acid}}\)) and C in carbohydrates (C\(_{\text{carbs}}\)) were higher and lower, respectively, than the content of fatty acids and carbohydrates in the whole biomass. After 3 days of batch growth, biomass C was approximately equally distributed between the fatty acid and carbohydrate pools at about 40% (w/w) each, in contrast to the relatively higher quantity of carbohydrate compared to fatty acids at steady-state (Fig. 1A).

At steady-state, the biomass productivity was calculated as the product of the biomass concentration and the dilution rate (Fig. 2). Biomass productivity was highest at approximately 300 mg dw L\(^{-1} \text{d}^{-1}\) at dilution rates of 0.7–1.05 d\(^{-1}\) and fell by about 50% at 0.25 d\(^{-1}\). The production of additional biomass over the 3-day period of undiluted batch growth was estimated to be 130 mg dw L\(^{-1} \text{d}^{-1}\) (Fig. 2). Analysis of the relative protein, carbohydrate and fatty acid content described in Fig. 1 enabled the calculation of their respective rates of productivity. Protein productivity was maximum at about 120 mg L\(^{-1} \text{d}^{-1}\) and decreased in parallel to biomass productivity. Carbohydrate productivity increased by about 40% from a dilution rate of 1.05 d\(^{-1}\) to 0.5 d\(^{-1}\) to a maximum of about 130 mg L\(^{-1} \text{d}^{-1}\). The fatty acid productivity remained relatively constant at around 20 mg L\(^{-1} \text{d}^{-1}\) across all dilution rates, then increased to 36 mg L\(^{-1} \text{d}^{-1}\) averaged over 3 days of batch growth under nutrient-starved conditions.

Elemental analysis of algal particulate matter collected from the chemostats at steady-state was also conducted (Table 1). The particulate N results confirmed the analysis of protein content shown in Fig. 1. Cellular N content decreased in linear, step-wise fashion from a maximum of 7.76% (w/w) to a low of 2.18% under nutrient starvation after 3 days of batch growth. Cellular P content decreased in parallel to N (Table 1). Cellular C content was not significantly different across all growth rates, indicating the maintenance of a proportionately higher rate of net C assimilation compared to N and P. The calculated N and P
supply rates were plotted against the net N and P assimilation rates, calculated as the product of the biomass productivity (Fig. 2) and ec cellular N and P contents (Table 1) (Fig. 3). Both N and P assimilation rates were tightly controlled by their respective rates of supply to the cultures, indicating classical chemostat nutrient limitation kinetics [45] (Fig. 3). Filtered, cell-free samples of chemostat effluent were assayed for free NH₃ and PO₄³⁻ under the different dilution rates used for growth. Greater than 99.5% of the initial NH₃ was taken up by the microalgae in the initial 2-days of batch culture before continuous cultivation was started. Neither free NH₃ nor PO₄³⁻ was detected at steady-state in any of the samples under any growth condition (lower detection limit of assay: NH₃–N LDL=0.015 mg L⁻¹; PO₄³⁻ LDL=0.15 mg L⁻¹). The results shown in Fig. 3 and the analysis of free NH₃ and PO₄³⁻ together indicated that continuous cultivation of microalgae is a useful and practical strategy for the complete removal of nutrients from municipal wastewaters.

### 3.2. FAME dynamics as a function of dilution rate

The saturation state of FAME was also influenced by the growth rate (Table 2). The relative content of polyunsaturated fatty acids (PUFAs) (primarily linolenic, linoleic, hexadecatetraenoic and stearidonic acids) decreased approximately 60% while monounsaturated fatty acids (MUFAs) (primarily oleic acid and heptadecanoic acid) increased by...
approximately 2.5 fold from the highest growth rate to nutrient starved cells (Table 2). The relative content of saturated fatty acids (SFAs) (primarily palmitic acid) was essentially unchanged at around 20–25% of the fatty acid pool at all dilution rates and in batch phase.

