Water Research 92 (2016) 61–68

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Nutrient removal from horticultural wastewater by benthic filamentous algae *Klebsormidium* sp., *Stigeoclonium* spp. and their communities: From laboratory flask to outdoor Algal Turf Scrubber (ATS)

Junzhuo Liu [a, b, *], Bram Danneels [b], Pieter Vanormelingen [b], Wim Vyverman [b]

[a] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, 71 East Beijing Road, Nanjing, 210008, China
[b] Laboratory of Protistology and Aquatic Ecology, Department of Biology, Ghent University, Krijgslaan 281-S8, 9000, Ghent, Belgium

ARTICLE INFO

Article history:
Received 9 November 2015
Received in revised form
15 January 2016
Accepted 21 January 2016
Available online 23 January 2016

Keywords:
Algal Turf Scrubber
Benthic filamentous algae
Flow rate
Horticultural wastewater
Nutrient removal

ABSTRACT

Benthic filamentous algae have evident advantages in wastewater treatment over unicellular microalgae, including the ease in harvesting and resistance to predation. To assess the potentials of benthic filamentous algae in treating horticultural wastewater under natural conditions in Belgium, three strains and their mixture with naturally wastewater-borne microalgae were cultivated in 250 ml Erlenmeyer flasks in laboratory as well as in 1 m^2 scale outdoor Algal Turf Scrubber (ATS) with different flow rates. *Stigeoclonium* competed well with the natural wastewater-borne microalgae and contributed to most of the biomass production both in Erlenmeyer flasks and outdoor ATS at flow rates of 2–6 L min^{-1} (water velocity 3–9 cm s^{-1}), while *Klebsormidium* was not suitable for growing in horticultural wastewater under the tested conditions. Flow rate had great effects on biomass production and nitrogen removal, while phosphorus removal was less influenced by flow rate due to other mechanisms than assimilation by algae.

1. Introduction

In the last half century the exponential increases of human population and activities have resulted in the production of tremendous volumes of domestic, agricultural and industrial wastewater which has greatly increased the input of nutrients and other pollutants into natural water bodies (Abdel-Raouf et al., 2012; Boelee et al., 2014). One of the main problems is that high concentration of inorganic nutrient in agricultural wastewater and the effluent from wastewater treatment systems, cause environmental problems in natural water ecosystems (de-Bashan and Bashan, 2004). Several studies have highlighted the potential of algae in removing inorganic nitrogen and phosphorus from domestic, agricultural and dairy wastewaters (Arbib et al., 2014; Mennaa et al., 2015; Van den Hende et al., 2014).

However, in the practical wastewater treatment, chemical compositions of different wastewater sources vary greatly (Abdel-Raouf et al., 2012). For example, the wastewater produced from animal farms is usually rich in ammonium and organic nitrogen, while municipal wastewater has less nitrogen and phosphorus but more heavy metals than agricultural wastewater (Cai et al., 2013). Furthermore, each microalgae strain has its favorable growth conditions, such as pH, light, temperature, salinity, and preferred nitrogen form and N/P ratio (Besson and Guiraud, 2013; Cai et al., 2013; Lee et al., 2015; Liu and Vyverman, 2015; Markou and Georgakakis, 2011). In addition to the physiological factors, the interactions between the cultivated algae and other (micro) organisms, especially grazing by heterotrophic protists and small animals and competition with other native microalgae in wastewater should be taken into consideration (Kesaano and Sims, 2014). For instance, filamentous algae with a large cell or colony size and indigestible cell wall are potentially more resistant to predation by grazers than the unicellular species (Guo et al., 2014; Wellnitz and Ward, 1998) and thus can improve the biomass production and nutrient removal efficiency (Kesaano and Sims, 2014). Besides

* Corresponding author. State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, 71 East Beijing Road, Nanjing, 210008, China.
E-mail address: liujzh04@aliyun.com (J. Liu).

http://dx.doi.org/10.1016/j.watres.2016.01.049
0043-1354/

removing nutrient, wastewater grown algae can also produce biomass for biofuel or high-value extracts (Mulbry et al., 2010). For example, some *Ulothrix* strains have high amounts of polyunsaturated fatty acids C16:4ω3 and C18:3ω3 (Van den Hende et al., 2014). In the authors' previous study (Liu et al., 2016), *Klebsormidium* spp. and *Stigeoclonium* sp. have high C18:2ω6 and C18:3ω3 contents respectively. Therefore, the selection of appropriate strains and a good understanding of the ecology of algal communities are critical in cleaning up a certain type of wastewater and producing valuable biomass (Roeselers et al., 2008).

Moreover, for the sustainable wastewater treatment using algae, it is required to get a high biomass recovery with a cost-efficient biomass harvesting method and discharge of biomass-free effluent (Van den Hende et al., 2014). Benthic filamentous algae with large cell or colony sizes such as *Cladophora*, *Oedogonium* and *Spirogyra* have the ability to grow attached to substrates, and are easy to harvest with low energy consumption (Adey et al., 2011; Mulbry et al., 2010). Accordingly, several cultivation systems including Algal Turf Scrubber (ATS) and Rotating Algal Biofilm Reactor have been developed to make use of the above mentioned characteristics of benthic filamentous algae in wastewater treatment (Craggs et al., 1996; Gross et al., 2015; Kesaano and Sims, 2014).

ATS is a controlled ecosystem for wastewater treatment by flowing wastewater over an inclined surface which is covered by seeded periphyton biofilms. It has already been applied in treating polluted river water, agricultural and dairy manure wastewater with a growing area of 1–1000 m^2 (Adey et al., 2011; Craggs et al., 1996; Mulbry et al., 2008; Sandefur et al., 2011). ATS is designed to promote biological wastewater treatment using benthic algae, by driving their photosynthesis to high levels, and harvesting the biomass periodically to remove the assimilated nutrients and stimulate further production (Adey et al., 2013; Sandefur et al., 2011). Accordingly, on an ATS flow rate is a vital factor of determining the ability of water to hold and transport suspended solids (Godillot et al., 2001). It can also facilitate nutrient uptake by bringing metabolites to reaction sites and carrying away the metabolic waste (Craggs et al., 1996). Zippel et al. (2007) reported that under the same light and temperature conditions, the biomass production was significantly higher at a flow rate of 100 L h^{-1} than at 25 L h^{-1}. However, the shear stress caused by water flow can break the attachment of benthic algae onto the supporting substrate and a weak shear stress or low flow rate/water velocity is usually preferred (Larned, 2010; Sushchik et al., 2010). Therefore, flow rate could be a critical factor in determining algal community, biomass production and nutrient removal performance of an ATS.

In this study, *Klebsormidium* sp. and *Stigeoclonium* spp. were cultivated in laboratory individually and in mixture with the horticultural wastewater-borne algae respectively. Then a mixture of them was inoculated to 1 m^2 outdoor ATS systems with different flow rates. The main objectives were to investigate: (1) the potential of *Klebsormidium* and *Stigeoclonium* in nutrient removal from horticultural wastewater and their competition with wastewater-borne algae under controlled conditions; (2) the effect of flow rate on benthic algal community, biomass production and nutrient removal of an outdoor ATS inoculated with *Klebsormidium* and *Stigeoclonium*. Moreover, the transferability of benthic filamentous algae in wastewater treatment from controlled laboratory conditions to outdoor conditions was evaluated.

