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## Polishing of anaerobic secondary effluent by *Chlorella vulgaris* under low light intensity

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**Abstract:** To investigate anaerobic secondary effluent polishing by microalgae (*Chlorella vulgaris*) under low light intensity ( $14 \mu\text{mol}/\text{m}^2/\text{s}$ ), bubbling column reactors were operated in batches of 8 d with initial ammonium nitrogen 10-50 mg/L, initial phosphate phosphorus 2-10 mg/L and microalgal seed 40 mg/L. Maximum microalgal biomass and minimum generation time were 370.9 mg/L and 2.5 d, respectively. Nitrogen removal (maximum 99.6%) was mainly attributed to microalgal growth rate, while phosphorus removal (maximum 49.8%) was related to microalgal growth rate, cell phosphorus content (maximum 1.5%) and initial nutrients ratio. Dissolved microalgal organics release in terms of chemical oxygen demand (maximum 63.2 mg/L) and hexane extractable material (i.e., oil and grease, maximum 8.5 mg/L) was firstly reported and mainly affected by nitrogen deficiency and deteriorated effluent quality. Ultrafiltration critical flux ( $16.6\text{-}39.5 \text{ L}/\text{m}^2/\text{h}$ )

showed negative linear correlation to microalgal biomass. Anaerobic membrane bioreactor effluent polishing showed similar results with slight inhibition to synthetic effluent.

**Keywords:** Anaerobic effluent, Hexane extractable material, Microalgae, Nitrogen and phosphorus ratio, Nutrients deficiency

## 1 Introduction

Wastewater phycoremediation by microalgae has been investigated for over seven decades (Oswald, 2003) and is booming in recent years with several bench scale photo-bioreactor (PBR) studies (Judd et al., 2015). Wastewater rich in organic carbon (easily converted to CO<sub>2</sub> via biological treatment), nitrogen and phosphorus may serve as a convenient carbon and nutrients source for year round microalgal biofuel production (Schenk et al., 2008), which is the most practical commercial solution temporarily to fulfill higher biomass plus oil productivity (Brennan & Owende, 2010), lower cost per yield (Stephens et al., 2010), less freshwater (Dismukes et al., 2008) or land demand than biodiesel derived from food crops (Rodolfi et al., 2009).

Several unicellular microalgae genus, such as *Chlorella* and *Scenedesmus*, are found highly tolerant to wastewater environments and efficient in removing nutrients (Ruiz-Marin et al., 2010), especially from anaerobic effluents rich in ammonium due to their preference of utilizing ammonium rather than nitrate in photoautotrophic metabolism (Wang et al., 2010). Hence, microalgal cultivation coupled with anaerobic secondary effluent polishing has been proposed as a promising low-cost and environmentally friendly approach for

nutrient remediation, requiring less aeration than conventional biological nutrients remediation with zones of various dissolved oxygen (DO) concentration (Judd et al., 2015), generating no additional wastes like sludge by-products (Pittman et al., 2011), and presenting nutrient recovery solutions via microalgal biomass processing (Wilkie & Mulbry, 2002). However, there is limited information available about microalgae polishing of anaerobic effluents with different nutrients ratios.

The submerged bubbling ultrafiltration (UF) process, which involves least biomass washout or chemical contamination (Boonchai & Seo, 2015), less shear stress than crossflow UF separation, and less sorption or irreversible fouling than microfiltration (MF) process (Bilad et al., 2014), is an appropriate microalgae-water separation step after polishing to provide high quality treated water for fit-for-purpose reuse as well as high microalgal biomass for microalgal post-utilization. However, there is limited information on UF systems application for microalgae-water separation so far.

Light availability affects phycoremediation behavior. The growth of microalgae is slower when receiving low light intensity, which may be a challenge in PBRs with compact configuration designs or during nighttime operation (Boelee et al., 2012). One approach is to enhance the microalgal polishing capacity by applying artificial low light intensity during the nighttime. Coupled with compact PBRs, improved water quality of the wastewater effluent may be achieved, since microalgal cells receiving low light intensity may have higher chlorophyll content and lower C/N ratios (Krause-Jensen et al., 1996), leading to potentially higher nutrients removal and lower microalgal metabolites release (i.e., lower organics contribution to the treated water).

To investigate anaerobic secondary effluent phycoremediation by microalgae under low light intensity, the present study operated bench scale bubbling column reactors fed by synthetic anaerobic secondary effluent (with different nutrients concentration) and anaerobic membrane bioreactor (AnMBR) effluent in batch modes to assess the polishing capacity (mainly nitrogen and phosphorus removal) by *Chlorella vulgaris* UTEX-259. This unicellular microalgae is characterized as oleaginous and might deteriorate final effluent quality by releasing metabolites (e.g., lipids) (Hultberg et al., 2016; Lutzu et al., 2016). Therefore, dissolved organics in terms of chemical oxygen demand (COD) and oil and grease in terms of hexane extractable material (HEM) were measured to assess the impact on the final effluent water quality. The applicability of UF filtration as a microalgae-water separation step was also investigated via critical flux evaluation at the end of the batch tests.

## 2 Material and Methods

### 2.1 Inoculum preparation

The stock of *Chlorella vulgaris* (UTEX 259) was prepared by batch cultivation in Bold's Basal Medium (BBM, from Sigma-Aldrich) with the recipe shown in Table 1. Membrane pre-filtration (nominal pore size 0.03  $\mu\text{m}$ ) of BBM was employed prior to seeding to minimize contamination by microorganisms.

### 2.2 PBR batch operation

Experiments were set in batch mode of 8 days at 20 °C. Treatment was performed in graduated glass cylinders (Kimble™ KIMAX™ Class B Mixing Cylinders, 2 L) with aerators to mimic a bubbling column PBR. Aeration was performed with air (0.02 µm filtered) via a diffuser placed at the bottom of cylinder at a rate of 1 vvm (2 L air volume/2 L reactor volume/min), controlled by flowmeters (Dwyer Instruments RMA-21-SSV), to provide excessive carbon source and sufficient mixing, as well as to maintain a pH balance.