3.3. Indirect estimation of cellular protein by particulate N content

Algal protein content is often determined indirectly by multiplying particulate N values by a conversion factor taken from the literature. Using a conversion factor may allow for better protein comparisons among species and researchers as it eliminates discrepancies observed due to extraction and protein determination methodologies. However, the conversion factors that are employed, such as the broadly used factor of 6.25, are often averages of data from many strains obtained under different growth conditions and therefore may not relate well to the protein content under a given set of conditions [46]. In addition, Gonzalez Lopez et al. [47] found that the method for determining particulate nitrogen influences the magnitude of the protein conversion factor. The relationship between nitrogen content and protein was therefore examined to determine whether or not an appropriate factor could be employed in the future using the protein assay used here. There was no significant difference in protein factors among the dilution rates examined and the rates of N and P assimilation at all dilution rates were high and were not significantly different during continuous growth at all dilution rates. After discontinuing continuous flow and moving from nutrient limitation to starvation, the Fv/Fm decreased from 0.9 to 0.25 d⁻¹ (Fig. 4). However, neither chlorophyll b nor total carotenoid content varied significantly in response to growth rate. The chl:a:C ratio decreased from a maximum of 0.025 to 0.015 as the growth rate decreased (Fig. 4). As cellular nitrogen content decreased, the cultures became visibly chlorotic, changing from very dark green to yellowish-green in color.

3.5. Steady-state fluorescence analysis of chemostat cultures

Fv/Fm measurements obtained with a fast repetition rate fluorometer were high and were not significantly different during continuous growth at all dilution rates. After discontinuing continuous flow and moving from nutrient limitation to starvation, the Fv/Fm decreased from 0.71 ± 0.01 (averaged from all dilution rates; p > 0.05) to 0.45 ± 0.02.

4. Discussion

The objectives of this study were twofold: To assess the applicability of continuous chemostats as a method for wastewater treatment coupled to biomass and biofuel production (1) and to determine the optimal cultivation management parameters (i.e. dilution rate, HRT etc.) for the production of biomass of defined compositions (2).

Municipal wastewater was used for the controlled and balanced growth of the freshwater chlorophyte Scenedesmus sp. AMDD in chemostats. Complete removal of N and P by algal growth was found at all dilution rates examined and the rates of N and P assimilation into biomass very closely matched the corresponding rates of N and P supply (Fig. 3). At intermediate rates of dilution, there appeared to be more N assimilated than available in the wastewater (Fig. 3 at 0.5–0.9 d⁻¹ dilution rates). This was possibly due to the assimilation of other sources of dissolved nitrogen present in the wastewater in addition to ammonia but which were not assayed for. It is probable that the chemostats are nitrogen-limited and cells have a ‘luxury’ storage of phosphate since the N:P ratio at which nitrogen-limitation switches to phosphate-limitation is 30 for Scenedesmus sp. [34]; higher than the N:P ratio of the inflow supply (Table 1). Continuous cultivation was maintained in the chemostats for more than 45 days and a maximum biomass productivity rate of about 300 mg dw L⁻¹ d⁻¹ was obtained, more than double that obtained by McGinn et al. [7] in batch cultures grown in wastewater from the same plant and higher than or comparable to the biomass productivities for Scenedesmus sp. in various types of wastewater reported by others [3,19,36] (Fig. 2). Optimal wastewater remediation efficiency is

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**Table 1**

Elemental and ash composition of Scenedesmus sp. AMDD at 5 steady-state dilution rates and in batch culture mode (0) after 3 days of stopping inflow. Percentages are based on dry weight biomass. Numbers in parentheses = SD, n≥24.