2. Materials and methods

2.1. Algal cultures

Three benthic filamentous green algae *Klebsormidium* sp., *Stigeoclonium* sp. LJ1 and *Stigeoclonium* sp. LJ2 used in this study were isolated as described previously (Liu and Vyverman, 2015). These three species were identified with their DNA sequences (Text S1), and the newly generated sequences were deposited in GenBank (accession numbers: KP165132, KR002183 and KR422334). All the strains were submitted to the BCCM/DCG culture collection (www.bccm.belspo.be, accession numbers: DCG0641, DCG0642 and DCG0643). Naturally occurring microalgae in the wastewater were collected by filtering the fresh horticultural wastewater through Whatman Grade 6 paper filters (pore size 3 μm) and were subsequently enriched in WC medium (Guillard and Lorenzen, 1972), but without vitamin addition and pH adjustment. *Klebsormidium* sp., *Stigeoclonium* spp. and the natural wastewater microalgae were cultivated in WC medium under a 16:8 h light/dark cycle at a light intensity of 80–90 μmol photons m^{-2} s^{-1} at 23 °C in a climate room. Culture conditions were the same throughout the study.

2.2. Laboratory experiment

To investigate the potential of benthic filamentous algae in nutrient removal from horticultural wastewater, two different culture media were tested under laboratory conditions. First, the horticultural wastewater was collected from a horticultural company (Proefcentrum voor Sierteelt vzw) in Destelbergen, Belgium and filtered through Whatman Grade 6 paper filters and stored at 4 °C prior to the experiment. The chemical compositions in the horticultural wastewater were: NO_3^--N 47.2 mg L^{-1}, PO_4^{3-}-P 11.6 mg L^{-1}, Cl^- 6 mg L^{-1}, SO_4^{2-} 135 mg L^{-1}, Mg^{2+} 18 mg L^{-1}, Ca^{2+} 96 mg L^{-1}, K^+ 34 mg L^{-1}, Na^+ 10 mg L^{-1}, B 0.13 mg L^{-1}, Mn^{2+} 0.08 mg L^{-1}, Cu^{2+} 0.15 mg L^{-1}, Fe^{3+} 1.4 mg L^{-1}, Zn^{2+} 0.15 mg L^{-1}. The second medium was a synthetic wastewater based on WC medium, to which additional NO_3^--N and PO_4^{3-}-P were added to a final concentration of 47.2 mg NO_3^--N L^{-1} and 11.6 mg PO_4^{3-}-P L^{-1} to match the nitrogen and phosphorus concentrations of the horticultural wastewater used in this study. The pH of the synthetic wastewater was adjusted to 7.0 (the same value as the horticultural wastewater) by adding 4% HCl after autoclaving.

Algal biomass from exponentially growing cultures (3 days after inoculation) of *Klebsormidium* sp., *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and the enriched natural wastewater microalgae were filtered through a GF/F filter and washed with distilled water three times to remove any nutrients from the original medium. Next, 0.04 g of wet algal biomass from each of the four algal cultures was weighed and inoculated separately to 100 ml of the horticultural wastewater and synthetic wastewater respectively in 250 ml Erlenmeyer flasks in 9 replicates. A fifth inoculum consisted of a mixture of 0.01 g wet biomass of each of the four algal cultures. An additional subsample of the wet algal biomass was dried at 60 °C for 24 h to measure its moisture content to determine their initial dry weight. Of the 9 replicate flasks, 3 were used for monitoring nutrient changes and harvested on day 9, 3 for harvesting on day 3 and 3 for harvesting on day 6 to get the growth curve (more details in section 2.5). Erlenmeyer flasks were manually shaken and replaced randomly on the shelf twice a day. The experiment lasted for 9 days by which time the concentration of NO_3^--N decreased below 1 mg L^{-1} under some cases.

2.3. Outdoor ATS

The ATS used in this study consisted of a water pump, a 120 L tank, a flow meter and a rectangular lane covered with plastic pond liner (a sketch drawing in Fig. 1). It was located under the natural conditions of Ghent, Belgium. The size of the lane was 0.39 m in width and 2.50 m in length (area 0.975 m^2), and it was set at a slope

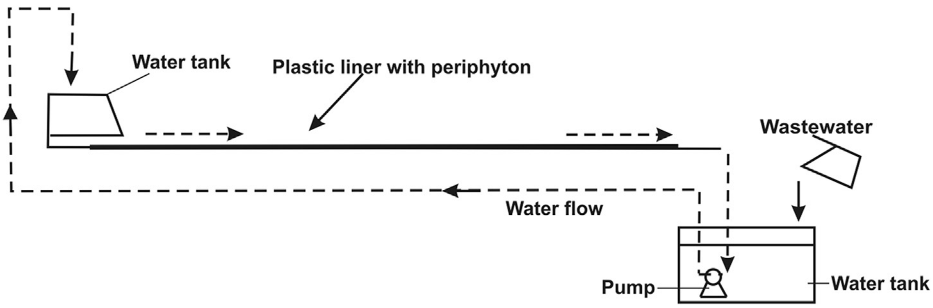

Fig. 1. Schematic drawing of the outdoor Algal Turf Scrubber used in this study.

of 1%. Four ATS units including three lanes each were set up, giving a total of 12 replicate lanes. The same horticultural wastewater as in section 2.2 was added to it. Then, 10 g wet biomass of a mixture of *Klebsormidium* sp. and *Stigeoclonium* spp. with equal amount from culture was inoculated to 12 individual lanes of the ATS. After a period of two weeks in which the algae could settle down, attach and form a biofilm community, the bioreactor was started to run with four different flow rates of 2, 4, 6 and 8 L min^{-1}(equal to water velocities of 3, 6, 9 and 12 cm s^{-1}) using horticultural wastewater, with triplicate lanes on three ATS units for each flow rate. After running for a week, the periphyton biomass was scrapped and discarded. From then on, the biomass was harvested weekly to monitor the algal community compositions and biomass productivity. For each lane, the initial wastewater volume was 65 L, and distilled water was added regularly to compensate the evaporation. The wastewater was continuously recirculated and refreshed when the nutrient decreased to zero under some conditions. After each heavy rain, excess water was removed to 65 L level and the removed volume recorded. This experiment lasted from April to June 2015.

2.4. Wastewater chemical analysis

For the measurement of nutrient concentrations of the wastewater, 2 ml of wastewater was collected daily from the Erlenmeyer flasks, while for the outdoor ATS 15 ml water was collected every two to three days after distilled water was added to the 65 L level. Fortunately, there was only one heavy rain during this operation period and no excess wastewater accumulated in the ATS tanks due to evaporation afterwards. The wastewater samples were filtered through Whatman grade 6 paper filters and the filtrate was used to measure pH, NO_3^--N and reactive phosphorus. pH was measured with a pH meter (PHM210, Unisense). NO_3^--N was measured with a spectrophotometer (Shimazu UV-1601, Japan) at 220 and 275 nm following the ultraviolet spectrophotometric screening method (APHA, 1998). The reactive phosphorus was measured following the Vanadomolybdophosphoric acid colorimetric method with spectrophotometer at 400 nm (APHA, 1998).