Common cool daylight fluorescent lamps (Philips TL-D 36W/54-765) for normal indoor night lighting applications were installed as the low-intensity light source, providing 24 h illumination. Light intensity was measured with a lux meter (Testo 435) and converted to a photosynthetically active radiation value of 14 µmol/m<sup>2</sup>/s. Compared to other studies applying a light intensity around 50-1600 µmol/m<sup>2</sup>/s (Atta et al., 2013; Liu et al., 2013; Yan et al., 2013), the light intensity used in this study is really low and representative of normal night lighting. The saturation point of cell density pre-measured by batch cultivation at this light intensity was around 650 mg/L in terms of volatile suspended solids (VSS).

The recipe modified from BBM, shown in Table 1, simulates anaerobic secondary effluent since anaerobic digestion is the preferred pretreatment for post polishing by microalgae (Wang et al., 2010). Ammonium nitrogen was set at 10, 20, 30, 40 and 50 mg/L and phosphate phosphorus at 2, 4, 6, 8 and 10 mg/L, respectively. Thus, a total of 25 batches with different nitrogen and phosphorus combinations to cover most real anaerobic secondary effluent qualities as possible were tested. In each batch, 40 mg/L of *Chlorella vulgaris* was seeded into a PBR before starting the batch test. In addition, real effluent of an AnMBR treating synthetic municipal wastewater (Wei et al., 2014), with COD of 25 mg/L,

ammonium nitrogen of 28.6 mg/L, phosphate phosphorus of 10 mg/L, was tested in one batch phycoremediation experiment, with all other parameters kept the same as for the tests with synthetic anaerobic secondary effluent.

A polyvinylidene fluoride (PVDF) hollow fiber UF membrane, with pore size of 0.03  $\mu\text{m}$  and a surface area of 48.4  $\text{cm}^2$ , was selected for the microalgae-water separation at the end of each batch. Critical flux (CF) was first measured as a filterability assessment of a microalgal sample with the critical transmembrane pressure (TMP) increase set at 20 Pa/min. The improved flux-stepping method with relaxation between each step, as reported in the literature (Bilad et al., 2012), was adopted for the CF measurement. After CF assessment, a sub-critical flux was selected to run a constant-flux filtration of the batch sample to produce a final polished effluent. Bubbling by diffuser was kept at 1 vvm, equal to the same flow rate during the batch test, to provide air-scouring along the UF membrane module for fouling mitigating to remove reversible fouling caused by cell deposition, as assumed to be the major fouling for the UF filtration.

### *2.3 Water quality analysis*

The pH and DO were recorded daily by a CyberScan pH 6000 and inoLab® Multi 9430 IDS, respectively. VSS was measured following protocols in Standard Methods for the Examination of Water and Wastewater (Clescerl et al., 1999). Microalgal biomass was calculated by optical density (OD), measured at 691.5 nm by UV-Vis spectrophotometer (Shimadzu UV-2550) via a pre-calibrated  $\text{MLVSS-OD}_{691.5\text{nm}}$  linear relationship (shown in

Fig. S1 in Supplementary Data 1). COD, ammonium nitrogen ( $\text{NH}_4^+\text{-N}$ ), and phosphate phosphorus ( $\text{PO}_4^{3-}\text{-P}$ ) were measured by HACH kits (TNT products) and UV-Vis spectrophotometer DR5000 according to suitable assay range. The size distribution of microalgal cells was measured with a particle analyzer (Malvern Mastersizer).

Hexane extractable material (HEM), as a parameter characterizing oil and grease in water, was measured at the end of five batch tests treating synthetic anaerobic secondary effluents with initial nitrogen of 10 mg/L and initial phosphorus of 2, 4, 6, 8, 10 mg/L, respectively. This was based on the report by [Li et al. \(2010\)](#) that a limited nitrogen medium would result in a higher lipid content in microalgae biomass, which might lead to lipid release and thus higher HEM in the treated water. As a potential limit-exceeding parameter, HEM was also measured for the AnMBR effluent polishing test.

HEM was analyzed gravimetrically following the modified EPA method 1664 (EPA, 2010) with vendor validated solid phase extraction (SPE) protocol (shown in Supplementary Data 2), in which Dionex™ SolEx™ C18 silica-based SPE cartridges installed on Dionex™ AutoTrace™ 280 were sequentially conditioned by hexane, methanol and acidified water (pH 2), then loaded with sample of 1 L, dried by nitrogen gas, eluted by hexane as fraction 1, washed by methane, eluted by hexane-tetrahydrofuran (v/v=1:1) as fraction 2. Both fractions were then evaporated at 70 °C for later gravimetric determination by a Mettler Toledo Balance XP2U with weighting precision down to 0.1 mg. Based on the microalgal sample preparation step, HEM was further classified as total HEM, dissolved HEM in 0.45 µm filtrate and in 0.03 µm filtrate. The microalgal sample was first acidified to pH 2 to break the microalgal cells to release intracellular HEM, and then



filtered by 0.45  $\mu\text{m}$  Whatman® glass microfiber syringe filters for removing microalgal cell debris to produce total HEM sample. The dissolved HEM in 0.45  $\mu\text{m}$  filtrate was obtained by first filtering the microalgal sample with 0.45  $\mu\text{m}$  Whatman® glass microfiber syringe filters to remove the microalgal cells and then acidified to pH 2. The sample of dissolved HEM in 0.03  $\mu\text{m}$  filtrate was obtained by filtering the microalgal sample with a UF membrane (pore size 0.03  $\mu\text{m}$ ) and then acidification to pH 2.