<table>
<thead>
<tr>
<th>% dw</th>
<th>Steady-state dilution rate (d⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>1.05</td>
</tr>
<tr>
<td>Ash</td>
<td>2.99(0.37)</td>
</tr>
<tr>
<td>N</td>
<td>7.29(0.26)</td>
</tr>
<tr>
<td>C</td>
<td>48.74(0.84)</td>
</tr>
<tr>
<td>P</td>
<td>0.84(0.05)</td>
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</table>

mol:mol

<table>
<thead>
<tr>
<th>C:N</th>
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<td>19.19</td>
<td>149.4</td>
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<td>7.42</td>
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<td>369.4</td>
</tr>
<tr>
<td>27.61</td>
<td>17.82</td>
<td>491.8</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Steady-state nitrogen and phosphorus assimilation rates (open symbols) by Scenedesmus sp. AMDD vs. calculated nitrogen and phosphorus supply rates (closed symbols) in continuous cultures growing in municipal wastewater. Error bars = SD, n≥24.
achieved under conditions of complete nutrient removal with the shortest possible HRT. This occurred in the present experiments at the highest dilution rate employed (HRT of ~1 day), which also yielded the highest biomass productivity (Fig. 2).

Biomass composition (Fig. 1) and macromolecular productivity (Fig. 2) were strongly influenced by the dilution rate. The relative proportions of lipid, protein and carbohydrate have been demonstrated to change under different environmental conditions [48,49]. It is well known that the cellular content of nitrogen decreases with increasing nitrogen limitation or starvation corresponding to a decrease in protein content [49]. In some species, this leads to a corresponding increase in lipids [50]. In nitrogen-limited chemostats at low dilution rates, there is a decline in cellular protein under increasing N-limitation leading to a relatively greater increase in carbohydrates compared to fatty acids. These results differed from the pattern seen in nitrogen-limited continuous cultures of *Nannochloropsis grunulata*, where the cellular lipid content doubled at the expense of protein as the dilution rate decreased [32]. Our results corroborate those obtained in *Scenedesmus obliquus* by Makulla [50] which showed that fatty acid content remained fairly constant in N-limited chemostats at low dilution rates. This suggests that N-limited *Scenedesmus* sp. accumulate carbohydrates more readily than fatty acids (Fig. 1). It is not clear why some species accumulate one form of storage compound over another or what physiological role this may play [51].

Fatty acid productivity remained constant at about 20 mg L$^{-1}$ d$^{-1}$, comparable to productivities achieved with *Scenedesmus* sp. in batch culture [52–54] (Fig. 2). In the present study, the greatest accumulation of fatty acids occurred after nutrient starvation was imposed by discontinuing the flow of wastewater to the chemostats. After three days of nutrient starvation the fatty acid content increased to 28% of biomass (or about 35% when normalized to biomass carbon, Fig. 1B). Richardson et al. [55] reported a similar trend with *Chlorella* sp.; fatty acid content remained constant at all dilution rates tested until cellular nitrogen content decreased below 3%, after which the fatty acid content increased significantly. Since nutrient starvation often triggers fatty acid accumulation, a biofuel production strategy consisting of a two-stage cultivation system in which biomass from continuous culture is transferred to and stressed in a second stage to induce triacylglycerol (TAG) accumulation is conceivable and has been proposed [28,56]. In this study, lipid productivity almost doubled to 36 mg L$^{-1}$ d$^{-1}$ after three days of nutrient starvation compared to 20 mg L$^{-1}$ d$^{-1}$ in steady-state, confirming the potential utility of a two-stage process for biofuel production. F$_v$/F$_m$ could be a useful tool for establishing initiation of nutrient starvation in a two-stage process. Our results support the findings of Parkhill et al. [39] that under nutrient limited steady-state conditions F$_v$/F$_m$ remains high and varies little among dilution rates, and declines during nutrient starvation and indicates nutrient stress. Hence, F$_v$/F$_m$ cannot be used to determine an effect of nutrient limitation but can be used as a measure to determine nutrient starvation and therefore stress.