2.5. Biomass harvesting and preparation

For the laboratory experiment, the algal growth curve was determined by separately harvesting the biomass of three replicate flasks every three days and measuring the dry weight (DW). Algal dry weight was determined by filtering the biomass through pre-weighed Whatman GF/F filters, freeze-drying overnight and weighing the dried filters with algal biomass the next day. The mean algal biomass production during the whole experiment period was calculated by Equation (1).

$$\text{Mean biomass production}\left(P_0,\ \text{mg DW L}^{-1}\,\text{d}^{-1}\right)\frac{DW_t - DW_0}{t*V}\times \tag{1}$$

In Equation (1), DW_t represents the dry weight of the algae on day t, mg; DW_0 is the dry weight on day 0, mg; t is the cultivation time, d; V is the volume of the medium, L.

For the ATS, it was harvested every week by scraping the biomass from the plastic linear using a metal scraper and collected to a pre-weighed bottle, followed by centrifuging the biomass for 5 min at 2000 *g*. Then the wet biomass was weighed and a subsample was collected to an aluminum cup and weighed to measure its moisture content by drying for 24 h to constant weight at 60 °C. The biomass production was calculated by Equation (2).

$$\text{Biomass production}\left(\text{g DW m}^{-2}\text{d}^{-1}\right) = \frac{(W_5 - W_3)(W_2 - W_1)}{(W_4 - W_3)*A*N} \tag{2}$$

In Equation (2), W_1 is the weight of the bottle, g; W_2 is the weight of bottle and wet biomass, g; W_3 is the weight of aluminum cup, g; W_4 is the weight of aluminum cup and wet biomass, g; W_5 is the weight of aluminum cup and dry biomass, g; A is the area of the bioreactor, m^2; N is the sampling interval, day.

Nitrogen and phosphorus contents of the harvested algal biomass were determined with the methods described previously (Liu and Vyverman, 2015). Nitrogen and phosphorus recovered rate in the biomass was calculated with Equation (3).

$$\text{N (P) recovery rate (\%)} = \frac{M*C_{N(P)}}{V*(C_0 - C_1)}*100\% \tag{3}$$

In Equation (3), M is the biomass produced between day t_0 and t_1, mg; $C_{N(P)}$ is nitrogen or phosphorus content of the biomass, % of DW; V is the volume of the wastewater, L; C_0 and C_1 are the nitrogen or phosphorus concentrations on day t_0 and t_1 respectively, mg N(P) L^{-1}.

2.6. Algal community structure

Subsamples for algal community analysis were collected on day 0 and 9 for Erlenmeyer flasks and every week for ATS, and fixed with an equal volume of 4% formalin. To assess the algal composition of the biofilms, 50 μl of the sample was transferred to a microscope slide and 9 photos were randomly taken at 400 × magnifications (Leitz Diaplan Microscope, Germany), and this was done in triplicate. Then the cell numbers of each genus on each photo were counted with Image J and converted to the biomass contribution by multiplying with their biovolume, which was calculated with the equations proposed by Hillebrand et al.

(1999) by measuring the sizes of 30–40 cells with Image J.

2.7. Statistical analysis

Two-way ANOVAs were used to test for statistical differences in biomass production or nutrients content of the biomass of the laboratory experiment with algal inoculum and growth medium as independent fixed factors (using STATISTICA 10.0). Post-hoc Tukey tests were used to determine significant pairwise differences. One-way ANOVA was used to test for statistical differences in biomass production or nutrient content of the biomass from the outdoor experiment with flow rate as independent fixed factor. A significance level of $p < 0.05$ was applied throughout.

3. Results and discussion

3.1. Algal composition and biomass production

3.1.1. Laboratory flasks

The naturally occurring wastewater microalgae were dominated by *Chlamydomonas* (8%), *Chlorella* (67%) and various filamentous cyanobacteria (25%). After 9 days cultivation, the filamentous algae *Stigeoclonium* competed well with the natural wastewater algae under the tested conditions and the algal compositions were relatively stable in both of horticultural and synthetic wastewater with a proportion of 62% on day 0 and 69% and 68% on day 9 respectively (Fig. 2), while *Klebsormidium* decreased from 26% to 13% and 11% in horticultural and synthetic wastewater respectively. For the natural wastewater algae, *Chlorella* grew well and its relative abundance increased from 67% to 75% in the horticultural wastewater and 90% in the synthetic wastewater (Fig. 2), while cyanobacteria decreased from 25% on day 0–14% and 1% on day 9 in the horticultural and synthetic wastewater respectively.

As shown in Fig. 3, the monocultures of *Klebsormidium* sp. and *Stigeoclonium* spp. had higher biomass production in the synthetic wastewater (36.0–63.3 mg L^{-1} d^{-1}) than in horticultural wastewater (13.5–50.8 mg L^{-1} d^{-1}). In contrast, the natural occurring wastewater algae and the mixture had higher biomass production in horticultural wastewater (53.7–57.1 mg L^{-1} d^{-1}) than in synthetic wastewater (46.5–49.0 mg L^{-1} d^{-1}). The two-way ANOVA showed that there was a significant effect of algae inoculums ($F = 166.2$, $p < 0.001$) and wastewater media ($F = 41.3$, $p < 0.001$) on the biomass production. Also the interaction of algae inoculums and wastewater media had a significant effect on biomass production ($F = 40.1$, $p < 0.001$). The Post-hoc Tukey test indicated that the inoculum effect on biomass production was mainly caused by the lower biomass production of *Klebsormidium* sp. and *Stigeoclonium* sp. LJ1 than the others, and the medium effect was mainly due to the lower biomass production of the monocultures in horticultural wastewater than in synthetic wastewater.

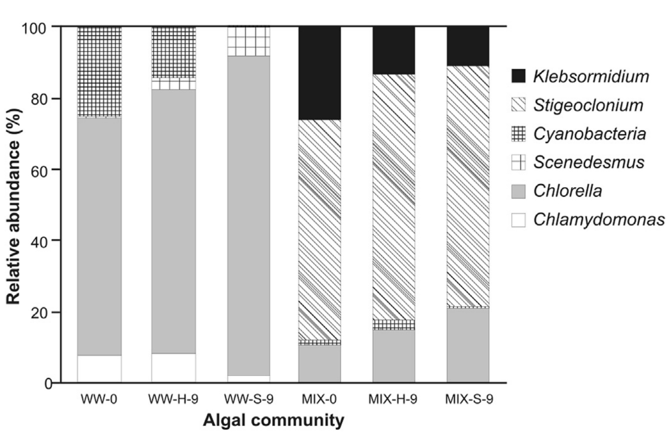

Fig. 2. Algal community composition of natural wastewater algae (WW) and the mixture of *Klebsormidium* sp., *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and natural wastewaterborne algae (MIX) in synthetic (S) or horticultural (H) wastewater on day 0 (0) and day 9 (9).

The monocultures of *Klebsormidium* sp. and *Stigeoclonium* sp. LJ1 had significantly lower biomass production (all $p < 0.001$) in the horticultural wastewater than *Stigeoclonium* sp. LJ2, the natural wastewater algae and the mixture. Thus, *Stigeoclonium* sp. LJ2 was more suitable for growing in the horticultural wastewater than *Klebsormidium* sp. and *Stigeoclonium* sp. LJ1.