#### 2.4 Data processing

Microalgal generation time was derived from a first-order growth kinetics assumption during the batch tests, and calculated according to Eq. (1), where  $t_2$  is generation time (d),  $k$  is the first-order growth coefficient (1/d), and  $T$  is growth time (i.e., the batch test duration) of 8 d. Nitrogen and phosphorus removal were calculated according to Eqs. (2) and (3), respectively. Specific nitrogen and phosphorus removal (sN and sP) were defined as the nitrogen and phosphorus removed per microalgal biomass growth (approximated to nitrogen and phosphorus assimilation) during the batch tests according to Eqs. (4) and (5), respectively. As an aggregate index characterizing released organics from microalgal cells, dissolved organics increase ( $\Delta\text{COD}$ ) was defined as the dissolved COD increase during the batch tests according to Eq. (6). Specific dissolved organics increase (sCOD) was defined as the dissolved COD increase per microalgal biomass growth during the batch tests according to Eq. (7). All contour plots were generated in MATLAB R2015a, with codes attached in the Supplementary Data 1. All other plots were generated with Microsoft Excel.

$$t_2 = \frac{\ln(2)}{k} = \frac{\ln(2)}{\frac{\ln(Final\ VSS) - \ln(Initial\ VSS)}{T}} \quad (1)$$

$$N\ removal\ efficiency = \frac{Initial\ N - Final\ N}{Initial\ N} \times 100\% \quad (2)$$

$$P\ removal\ efficiency = \frac{Initial\ P - Final\ P}{Initial\ P} \times 100\% \quad (3)$$

$$sN = \frac{Initial\ N - Final\ N}{Final\ VSS - Initial\ VSS} \times 100\% \quad (4)$$

$$sP = \frac{Initial\ P - Final\ P}{Final\ VSS - Initial\ VSS} \times 100\% \quad (5)$$

$$\Delta COD = Final\ COD - Initial\ COD \quad (6)$$

$$sCOD = \frac{\Delta COD}{Final\ VSS - Initial\ VSS} \times 100\% \quad (7)$$

### 3 Results and Discussion

#### 3.1 Microalgal growth and nutrients removal in synthetic anaerobic secondary effluent

During all the batch tests of treating synthetic anaerobic secondary effluent by *Chlorella vulgaris*, pH was maintained at  $5.2 \pm 1.0$  with sufficient aeration by air bubbling. At this pH value, the carbonate equilibrium is shifted towards carbon dioxide thus facilitating microalgal carbon uptake (Alcántara et al., 2015). In addition, ammonia is transformed into ammonium thus minimizing the potential of ammonia toxicity and ammonia stripping from

the aeration (Peccia et al., 2013), and phosphate phosphorus is kept in a dissolved state thus avoiding phosphate precipitation (Ruiz-Martinez et al., 2014). Subsequently, nutrients removal is predominantly through cell assimilation. DO values were kept at  $16.2 \pm 1.0$  mg/L in all batch tests, higher than the normal oxygen saturated point of 9.0 mg/L (at 20 °C), but still lower than 20 mg/L which is a common threshold reported in photo-oxygenation studies (Lee & Lee, 2003). This is probably due to the photo-oxygenation balance and mixing achieved at the set aeration flow up to 1 vvm applied in this study.

Microalgal growth is presented in Fig. 1a and Fig. 1b, in which final microalgal biomass (i.e., VSS) and generation time are contoured against initial nutrients concentration, respectively. The final VSS in the batch tests ranged from 60-360 mg/L (initial seed 40 mg/L), representing generation times varying between 3 and 10 days as a function of initial nutrients concentration. Phosphorus was observed to be the main limiting factor for microalgal growth for initial phosphorus concentration less than 4 mg/L, while nitrogen was observed to be the main factor for the other conditions tested. Generally, final VSS increased with initial nitrogen concentration up to 40 mg/L, beyond that final VSS decreased probably due to ammonium toxicity or inhibition at high concentrations (Markou, 2015). Similarly, final VSS increased with initial phosphorus concentration until around 8 mg/L, beyond that final VSS decreased probably due to nitrogen limitation. This resulted in a peak of around 360 mg/L centered at a coordinate of initial nitrogen and phosphorus concentration (40 mg/L, 8 mg/L) in Fig. 1a.

Final nitrogen concentration in the batch tests ranged from 5-45 mg/L, with most conditions less than 15 mg/L (Fig. 1d). Nitrogen removal was found to be good with results

mostly above 60% and two peaks over 90% (Fig. 1e). Initial phosphorus concentration was a primary factor affecting final nitrogen concentration as well as nitrogen removal, while initial nitrogen concentration played a minor role. When initial phosphorus concentration was less than 4 mg/L (i.e., a limiting factor for microalgal growth), there was a limited nitrogen removal (less than 60%). Final phosphorus concentration in the batch tests ranged from around 2-9 mg/L (Fig. 1g) and decreased with initial nitrogen concentration (particularly above 25 mg/L), showing the effects of initial nutrients ratio on phosphorus uptake by microalgae. Compared to nitrogen removal, both initial nitrogen and phosphorus concentrations affected phosphorus removal (Fig. 1h). For initial nitrogen concentration less than 25 mg/L, phosphorus removal was less than 15% regardless of initial phosphorus concentration. For initial nitrogen concentration in the range of 25-50 mg/L, a peak of 50% for phosphorus removal centered at initial nitrogen concentration of 40 mg/L and phosphorus concentration of 6 mg/L was observed.

sN (i.e., specific nitrogen removal) ranged from 8-20% (Fig. 1f). In general, sN was positively affected by initial nitrogen concentration, albeit slightly decreasing at initial nitrogen concentration of 40 mg/L, and was less affected by initial phosphorus concentration. Comparable studies using nitrate nitrogen at pH 8 reported the same positive trends but was hampered at higher nitrogen concentration of 50 mg/L (Beuckels et al., 2015), which could be likely due to a postponed ammonium toxicity effect based on required metabolism conversion from nitrate to nitrite then ammonium (Shen et al., 2015). Interestingly, a small valley was formed at initial nitrogen concentration of around 40 mg/L and initial phosphorus concentration of around 5-8 mg/L. This response correlated to the

peak of VSS (Fig 1a), implying that shading might have happened, resulting in lower energy input, lower metabolic activity and thus lower sN due to less activated synthesis of protein (Ruiz-Martinez et al., 2014). Furthermore, low final nitrogen concentration at the end of long batch test (i.e., 8 days) might affect sN because reports proved that low nitrogen concentration may decrease cell growth, chlorophyll content (Lin et al., 2011) and protein synthesis (Loladze & Elser, 2011). The initial phosphorus concentration for local maximum sN shifted from 6 to 10 mg/L for initial nitrogen of less than 35 mg/L to 5 mg/L at initial nitrogen of 50 mg/L. Phosphate uptake has been reported to be helpful in the assimilation of nitrogen (Ruiz-Martinez et al., 2014). Consequently, the sN contour might be a combined outcome from both negative effect of shading via VSS and positive effect of two neighboring peaks of sP (i.e., specific phosphorus removal) as shown in Fig. 1i.