Within the fatty acid pool, the relative contents of MUFAs and PUFAs were inversely correlated with one another (Table 2). As dilution rate decreased, and therefore algal growth is slowed, the requirement for the synthesis of new membrane compounds decreases [57]. A shift therefore occurs in lipid metabolism from glycerol-based membrane synthesis which typically have a high PUFA content to the synthesis and storage of TAGs (mostly composed of MUFAs and SFAs) [57,58]. This finding is important if biodiesel is a targeted endpoint. Changing the fatty acid composition or targeting a profile is essential for biodiesel production [59] and information on fatty acid compositions under different environmental conditions is limited [28]. The fatty acid methyl esters in this study were composed mainly of C16:0 (palmitic), C18:1 (oleic), C18:2 (linoleic) and C18:3 (linolenic) (Table 2) comparable to the most common fatty acids (palmitic, oleic, stearic, linoleic and linolenic) found in biodiesel [60]. A biodiesel feedstock should be as

![Fig. 4. Effect of steady-state dilution rate on the cellular abundance of chlorophyll a and b, total carotenoids (left ordinate axis) and on the chlorophyll a: cellular C ratio (right ordinate axis) in continuous cultures of *Scenedesmus* sp. AMDD growing in municipal wastewater. Error bars = SD, n ≥ 24.](image-url)
high as possible in MUFIA content with a mix of SFAs consisting mostly of short chains in order to have adequate ignition properties, cold-flow properties and viscosities [30]. In this study the quality of the fatty acid composition increased as dilution rate decreased and was most favorable in batch mode (Table 2). Linolenic acid for example decreased from 29.3% at the high dilution rate of 1.05 d⁻¹ to 17.1% at the lowest continuous dilution rate of 0.25 d⁻¹ to 14.9% in batch mode (Table 2). This 14.9% is much closer to the EN 14214 (2004) limit of 12% for biodiesel and can be more easily improved by blending with other sources of biodiesel [61]. The high oleic acid (C18:1) content found is optimal for combustion quality without adverse effects on biodiesel cold-flow properties and the low-levels of C18:2 and C18:3 add to the high oxidative stability of the fuel [62] (Table 2).

Based on our findings we suggest a couple of strategies that may be employed to maximize biofuels potential while considering wastewater treatment efficiency and cost.

Anaerobic digestion (AD) of algae biomass as a means to produce biofuel in the form of methane-enriched biogas eliminates the high energy costs of harvesting and chemical extraction [13,14] and may be ideal for continuous one-stage cultivation in wastewater. The gross biochemical composition of biomass can influence methane production through AD [63]. High lipid content has a higher methane potential, however lipids can also inhibit AD processes, due to their greater resistance to hydrolysis [64]. Algae high in protein release high concentrations of ammonia during digestion that has a toxic inhibitory effect on anaerobic bacteria [14,63]. Therefore the dilution rate (and HRT) of algae wastewater treatment systems should be carefully selected in order to ensure a biomass composition that is compatible with the requirements and tolerances of AD systems (preferably low protein, high carbohydrate and lipid). For example, at a dilution rate of 0.5 d⁻¹, although biomass productivity is less than maximum, carbohydrate productivity is greatest corresponding to decreased protein and intermediate fatty acid contents. Wastewater remediation rate and total biomass productivity are therefore compromised by an increased quality of biomass for anaerobic digestion.

Another strategy is to combine two processes wherein delipidated biomass left over from the production of biodiesel is digested anaerobically to biomethane [15]. This approach may be more amenable to the two-stage strategy described above wherein delipidated biomass enriched in carbohydrates (after removal of lipids) is a potentially more digestible substrate for an AD process. Cost and capacity for reservoir containers would have to be considered in conjunction with the dilution rate applied to the continuous stage.

Considered together, the results of this study support the proposal that municipal wastewater can be used efficiently for the continuous production of microalgal biomass and that the biochemical composition of the biomass can be controlled to some extent by manipulating the steady-state growth rate of the culture. In order to obtain the desired production rate of a targeted component of the biomass, careful selection of the dilution rate is necessary. To remediate large volumes of wastewater daily, shorter retention times with higher dilution rates should be chosen. Biomass productivity is greatest at high dilution rates however maximum biomass concentration is achieved at low dilution rates. Intermediate dilution rates should be chosen for biogas production and low dilution rates for biodiesel production. Enhanced fatty acid productivity and quality can be achieved by imposing a two-stage strategy of cultivation.

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