3.1.2. Outdoor ATS

Triplicate ATS lanes (Fig. 1) were operated at flow rates of 2–8 L min^{-1} from May to June 2015. As shown in Fig. 4, *Klebsormidium* only had a low abundance (0–4%) in the first two weeks of the experiment and then disappeared. For *Stigeoclonium*, it had an abundance of 66–73% in the beginning and then its abundance at the flow rate of 8 L min^{-1} decreased sharply to 8% by week 5 (Fig. 4D), while its abundance remained 46% at flow rate of 2 L min^{-1} and 20–31% at 4–6 L min^{-1} by week 5 (Fig. 4A, B and C). For the unicellular algae, *Chlamydomonas*, *Desmodesmus* and *Scenedesmus* were the main genera and showed a gradually increase in their relative abundance. Especially at the flow rate of 8 L min^{-1}, the abundance of *Desmodesmus* greatly increased from 5% in the beginning to 71% by week 5 (Fig. 4D). In summary, the benthic filamentous algae were susceptible to flow rate and the results of this study were in accordance with the reports of Ahn et al. (2013) and Dodds (1991). Additionally, under outdoor conditions, the temperature variation (weekly average 13–17 °C) during the operation period could be a potential factor affecting algal community (Adey et al., 2013; Breeman et al., 2002). Variation in solar irradiance (4.5–6.0 kWh m^{-2} d^{-1}) was observed during the operation period, but without significant difference between the five weeks (One-way ANOVA, $p < 0.01$).

The biomass production showed an increasing trend from May to June (Fig. 5), following a gradual rise of weekly average temperature from 13 to 17 °C (KMI, http://www.meteo.be/). One-way ANOVA indicated that there was a significant positive effect of flow rate on biomass production ($p = 0.016$). Specifically, the lowest biomass production (0.8–1.7 g DW m^{-2} d^{-1}) was observed at the lowest flow rate of 2 L min^{-1}, while the highest (1.2–2.0 g DW m^{-2} d^{-1}) was produced at the flow rate of 8 L min^{-1}.

3.2. Nitrogen and phosphorus removal

3.2.1. Laboratory flasks

For the experiment studying the nutrient removal by different algal inoculums from the horticultural and synthetic wastewater (Fig. 6A, B), phylogenetic difference in nutrient removal efficiency was observed among the selected three species. For instance, the monoculture of *Stigeoclonium* sp. LJ2 had the highest daily nitrogen removal rate (Table 1, maximally 8.0 and 8.6 mg NO_3^--N L^{-1} d^{-1} in the horticultural and the synthetic wastewater respectively), while the monoculture of *Klebsormidium* sp. had the lowest nitrogen removal rate (maximally 4.5 and 5.7 mg NO_3^--N L^{-1} d^{-1}). In terms of phosphorus removal, the monoculture of *Stigeoclonium* sp. LJ2 had the highest daily phosphorus removal rate (maximally 5.4 mg PO_4^{3-}-P L^{-1} d^{-1}) in the horticultural wastewater, while the wastewater-borne algae had the lowest (maximally 3.8 mg PO_4^{3-}-P L^{-1} d^{-1}).

After 9 days cultivation, the nitrogen removal efficiency of the synthetic wastewater (59–99%) was slightly higher than the horticultural wastewater (20–86%) for the five algal inoculums (Table 1, Fig. 6A). From Fig. 6A, it can be concluded that nitrogen

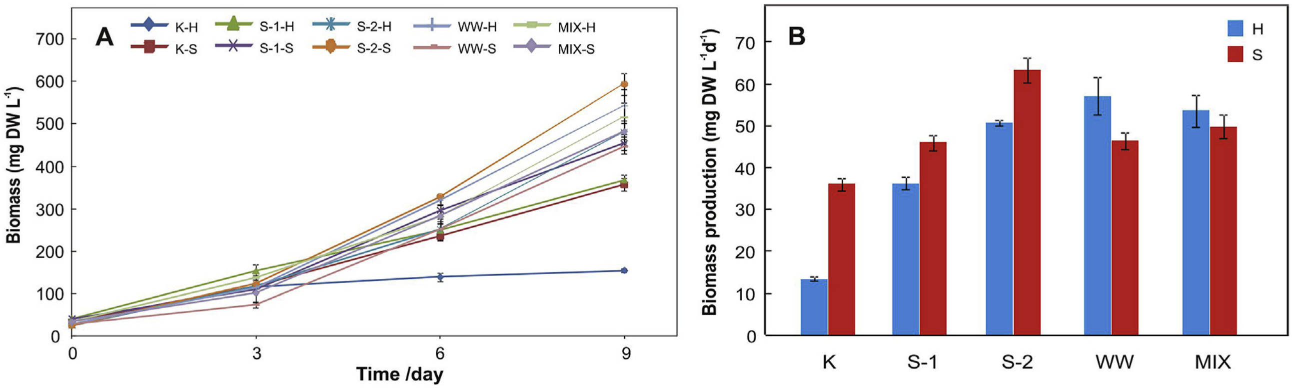

Fig. 3. A: Average biomass (mg DW L-1) accumulated over time of *Klebsormidium* sp. (K), *Stigeoclonium* sp. LJ1 (S-1), *Stigeoclonium* sp. LJ2 (S-2), natural wastewater-borne algae (WW) and mixture (MIX) respectively in indoor flasks with horticultural (H) and synthetic wastewater (S); B: Average biomass production (mg DW L^{-1} d^{-1}) of the five algal inoculums in indoor flasks with horticultural and synthetic wastewater.

was removed at a higher rate from synthetic wastewater than from horticultural wastewater by all five algal inoculums, especially the monoculture of *Klebsormidium* sp. and both *Stigeoclonium* strains. For example, the NO_3^--N concentration of horticultural wastewater treated by *Klebsormidium* sp. remained around 40 mg L^{-1} from day 2, while it was reduced to 20 mg L^{-1} in synthetic wastewater by day 9. Moreover, the NO_3^--N removal process from horticultural and synthetic wastewater by the natural wastewater algae was quite similar (Table 1, Fig. 6A). It indicated that the natural wastewater-borne algae were well adapted to the horticultural wastewater and could efficiently assimilate nitrogen from the wastewater. The monoculture of *Stigeoclonium* sp. LJ2 showed higher nitrogen removal efficiency from both horticultural and synthetic wastewater than *Klebsormidium* sp. and *Stigeoclonium* sp. LJ1. It indicated that *Stigeoclonium* sp. LJ2 had a good capacity of assimilating nitrogen from the horticultural wastewater.

For phosphorus removal, there was a sharp decrease in the horticultural wastewater after the first day, during which the PO_4^{3-}-P concentration decreased from 11.6 mg L^{-1} to 6.1–7.7 mg L^{-1}. Accordingly, it resulted in a maximal phosphorus removal rate of 4.1, 5.0, 5.4, 3.8 and 5.2 mg PO_4^{3-}-P L^{-1} d^{-1} for the monoculture of *Klebsormidium* sp., *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2, natural wastewater algae and the mixture respectively. Compared to phosphorus removal process of horticultural wastewater, both phosphorus removal rate and efficiency from synthetic wastewater were lower for the five algal inoculums (Fig. 6B and Table 1). Their maximal phosphorus removal rates were 1.0, 1.3, 1.6, 1.8 and 1.3 mg PO_4^{3-}-P L^{-1} d^{-1} for the monoculture of *Klebsormidium* sp., *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2, natural wastewater algae and the mixture respectively.