For all conditions tested, sP varied from 0.4 to 1.4% (Fig 1i). The maximum value measured well below 3% (i.e., the critical value for luxury uptake (Ruiz-Martinez et al., 2014)) indicated that the phosphorus removal mechanism observed in this study could be ascribed to microalgal cell assimilation. The graph largely followed the trend observed for VSS and generation time (Fig 1a, 1b) where growth was predominantly limited by initial phosphorus concentration when below 4 mg/L, then by initial nitrogen concentration. Nitrogen has a positive effect on the accumulation of phosphorus (Beuckels et al., 2015), and the mentioned shading effect would also reduce cellular ribosomal ribonucleic acid content and phosphorus removal (Sterner & Elser, 2002), thus the two peaks in sP might be shaped by both VSS and sN.

Nutrients removal ratio (i.e.,  $sN/sP$ ) varied from 14 to 38 (Fig. 1c), falling within the range reported in the literature (Boelee et al., 2012). Initial phosphorus concentration was a limiting parameter and negatively correlated when less than 4 mg/L, while for all other combinations studied initial nitrogen concentration together with shading impacted the results. The relationship between  $sN$  and  $sP$  (shown in Fig. S2 Supplementary Data 1) showed an overall weak positive correlation, suggesting a potential synergetic nitrogen and phosphorus removal process.

The plot of nitrogen removal followed the same trend as VSS (Fig. 1a), generation time (Fig. 1b), and final nitrogen (Fig. 1d), however, not for  $sN$  (Fig. 1f). These findings suggest that nitrogen removal is essentially contributed by microalgal growth rate rather than cell nitrogen content (i.e.,  $sN$ ). The plot of phosphorus removal was similar to that of VSS (Fig. 1a), generation time (Fig. 1b), and  $sP$  (Fig. 1i), but not final phosphorus (Fig. 1g). It can be inferred that phosphorus removal is regulated by both microalgal growth rate and cell phosphorus content (i.e.,  $sP$ ). Efficient phosphorus removal is, therefore, dependent on an optimized nutrients ratio in the influent.

Nutrients removal as a function of initial nutrients ratio is shown in Fig. 2. With the boundary value excluded (i.e., under initial phosphorus concentration of 2 mg/L), phosphorus removal (5-50%) correlated positively with initial N/P ratio. The effect of increase in initial N/P ratio on nitrogen removal (53-99%) was not evident, confirming that phosphorus removal was more susceptible to influent N/P ratio than nitrogen. For microalgal polishing of nutrients in wastewater, adjustment of the N/P ratio by adding sodium nitrate or industrial urea could be a solution (Boelee et al., 2012). The significantly

lower removals for both nitrogen and phosphorus under initial phosphorus concentration of 2 mg/L compared to the other conditions tested were mainly due to the lower microalgal biomass production (Fig. 1a), sN (Fig. 1f) and sP (Fig. 1i).

### *3.2 COD and HEM analysis in synthetic anaerobic secondary effluent polishing*

The increase of dissolved organics (i.e.,  $\Delta\text{COD}$ ) during the batch tests is plotted in Fig. 3a. All values measured ranged from 10 to 60 mg/L, where a positive value indicates the release of microalgal organic matter. Generally  $\Delta\text{COD}$  increased with initial phosphorus concentration (with an exception under initial nitrogen of around 30 and 50 mg/L), but fluctuated with initial nitrogen concentration, resulting in two peaks located close to the ones of VSS (Fig. 1a) and final nitrogen (Fig. 1d). This pattern could be expected based on the knowledge that when nutrients become limiting, microalgae accumulated carbon-rich compounds which include carbohydrates and lipids (González-Fernández & Ballesteros, 2012), are released into the water resulting in a COD increase. Furthermore, lipid production has been reported to correlate more with the growth rate of microalgal biomass than with cellular lipid content (Weldy & Huesemann, 2007). However, when shading effects occur, individual microalgal cells may receive fewer photons and might produce less lipid per unit biomass (Courchesne et al., 2009). Therefore, the  $\Delta\text{COD}$  peaks were centered where nitrogen was depleted (Fig. 1d), and VSS peaked (Fig. 1a), with one peak possessing a higher VSS of 360 mg/L and showing a relatively lower  $\Delta\text{COD}$  of 40 mg/L compared to the peak with a lower VSS of 150 mg/L but higher  $\Delta\text{COD}$  of 60 mg/L.

The specific dissolved organics increase (i.e., sCOD) during the batch tests varied from 0.1 to 0.8% (Fig. 3b). Generally sCOD was found to be negatively affected by initial nitrogen concentration, which is in agreement with studies reporting the effect of nitrogen limitation on lipids production (Beuckels et al., 2015; Shen et al., 2015; Weldy & Huesemann, 2007). Nonetheless, an increase of sCOD was observed at initial nitrogen concentration of 10-15 mg/L, probably owing to restricted photosynthesis from extreme nitrogen limitation causing inadequate protein and chlorophyll production (Lin et al., 2011). Moreover, initial phosphorus concentration might also negatively affect sCOD when below 4 mg/L, which could be related to carbohydrate production when extracellular phosphorus was deprived (Beuckels et al., 2015; Brányiková et al., 2011).

The endpoint of effluent polishing is typically required to have low nutrients concentration (e.g., nitrogen < 5 mg/L, phosphorus < 1 mg/L), and restrictions on COD values (e.g., < 50 mg/L). A challenge for microalgal polishing is the carbon-rich metabolites like carbohydrates and lipids that can be accumulated and released into the treated water, potentially exceeding the COD limit for discharge. Since biomass production is the driving force for nutrients removal by assimilation, further research on a solution to minimize sCOD (e.g., selection of non-oleaginous microalgae) is probably necessary.