The big difference in the maximal phosphorus removal rate between the horticultural and synthetic wastewater could probably be caused by the chemical precipitation of phosphorus. Chemical precipitation has been documented as an important phosphorus removal mechanism from the wastewater by algae (de-Bashan and Bashan, 2004). The pH of the wastewater can be prompted by the consumption of CO_2 through the algal photosynthesis and then result in the phosphorus precipitation (Craggs et al., 1996; Larsdotter et al., 2010; Roeselers et al., 2008). In this study, the pH increased greatly from 7.0 on day 0–8.5 on day 1 and then above 9.0 afterwards in both the horticultural and synthetic wastewater. However, in the synthetic wastewater, the chelating agent EDTA which can prevent phosphorus precipitation (de-Bashan and Bashan, 2004) was present and caused a lower phosphorus removal rate and thus a lower phosphorus removal efficiency than in horticultural wastewater.

3.2.2. Outdoor ATS

For the outdoor ATS with different flow rates, the lanes under a flow rate of 8 L min^{-1} had the highest nitrogen removal rate and efficiency while the lanes at 2 L min^{-1} had the lowest (Fig. 6C, Table 2). In terms of phosphorus removal, the lanes under the flow rate of 8 L min^{-1} had slightly higher phosphorus removal efficiency from day 2 on while the other three showed no difference (Fig. 6D).

As shown in Fig. 6C, the NO_3^--N removal process accelerated following the running of the ATS, and it decreased from 18 to 9 days to remove NO_3^--N from 32 mg L^{-1} to 0. This was in accordance with the biomass accumulation process (Fig. 5) and was most probably caused by the rise of temperature from May to June. Generally, the nitrogen removal rate at a flow rate of 8 L min^{-1} was higher than at 2–6 L min^{-1} and it was maximally 6.4 mg L^{-1} d^{-1} (Table 2), while it was 3.5–6.1 mg L^{-1} d^{-1} at flow rates of 2–6 L min^{-1}. For the ATS lanes with flow rate of 8 L min^{-1}, it took 9 days during the first cycle to reach the nitrogen discharge norm in Belgium (10 mg TN L^{-1}), while it took 14, 11 and 12 days for the lanes with flow rates of 2, 4 and 6 L min^{-1} respectively.

In terms of phosphorus removal, there was a sharp decrease in PO_4^{3-}-P concentration from 9.5 to 1.7–2.9 mg L^{-1} for all the lanes after running two days, which was similar to the phosphorus removal process of the laboratory experiment. It was known that the phosphorus assimilation by algal cells was through active transport which was highly related to the nitrogen availability of both algal cell tissues and the medium (Liu and Vyverman, 2015). In this study, the nitrogen removal rate was around 1–3 mg L^{-1} d^{-1}, but the phosphorus removal rate reached 3.4–3.9 mg L^{-1} d^{-1}, thus most probably other mechanisms had participated in phosphorus removal. The same as the laboratory experiment, great increase in pH was observed from 7.0 to over 8.5 in the outdoor ATS system two days after refreshing the wastewater. Thus, chemical precipitation must have occurred. Moreover, phosphorus can also be removed through surface adsorption by benthic algal community via the formation of hydroxyapatite (Lu et al., 2014; Sañudo-Wilhelmy et al., 2004). Accordingly, because of the diverse phosphorus removal processes, the effect of flow rate on phosphorus removal was suppressed.

3.3. Nutrient composition and recovery by algal biomass

For the laboratory experiment, nitrogen and phosphorus contents of the algal biomass were 3.3–6.4% and 0.7–2.5% of dry weight respectively (Table 1). Two-way ANOVA showed that there were significant effects of algal inoculums ($F = 85.0$, $p < 0.001$) and wastewater media ($F = 274.7$, $p < 0.001$) on nitrogen content and

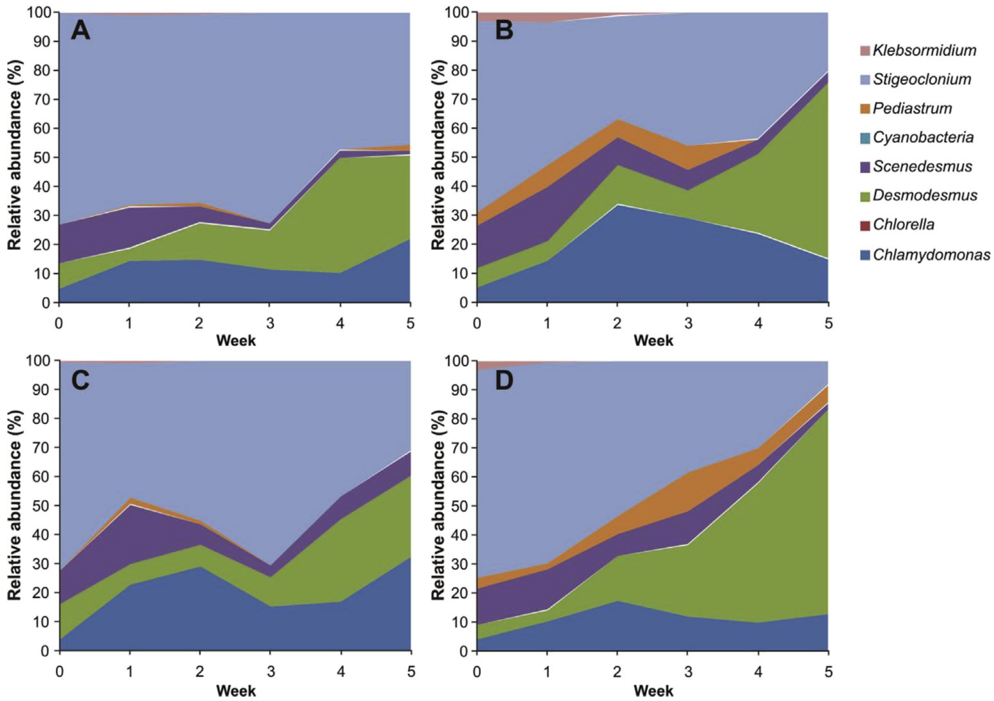

Fig. 4. A–D: Algal community composition changes over time of the outdoor ATS under flow rates of 2, 4, 6 and 8 L min^{-1} respectively.

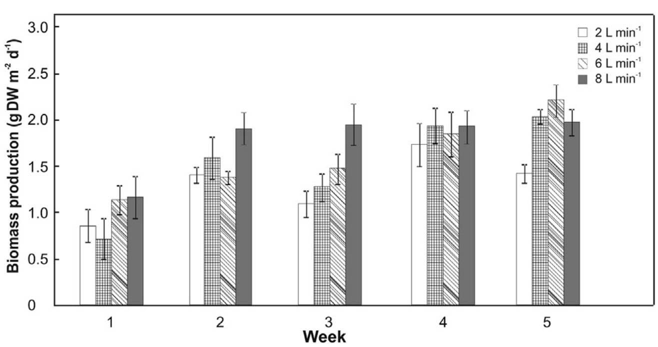

Fig. 5. The biomass production on the outdoor ATS (g DW m^{-2} d^{-1}) under flow rates of 2–8 L min^{-1}. The error bars correspond to the standard deviation of triplicates.

the interaction between algal inoculums and the wastewater media on nitrogen content was also highly significant ($F = 32.9$, $p < 0.001$). The Post-hoc Tukey test indicated that the effect of algae inoculum on nitrogen content was mainly caused by the lower nitrogen content of *Klebsormidium* sp. and the higher of wastewater-borne microalgae than the others. The effect of wastewater media was due to the lower nitrogen content of algal biomass from horticultural wastewater than from synthetic wastewater.