Fig. 4 shows the HEM results for the batches with initial nitrogen of 10 mg/L and initial phosphorus of 2-10 mg/L, where a nitrogen limiting condition may enhance lipid accumulation in microalgal cells and thus release it into the water. Dissolved HEM in 0.03  $\mu\text{m}$  filtrate was the major part (> 95%) of dissolved HEM in 0.45  $\mu\text{m}$  filtrate. The dissolved



HEM constituted more than half (56-59%) of total HEM, with an exception at initial phosphorus of 10 mg/L where nearly 90% of total HEM was dissolved.

Total HEM increased from 7.3 to 14.9 mg/L with increasing initial phosphorus concentration from 2 to 8 mg/L, corresponding to final nitrogen concentration decrease from 7.3 to 0.5 mg/L, while  $\Delta$ COD increased from 15.3 to 47.7 mg/L. Results demonstrated that with nitrogen deficiency microalgal cells produced more lipids, engendering the increase of HEM as well as  $\Delta$ COD. It has been reported that under nutrients limitation, microalgal cells can replenish their internal carbohydrate reservoirs, which can be later secreted as extracellular polymeric substance (EPS) (Barranguet et al., 2005; Markou, 2015). Moreover, when nutrients stress is severe, photosynthetic products tend to switch to lipids, as well as converting internally stored carbohydrates to lipids (Li et al., 2012; Rodolfi et al., 2009).

Considering the similar values for final nitrogen,  $\Delta$ COD and dissolved HEM in the batch tests with initial phosphorus of 6, 8 and 10 mg/L (initial nitrogen of 10 mg/L), one would anticipate that the total HEM for these batches should also be similar. However, a significant drop of total HEM from 14.4 and 14.9 mg/L (initial phosphorus of 6 and 8 mg/L) to 8 mg/L (initial phosphorus of 10 mg/L) was observed. This drop might be due to the precipitation of the particulate fraction or cellular content during sample preparation, which should be confirmed in future studies.

It should be noted that for most cases, dissolved HEM exceeded the 5 mg/L discharge limit (PME, 2001), risking the applicability of effluent polishing via oleaginous microalgae or a conceptual wastewater treatment process combined with biodiesel production.

### *3.3 Critical flux in synthetic anaerobic effluent polishing*

The contour map of critical flux (shown in Fig. S3 in Supplementary Data 1) was very similar to the final VSS map (Fig. 1a). The critical flux measurements varied as a function of operating conditions between 39.4-16.6 L/m<sup>2</sup>/h (LMH) and showed a strong negative linear relationship (correlation coefficient R of 0.96) with the corresponding microalgal VSS values between 69.1-370.9 mg/L (Fig. 5). Analysis of the microalgal cells showed a size distribution of diameters between 2 to 11 µm, with a median diameter of 4.5 µm (shown in Fig. S4 in Supplementary Data 1). This is far above the nominal pore size of 0.03 µm of the UF membranes. Therefore, during the short term critical flux measurements, microalgal cells deposited on the membrane to form a reversible fouling layer, acting as the major filtration resistance contributor (Castaing et al., 2011). By applying the same hydrodynamic conditions and microalgal strain, microalgal cell density (i.e., VSS) can be used to estimate the critical flux based on the classical critical flux theory (Bacchin et al., 2006).

### *3.4 Validation in AnMBR effluent polishing*

Batch treatment results of AnMBR effluent polishing are presented in Table 2, comparing the data derived from synthetic anaerobic secondary effluent with similar nutrients content to AnMBR effluent. DO and pH in the reactor with AnMBR effluent was within the range of the synthetic effluent. When comparing the AnMBR effluent to the

synthetic anaerobic secondary effluent, some parameters were found to be slightly lower (i.e., nitrogen removal, specific nitrogen removal, generation time and critical flux) and some slightly higher (i.e., phosphorus removal and specific phosphorus removal). However, all were within a 15% deviation, validating the applicability of the synthetic recipe used in this study to simulate AnMBR effluent.

As for microalgal biomass increase, inhibitory compounds to microalgal cell growth might be present in AnMBR effluent (Holmstrom & Kjelleberg, 2000; Rivas et al., 2010), which could explain the 18% lower biomass production than in synthetic anaerobic secondary effluent. Both  $\Delta$ COD and sCOD increase in the AnMBR effluent were significantly higher (42% and 52%, respectively) compared to the synthetic anaerobic secondary effluent. The ratio of dissolved HEM in 0.03  $\mu$ m filtrate over dissolved HEM in 0.45  $\mu$ m filtrate in the AnMBR effluent (49%) was significantly lower than in the synthetic anaerobic secondary effluent (over 95%, shown in Fig. 4). Microalgae cultivated in real wastewater have been observed to have higher carbon-rich metabolites content than those in synthetic wastewater (Órpez et al., 2009), thus, this case might be enhanced by lower VSS and less shading in AnMBR effluent than in synthetic anaerobic secondary effluent. The elevated secretion of EPS, composed mainly of polysaccharide rather than lipids, is probably a response to inhibitory compounds generated in the AnMBR process (Holmstrom & Kjelleberg, 2000).

#### 4 Conclusions

During anaerobic effluent polishing by microalgae (*Chlorella vulgaris*) assimilation under low light intensity, a relatively good nitrogen removal was achieved (mostly over 60%) but a lower phosphorus removal was observed (generally below 50%). A significant dissolved COD (maximum 63.2 mg/L) and HEM (maximum 8.5 mg/L) release was observed, mainly induced by nitrogen deficiency (e.g., below 10 mg/L), indicating the need for further research on maximizing cell productivity, sN, sP, and minimizing sCOD. Potential solutions may include increasing light intensity, adjusting the influent nutrients ratio, and screening microalgae strains with higher cell productivity, sN, sP and lower sCOD.