Similarly, the two-way ANOVA also indicated that there were significant effects of algal inoculums ($F = 9.5$, $p < 0.001$) and wastewater media ($F = 522.9$, $p < 0.001$) on phosphorus content and the interaction between algae inoculums and wastewater media was highly significant as well ($F = 87.3$, $p < 0.001$). The Post-hoc Tukey test indicated that the effect of algal inoculums on phosphorus content was mainly caused by the higher phosphorus content of *Klebsormidium* sp. and *Stigeoclonium* sp. LJ1 than the others, and the effect of wastewater media was caused by the higher phosphorus content of algal biomass from horticultural wastewater than from synthetic wastewater.

Nitrogen recovery rate by algal biomass of these five algae inoculums varied between 53% and 69%, while it was 46–99% for phosphorus (Table 1). Although the biomass produced from horticultural wastewater had higher phosphorus content than from synthetic wastewater, the phosphorus recovery rate of *Klebsormidium* sp., *Stigeoclonium* spp. and the mixture was higher in synthetic wastewater (90–99%) than in horticultural wastewater (46–81%). This further indicated that other process (e.g. chemical precipitation and adsorption) than assimilation participated in phosphorus removal from the horticultural wastewater.

Compared to the laboratory flasks, the nitrogen and phosphorus content of the biomass harvested from the outdoor ATS was more sensitive to nitrogen and phosphorus concentrations of the wastewater and it varied between 4.9% and 6.9%, 1.5% and 3.6% for nitrogen and phosphorus respectively (Table 2). It showed no significant effect of flow rates on biofilm nitrogen and phosphorus contents (p was 0.184 and 0.23 for nitrogen and phosphorus respectively). However, phosphorus recovery rates of the ATS increased greatly from $60 \pm 5\%$ to $86 \pm 21\%$ following the increase of flow rate from 2 to 8 L min^{-1}. That was most likely because of higher biomass production at higher flow rate, which produced more algal biomass to absorb and/or adsorb phosphorus from wastewater (Liu and Vyverman, 2015; Lu et al., 2014).

3.4. Potentials of benthic filamentous algae in outdoor bioreactor

In this study, *Stigeoclonium* grew well both in monoculture and mixture with the natural wastewater growing microalgae in horticultural wastewater and efficiently assimilated nitrogen and phosphorus from wastewater under controlled laboratory conditions. In the outdoor ATS, *Stigeoclonium* was successfully inoculated and maintained its dominance with a contribution of 46–73% to the total biomass at a low flow rate of 2 L min^{-1} (3 cm s^{-1}), while they gradually lost their dominance at high flow rates. *Klebsormidium* sp. had a much lower biomass and nutrient removal efficiency in the horticultural wastewater than *Stigeoclonium* spp. in the laboratory experiment. Similarly, *Klebsormidium* sp. only had a relative abundance of less than 4% in the beginning few weeks on the outdoor ATS. That was likely due to the presence of some inhibiting

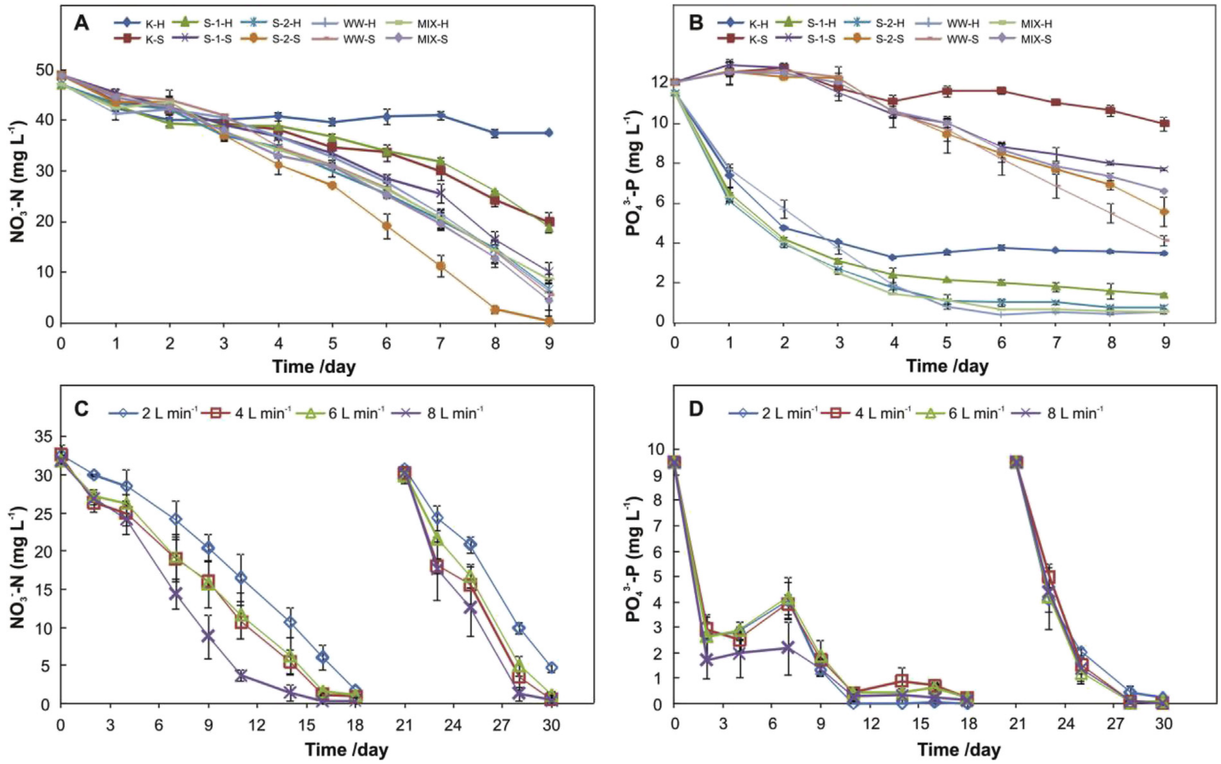

Fig. 6. A, B: NO3⁻-N and $PO4^{3-}$-P concentration (mg L^{-1}) changes process of *Klebsormidium* sp. (K), *Stigeoclonium* sp. LJ1 (S-1), *Stigeoclonium* sp. LJ2 (S-2), natural wastewater-borne algae (WW) and mixture (MIX) respectively in horticultural (H) and synthetic wastewater (S) in indoor flasks over time. C, D: $NO3^{-}$-N and $PO4^{3-}$-P concentration (mg L^{-1}) changes process of the outdoor ATS at flow rates of 2–8 L min^{-1} over time. The error bars correspond to the standard deviation of triplicates.