#### **Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version.

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### List of Figure captions

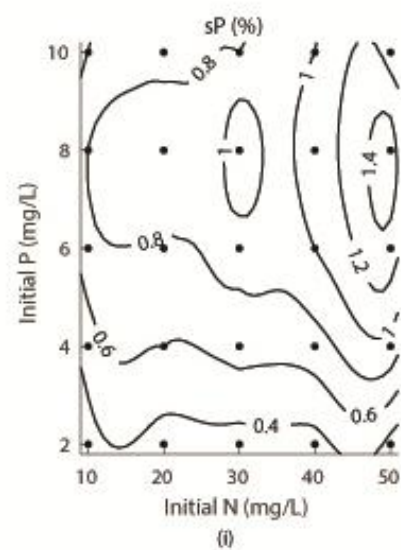
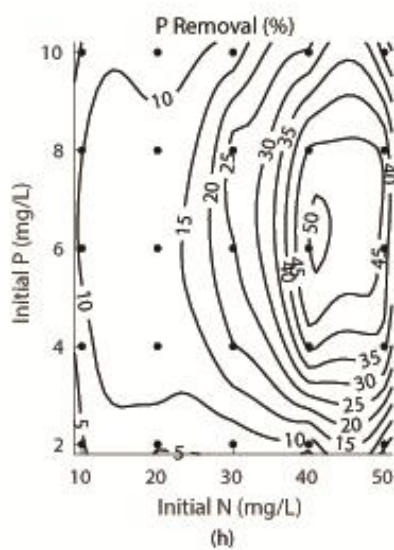
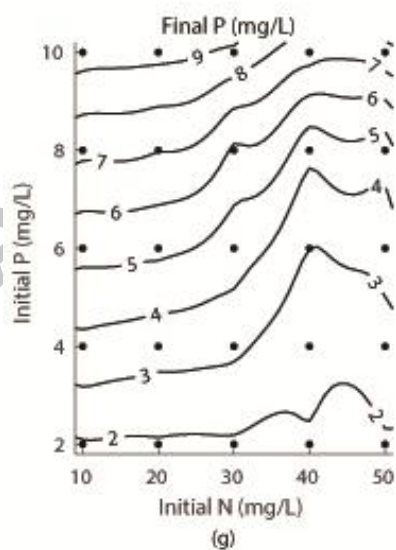
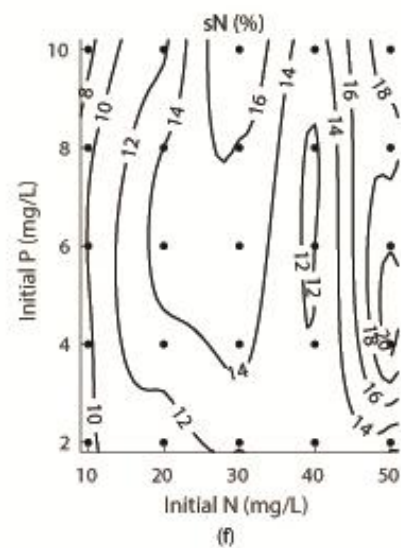
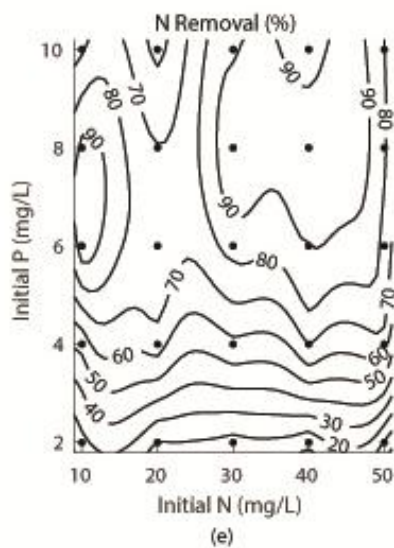
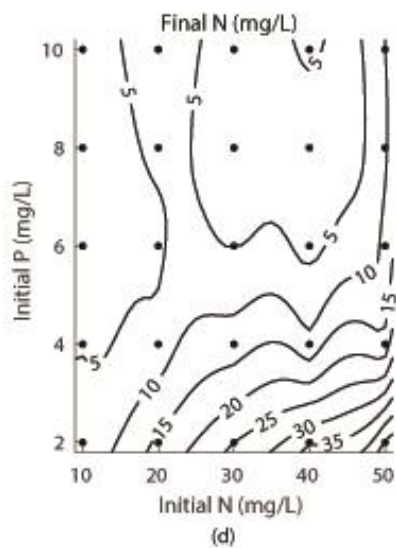
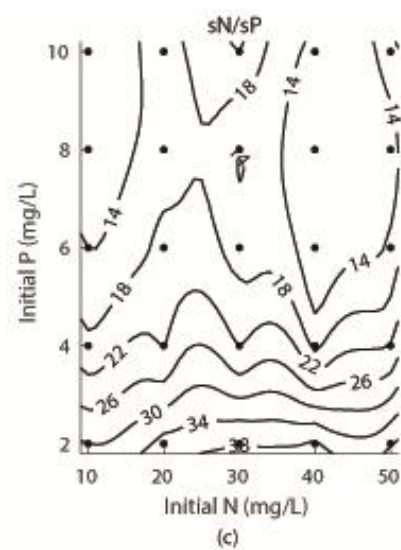
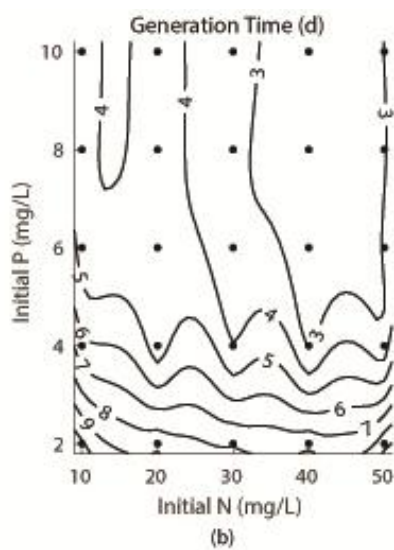
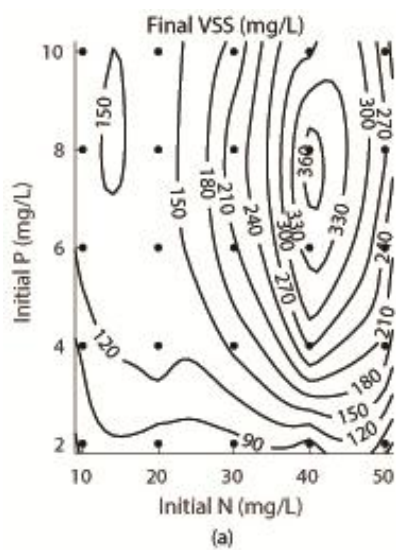
**Fig. 1.** Contour plots of microalgal biomass growth and nutrients removal versus initial nutrients concentration during synthetic anaerobic secondary effluent batch polishing by *Chlorella vulgaris*. (a) Final VSS, (b) Generation time, (c) sN/sP, (d) Final N, (e) N removal, (f) sN, (g) Final P, (h) P removal, (i) sP. “●” marks experimental data from batch tests and contour line derives from biharmonic spline interpolation of experimental data using MATLAB® V4 griddata method.