Table 1
Maximal NO_3^--N and PO_4^{3-}-P removal rate ($R_{max,\ N}$, $R_{max,\ P}$, mg N or P L^{-1} d^{-1}), the final nitrogen and phosphorus removal efficiency (RE_N, RE_P, %), nitrogen and phosphorus contents and recovery rates of Klebsormidium sp. (K), Stigeoclonium sp. LJ1 (S-1), Stigeoclonium sp. LJ2 (S-2), natural wastewater algae (WW) and mixture of the above four (MIX) in the horticultural (H) and synthetic wastewater (S) under laboratory conditions.

Algal inoculum	K		S-1		S-2		WW		MIX	
	H	S	H	S	H	S	H	S	H	S
$R_{max,\ N}$ (mg N L^{-1} d^{-1})	4.7	5.7	6.0	9.1	8.0	8.6	7.8	8.5	6.4	8.3
RE_N (%)	20	59	60	79	86	>99	86	89	82	91
N content (%)	3.3 ± 0.1	5.4 ± 0.1	4.7 ± 0.1	5.4 ± 0.2	5.6 ± 0.2	5.6 ± 0.3	5.2 ± 0.3	6.4 ± 0.1	5.2 ± 0.1	6.3 ± 0.1
N recovered (%)	53 ± 0.8	67 ± 1.2	61 ± 1.4	63 ± 1.4	66 ± 1.1	69 ± 0.1	69 ± 0.9	67 ± 2.9	69 ± 5.6	66 ± 2.6
$R_{max,\ P}$ (mg P L^{-1} d^{-1})	4.1	1.0	5.0	1.3	5.4	1.6	3.8	1.8	5.2	1.3
RE_P (%)	70	17	88	36	93	54	95	66	95	45
P content (%)	2.5 ± 0.1	0.7 ± 0.02	2.2 ± 0.1	1.2 ± 0.04	1.8 ± 0.1	1.2 ± 0.03	1.6 ± 0.09	1.4 ± 0.1	1.5 ± 0.05	1.3 ± 0.01
P recovered (%)	46 ± 6.2	93 ± 7.3	78 ± 2.6	96 ± 1.2	81 ± 5.0	90 ± 0.1	79 ± 10.5	72 ± 3.9	69 ± 6.9	99 ± 0.7

Table 2
Maximal NO_3^--N and PO_4^{3-}-P removal rate ($R_{max,\ N}$, $R_{max,\ P}$, mg N or P L^{-1} d^{-1}), nitrogen and phosphorus removal efficiency (RE_N, RE_P, %), nitrogen and phosphorus contents of harvested biomass and their recovery rates at flow rates of 2–8 L min^{-1}of outdoor ATS.

Flow rate (L min^{-1})	2	4	6	8
$R_{max,\ N}$ (mg N L^{-1} d^{-1})	3.5	6.1	5.9	6.4
RE_N (%)	88 ± 5.4	98 ± 1.4	96 ± 1.2	99 ± 1.2
N content (%)	6.2 ± 0.8	6.3 ± 0.3	6.8 ± 0.8	6.5 ± 0.3
N recovered (%)	86 ± 7.3	83 ± 12.1	73 ± 17.8	73 ± 17.7
$R_{max,\ P}$ (mg P L^{-1} d^{-1})	3.4	3.3	3.5	3.9
RE_P (%)	>99	>99	>99	>99
P content (%)	2.1 ± 0.5	2.1 ± 0.4	2.3 ± 0.8	2.3 ± 0.6
P recovered (%)	60 ± 5.1	72 ± 14.4	79 ± 12.2	86 ± 20.8

compounds present in the horticultural wastewater (e.g. pesticide) or its relatively low growth rate and nutrient uptake capacity as shown in the synthetic wastewater and in our previous study (Liu and Vyverman, 2015).

Flow rate or water velocity was proved to be a critical factor in determining algal community, biomass production and nutrient removal efficiency of ATS in this study. Specifically, a low flow rate enhanced the dominance of benthic filamentous algae in the periphyton biofilm, while a high flow rate promoted the mass exchange and consequently the algal biomass accumulation and nutrient removal efficiency. The findings of this study were in accordance with the previous reports (Ahn et al., 2013; Craggs et al., 1996; Dodds, 1991; Zippel et al., 2007). Therefore, selection of appropriate algal strains and optimization of flow rate for a proper algal community and efficient nutrient removal should be taken into consideration for the future work on benthic filamentous algae based bioreactors.

Additionally, in this study the biomass production was maximally 2.0 g DW m^{-2} d^{-1}, however, it can be 5–35 g DW m^{-2} d^{-1} (Craggs et al., 1996; Kebede-Westhead et al., 2003; Mulbry et al., 2010). Besides the phylogenetic difference and flow rate elucidated here, temperature and solar irradiance were also critical factors in algal photosynthesis and nutrient removal (Godillot et al., 2001; Kebede-Westhead et al., 2003; Richmond, 2004). For example, in the study of Sandefur et al. (2011), the monthly biomass production in May and June was 12 and 29 g DW m^{-2} d^{-1} respectively with average temperature of 19 and 22 °C, while in this study the monthly average temperature in May and June was 12.5 and 16.3 °C respectively. Moreover, the daily sum of irradiance of the operation period varied between 4.5 and 6.0 k Wh m^{-2}(KMI: http://www.meteo.be/), which was much lower than the place of Sandefur et al. (2011) (http://solargis.info/doc/free-solar-radiation-maps-GHI). Therefore, selection of species with high light utilization efficiency and preference of low temperature will benefit to improve biomass production and nutrient removal efficiency in the places with low solar irradiance, such as Belgium.

4. Conclusions

Stigeoclonium performed well in growth and removing nutrient from the horticultural wastewater both in laboratory flask and on outdoor Algal Turf Scrubber (ATS) with a low flow rate and can be good candidate for treating horticultural wastewater, while the *Klebsormidium* strain was not suitable. Relatively high flow rate had significant positive effect on the biomass production and nitrogen removal rate of the outdoor ATS, while phosphorus removal was less influenced, probably due to other processes than algal assimilation. This study provided an attempt to select the appropriate benthic algae strain and optimize the flow rate to improve biomass production and nutrient removal efficiency of an ATS.

Acknowledgments

This study was financially supported by Special Research Fund (Project BOF11/CHN/016) of Ghent University, Belgium and China Scholarship Council (CSC). The authors would like to thank Renaat Dasseville for helping to run the ATS, Tine Verstraete for doing the nitrogen analysis on the biomass and Dr. Yonghong Wu for revising the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.01.049.