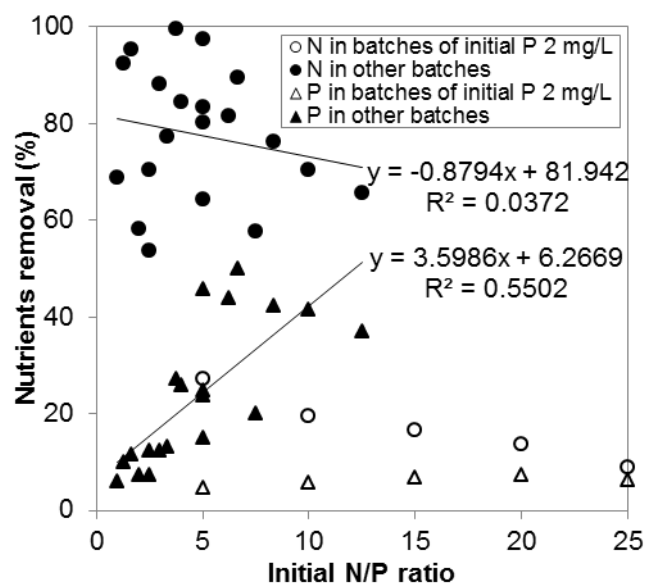
**Fig. 2.** Nutrients removal versus initial N/P ratio during synthetic anaerobic secondary effluent batch polishing by *Chlorella vulgaris*.

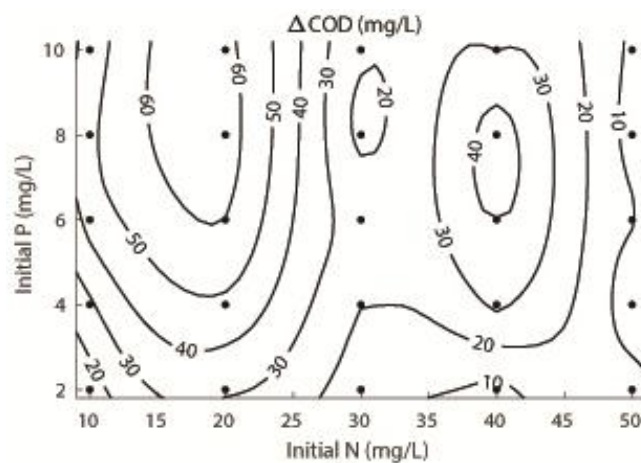
**Fig. 3.** Contour plots of dissolved organics increase versus initial nutrients concentration during synthetic anaerobic secondary effluent batch polishing by *Chlorella vulgaris*. (a)  $\Delta$ COD, (b) sCOD. “●” marks experimental data from batch tests and contour line derives from biharmonic spline interpolation of experimental data using MATLAB® V4 griddata method.

**Fig. 4.** Oil and grease in terms of HEM (hexane extracted material) produced in batches under initial nitrogen of 10 mg/L during synthetic anaerobic secondary effluent batch polishing by *Chlorella vulgaris*.

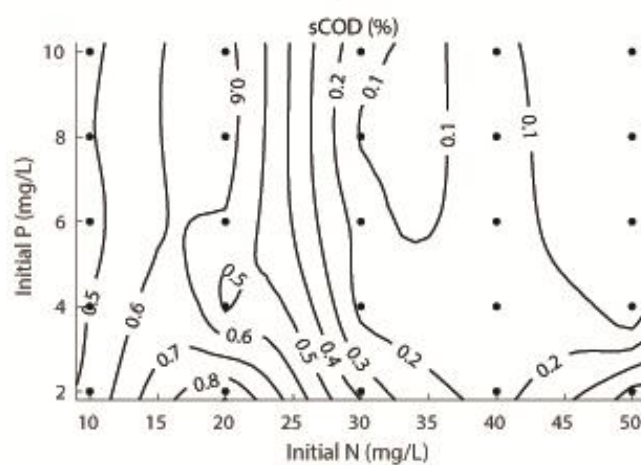
**Fig. 5.** Critical flux versus final microalgal VSS during synthetic anaerobic secondary effluent batch polishing by *Chlorella vulgaris*.