References

Abdel-Raouf, N., Al-Homaidan, A.A., Ibraheem, I.B.M., 2012. Microalgae and wastewater treatment. Saudi J. Biol. Sci. 19 (3), 257–275.
Adey, W.H., Kangas, P.C., Mulbry, W., 2011. Algal turf scrubbing: cleaning surface waters with solar energy while producing a biofuel. Bioscience 61 (6), 434–441.
Adey, W.H., Laughinghouse, H.D., Miller, J.B., Hayek, L.A.C., Thompson, J.G., Bertman, S., Hampel, K., Puvanendran, S., 2013. Algal turf scrubber (ATS) floways on the great wicomico River, Chesapeake Bay: productivity, algal community structure, substrate and chemistry. J. Phycol. 49 (3), 489–501.
Ahn, C.H., Song, H.M., Lee, S., Oh, J.H., Ahn, H., Park, J.-R., Lee, J.M., Joo, J.C., 2013. Effects of water velocity and specific surface area on filamentous periphyton biomass in an artificial stream mesocosm. Water 5 (4), 1723–1740.
APHA, 1998. Standard Methods for the Examination of Water and Wastewater, twentieth ed. American Public Health Association/American Water Work Association/Water Environmental Federation, Washington, DC.
Arbib, Z., Ruiz, J., Álvarez-Díaz, P., Garrido-Pérez, C., Perales, J.A., 2014. Capability of different microalgae species for phytoremediation processes: wastewater tertiary treatment, CO2 bio-fixation and low cost biofuels production. Water Res. 49 (0), 465–474.
Besson, A., Guiraud, P., 2013. High-pH-induced flocculation-flotation of the hypersaline microalga *Dunaliella salina*. Bioresour. Technol. 147, 464–470.
Boelee, N., Janssen, M., Temmink, H., Shrestha, R., Buisman, C., Wijffels, R., 2014. Nutrient removal and biomass production in an outdoor pilot-scale phototrophic biofilm reactor for effluent polishing. Appl. Biochem. Biotechnol. 172 (1), 405–422.
Breeman, A.M., Oh, Y.S., Hwang, M.S., Van den Hoek, C., 2002. Evolution of temperature responses in the Cladophora vagabunda complex and the C-albida/sericea complex (Chlorophyta). Eur. J. Phycol. 37 (1), 45–58.
Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew. Sustain. Energy Rev. 19 (0), 360–369.
Craggs, R.J., Adey, W.H., Jessup, B.K., Oswald, W.J., 1996. A controlled stream mesocosm for tertiary treatment of sewage. Ecol. Eng 6 (1–3), 149–169.
de-Bashan, L.E., Bashan, Y., 2004. Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997–2003). Water Res. 38 (19), 4222–4246.
Dodds, W.K., 1991. Factors associated with dominance of the filamentous green alga *Cladophora glomerata*. Water Res. 25 (11), 1325–1332.
Godillot, R., Caussade, B., Ameziane, T., Capblancq, J., 2001. Interplay between turbulence and periphyton in rough open-channel flow. J. Hydraul. Res. 39 (3), 227–239.
Gross, M., Jarboe, D., Wen, Z., 2015. Biofilm-based algal cultivation systems. Appl. Microbiol. Biotechnol. 99 (14), 5781–5789.
Guillard, R., Lorenzen, C.J., 1972. Yellow-green algae with Chlorophyllide C. J. Phycol. 8 (1), 10–14.
Guo, F., Wang, H., Wang, J., Zhou, W., Gao, L., Chen, L., Dong, Q., Zhang, W., Liu, T., 2014. Special biochemical responses to nitrogen deprivation of filamentous oleaginous microalgae *Tribonema* sp. Bioresour. Technol. 158 (0), 19–24.
Hillebrand, H., Dürselen, C.D., Kirschtel, D., Pollingher, U., Zohary, T., 1999. Biovolume calculation for pelagic and benthic microalgae. J. Phycol. 35 (2), 403–424.
Kebede-Westhead, E., Pizarro, C., Mulbry, W.W., Wilkie, A.C., 2003. Production and nutrient removal by periphyton grown under different loading rates of anaerobically digested flushed dairy manure. J. Phycol. 39 (6), 1275–1282.
Kesaano, M., Sims, R.C., 2014. Algal biofilm based technology for wastewater treatment. Algal Res. 5, 231–240.
Larned, S.T., 2010. A prospectus for periphyton: recent and future ecological research. J. North. Am. Benthol. Soc. 29 (1), 182–206.
Larsdotter, K., la Cour Jansen, J., Dalhammar, G., 2010. Phosphorus removal from wastewater by microalgae in Sweden–a year-round perspective. Environ. Technol. 31 (2), 117–123.
Lee, C.S., Lee, S.-A., Ko, S.-R., Oh, H.-M., Ahn, C.-Y., 2015. Effects of photoperiod on nutrient removal, biomass production, and algal-bacterial population dynamics in lab-scale photobioreactors treating municipal wastewater. Water Res. 68 (0), 680–691.
Liu, J., Vanormelingen, P., Vyverman, W., 2016. Fatty acid profiles of four filamentous green algae under varying culture conditions. Bioresour. Technol. 200, 1080–1084.
Liu, J., Vyverman, W., 2015. Differences in nutrient uptake capacity of the benthic filamentous algae *Cladophora* sp., *Klebsormidium* sp. and *Pseudanabaena* sp. under varying N/P conditions. Bioresour. Technol. 179, 234–242.
Lu, H., Yang, L., Shabbir, S., Wu, Y., 2014. The adsorption process during inorganic phosphorus removal by cultured periphyton. Environ. Sci. Pollut. R. 21 (14), 8782–8791.
Markou, G., Georgakakis, D., 2011. Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: a review. Appl. Energy 88 (10), 3389–3401.
Mennaa, F.Z., Arbib, Z., Perales, J.A., 2015. Urban wastewater treatment by seven species of microalgae and an algal bloom: biomass production, N and P removal kinetics and harvestability. Water Res. 83 (0), 42–51.
Mulbry, W., Kangas, P., Kondrad, S., 2010. Toward scrubbing the bay: nutrient removal using small algal turf scrubbers on Chesapeake Bay tributaries. Ecol. Eng. 36 (4), 536–541.
Mulbry, W., Kondrad, S., Pizarro, C., Kebede-Westhead, E., 2008. Treatment of dairy manure effluent using freshwater algae: algal productivity and recovery of manure nutrients using pilot-scale algal turf scrubbers. Bioresour. Technol. 99 (17), 8137–8142.
Richmond, A., 2004. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Science, Ames, Iowa, USA.
Roeselers, G., van Loosdrecht, M.C.M., Muyzer, G., 2008. Phototrophic biofilms and their potential applications. J. Appl. Phycol. 20 (3), 227–235.
Sandefur, H.N., Matlock, M.D., Costello, T.A., 2011. Seasonal productivity of a periphytic algal community for biofuel feedstock generation and nutrient treatment. Ecol. Eng. 37 (10), 1476–1480.
Sañudo-Wilhelmy, S.A., Tovar-Sanchez, A., Fu, F.-X., Capone, D.G., Carpenter, E.J., Hutchins, D.A., 2004. The impact of surface-adsorbed phosphorus on phytoplankton redfield stoichiometry. Nature 432 (7019), 897–901.
Sushchik, N.N., Gladyshev, M.I., Ivanova, E.A., Kravchuk, E.S., 2010. Seasonal distribution and fatty acid composition of littoral microalgae in the Yenisei River. J. Appl. Phycol. 22 (1), 11–24.
Van den Hende, S., Beelen, V., Bore, G., Boon, N., Vervaeren, H., 2014. Up-scaling aquaculture wastewater treatment by microalgal bacterial flocs: from lab reactors to an outdoor raceway pond. Bioresour. Technol. 159, 342–354.
Wellnitz, T.A., Ward, J., 1998. Does light intensity modify the effect mayfly grazers have on periphyton? Freshw. Biol. 39 (1), 135–149.
Zippel, B., Rijstenbil, J., Neu, T.R., 2007. A flow-lane incubator for studying freshwater and marine phototrophic biofilms. J. Microbiol. Methods 70 (2), 336–345.