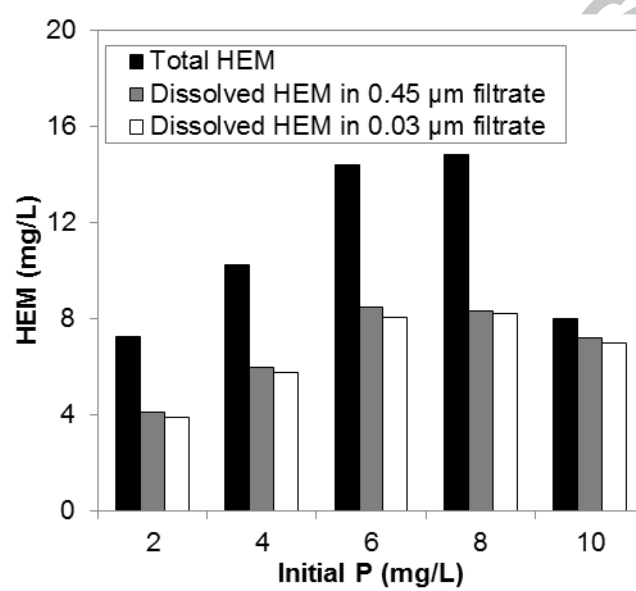


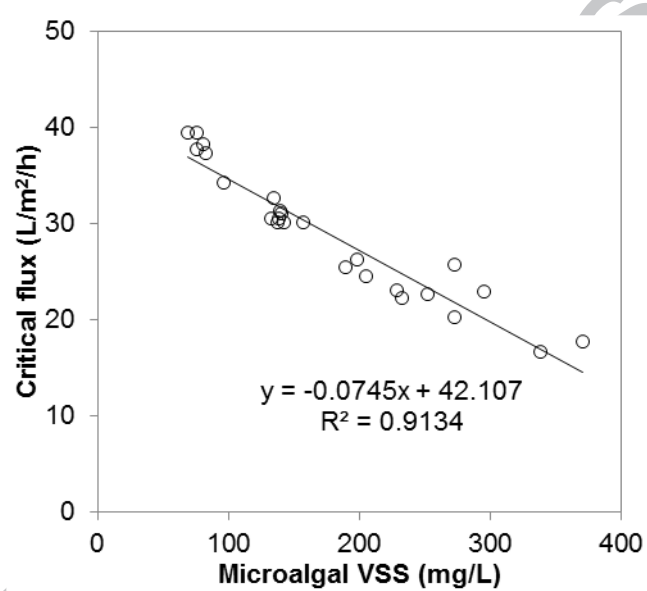


(a)



(b)







**Table 1** Recipe of BBM and synthetic anaerobic secondary effluent.

Component (mg/L)	BBM	Synthetic anaerobic secondary effluent
NH <sub>4</sub> Cl	-	10, 20, 30, 40, 50 in terms of N
H <sub>3</sub> BO <sub>3</sub>	11.42	11.42
CaCl <sub>2</sub> ·2H <sub>2</sub> O	25	25
CoNO <sub>3</sub> ·6H <sub>2</sub> O	0.49	-
CuSO <sub>4</sub> ·5H <sub>2</sub> O	1.57	1.57
EDTA (free acid)	50	-
FeSO <sub>4</sub> ·7H <sub>2</sub> O	4.98	4.98
MgSO <sub>4</sub>	36.628	36.628
MnCl <sub>2</sub> ·4H <sub>2</sub> O	1.44	1.44
MoO <sub>3</sub>	0.71	-
NiSO <sub>4</sub> ·6H <sub>2</sub> O	0.003	0.003
KOH	31	31
KIO <sub>3</sub>	0.004	0.004
KH <sub>2</sub> PO <sub>4</sub>	175	2, 4, 6, 8, 10 in terms of P
K <sub>2</sub> HPO <sub>4</sub>	75	-
NaCl	25	25
NaNO <sub>3</sub>	250	-
Na <sub>2</sub> SeO <sub>3</sub>	0.002	-
SnCl <sub>4</sub>	0.001	-
VOSO <sub>4</sub>	0.002	-
ZnCl <sub>2</sub>	4.181	4.181

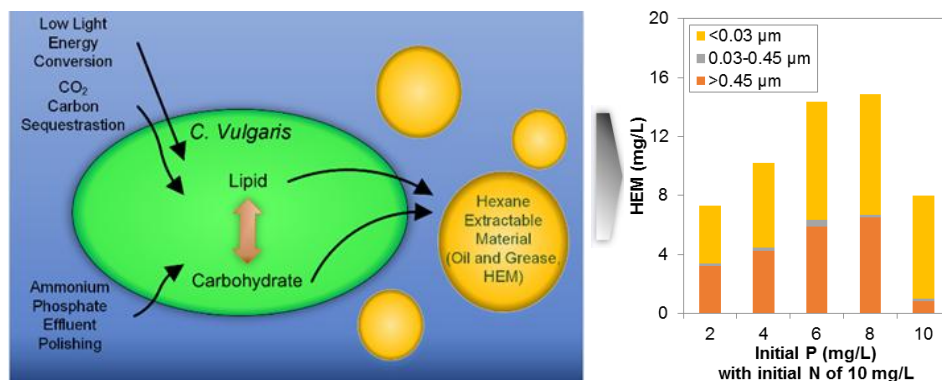
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**Table 2** AnMBR effluent polishing results and comparison with corresponding synthetic effluent polishing.

Batch polishing test		AnMBR effluent		Synthetic effluent	
		Initial	Final	Initial	Final
NH <sub>4</sub> <sup>+</sup> -N	(mg/L)	28.6	1.9	30.0	3.6
PO <sub>4</sub> <sup>3-</sup> -P	(mg/L)	10.0	8.9	10.0	8.8
N removal	(%)	-	93.5	-	88.2
P removal	(%)	-	11.1	-	12.0
Microalgal VSS	(mg/L)	40.0	166.3	40.0	189.4
Generation time	(d)	-	3.9	-	3.6
sN	(gN/gVSS)	-	0.21	-	0.18
sP	(gP/gVSS)	-	0.008	-	0.009
COD	(mg/L)	25.0	64.7	25.5	47.7
sCOD	(gCOD/gVSS)	-	0.31	-	0.15
	Total	-	10.3	-	-
HEM (mg/L)	0.45µm filtrate	-	4.5	-	-
	0.03µm filtrate	-	2.2	-	-
Critical flux	(L/m <sup>2</sup> /h)	-	28.0	-	25.4

-: data not available

## Graphical Abstract



**Highlights:**

- ✓ Microalgal batch cultivation under low light intensity and various nutrient ratios.
- ✓ N removal ascribed to growth rate and P removal to growth rate and cell P content.
- ✓ Hexane extractable material (max. 8.5 mg/L) release due to N deficiency.
- ✓ Ultrafiltration critical flux showed negative linearity to microalgal biomass.
- ✓ Real effluent showed similar results to synthetic effluent with slight inhibition.