Phycoremediation of tertiary municipal wastewater: a new insight of filamentous algae coculture in Nordic country

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Abstract

This study demonstrated that the application of filamentous co-culture could be a promising supplementary approach to further purify municipal tertiary wastewater in Nordic country. Initial screening of 25 algae strains across multiple genera revealed that *Spirogyra* sp. and *Klebsormidium* sp. were suitable for use as a coculture for phycoremediation of the tertiary effluent from a wastewater treatment plant, and this result was validated in three consecutive outdoor pilot tests at 10–15 ^{o}C. In the first two batches of pilot tests, the total prosperous and ammonium were depleted close to zero in 24 hours, while the pH in the wastewater increased from 7 to 9. In the 3rd batch, CO_2 was thereby added for pH control. Macronutrients (N and P) were successfully removed from the treated wastewater. The total algae biomass increased 2 to 3 times over 7 days with average algae productivity of 1.68 g m^2 d^{-1}. Meanwhile, the produced algae biomass accumulated notable mineral elements (Ca, Mg, K, Fe and Al) and some heavy metals at levels of g kg^{-1} and mg kg^{-1}, respectively. In light of circular economy concept, the produced biomass could be used for different valorizations based on the analytical analysis. This study provides a new insight of phycoremediation for further purification of municipal treated wastewater, by effectively using filamentous algae coculture. Given a great potential for further optimization and improvement, this proof of concept will benefit to the green transition of wastewater treatment plants in Nordic country.

Introduction

Wastewater management has emerged as an important component in the concept of circular economy. Waste streams should no longer be treated as a problem but as a resource to recover for valorization. This has become a new consensus in the green transition of wastewater treatment plants (WWTPs). With the incentive to neutralize carbon emissions, new technologies and solutions are highly demanded for WWTPs to alleviate their environment footprints as part of the green transition. Algae, robust photosynthetic organisms able to thrive in various waste streams, represent a promising tool to recover and recycle the nutrient residuals from wastewater into valorized bioproducts, while simultaneously coupling with carbon abatement. Therefore, the application of algae-based treatment process (or phycoremediation) may become a vital approach to facilitate circular economy development for WWTPs.

Phycoremediation is renowned as a solar-power driven, ecological friendly, and sustainable reclamation strategy (Xiong et al. 2018). The cost for algae scalable cultivation can be subsidised by recovering the nutrients in the wastewater and sequestrating CO_2 released by WWTPs (Whitton et al. 2015). It is already highlighted that using microalgae for nutrient recovery in municipal wastewater water can remove up to 99% of nitrogen (N) and phosphorous (P), and reduce the nutrient concentration below 1 mg/L (Silkina et al. 2019). More importantly, the produced algal biomass can be utilised for different valorisation purposes, from niche markets on special materials (e.g., biopolymers and coatings) to the large-scale uses of fertilizer (Liu et al. 2020). Albeit some applications for feed, food and/or high value bioproducts

will need further clarification due to the safety/legislation concerns, how to valorize the yield of algal biomass stands for a new value chain for the wastewater treatment process.

Actually, using microalgae for wastewater treatment was initiated in the 1950s (Oswald et al. 1957). Such a phycoremediation concept has been proved to be technically possible at lab scale, pilot scale and demonstration scales, but not economically feasible. Particularly, harvesting of small microalgal cells is a major obstacle to hinder the popularity and scalability of phycoremediation. To an operational facility, this is an energy intensive and expensive process that inevitably can occupy over 90% of capital expenses (Amer et al. 2011) and above 20–30% of the overall production cost (Rawat et al. 2011). Meanwhile, the fluctuation of wastewater biotic and abiotic conditions is an inevitable challenge and threat to the microalgae cultivation especially to the commonly proposed monoculture as pointed by Liu et al. (2020). Apparently, this fact will make both algae productivity and wastewater treatment efficiency unsecured. Therefore, even the microalgae have a great potential for wastewater treatment, there is still an ongoing debate on the value of the outcome compared to the input for the scalable application.

From this point of view, using filamentous algae for wastewater treatment offers a better solution. Species from the genera *Oedogonium, Cladophora, Spirogyra, Klebsormidium* and *Stigioclonium* have been approved as optimal candidates for wastewater treatment applications (Liu et al. 2020). These filamentous algae have several inherent advantages in wastewater treatment, such as robust ability to uptake nutrients from wastewater to achieve about 60% increase in dry biomass per day (Auer and Canale 1982), simplicity of harvesting, stronger resistance to a variety of aquatic grazers and competing organisms, as well as better adaptation to changeable aquatic conditions (Grayburn et al. 2013). Even in the environment with varying N:P ratios, the filamentous algae still can remove 99% of N and P simultaneously (Liu and Vyverman 2015). Moreover, these filamentous algae can naturally grow and bloom in a broad spectrum of waste streams including ash dam water (Sternberg and Dorn 2002). Like microalgae, they can also form a symbiotic relation with bacterial consortia for oxygen and CO_2 supplements to improve the productivity and yield of algae biomass in wastewater treatment (Liu et al. 2020). Hence, the use of filamentous algae for phycoremediation becomes an emerging area for wastewater treatment research.

In past decades, there are numerous attempts to employ phycoremediation technologies for the treatment of primary or secondary effluents, whilst less attention has been given to tertiary treated effluents. In fact, the final treated effluents still contain considerable amount of N and P which may cause eutrophication in natural water bodies as secondary pollution. In Norway, for example, WWTPs release more than 900 tons of phosphorus and about 15000 tons of nitrogen per annum (Norskeutslipp 2022), which are equivalent to approx. 5% and 15% of agriculture P and N fertilizer consumptions in 2017 (FAOSTAT 2017). Apparently, it will be beneficial to recover these nutrients from the wastewater instead of releasing them to the environment as pollutants. In contrast to primary and secondary wastewaters, tertiary treated wastewater has less turbidity and relatively stable pH range, which are more compatible conditions for algae growth. In modern WWTPs, when the biological oxidation and denitrification remove most of the organic macronutrients, the inorganic nutrients and less bioavailable organic compounds will be main

nutrient basis in the tertiary treated wastewater with relatively high carbon saturation. These are also preferable factors to facilitate algae photosynthetic activity. Hence, it is conceived that using filamentous algae to purify tertiary wastewater could represent a new win-win strategy for WWTPs. As a complementary process, this can promote the green transition of WWTP towards “zero” waste emission with new algae-based value chain development.

The objective of this study was to investigate the potentials of using filamentous algae species to treat the municipal tertiary effluent from a Norwegian WWTP. Collection, screening and characterisation of appropriate filamentous algae are a crucial preliminary step in this phycoremediation study. Instead of the conventional monoculture approach, new algae coculture was prepared to enhance the adaptability and strengthen the resilience of phycoremediation to fluctuations in the wastewater, including the potential impacts from microbial contamination (Van Den Hende 2014). Since the process of transferring the results from lab-scale R&D to pilot scale is a critical link to the final production scale, we employed both laboratory research and pilot scale tests to evaluate the feasibility of the proposed proof of concept. High Rate Algal Pond (raceway) was used in the pilot study as it is an efficient system for algae cultivation in wastewater treatment (Park et al. 2011). The depletion of nutrients was monitored to ensure appropriate treatment standards matched. The biochemical and elemental contents of the produced algae biomass were characterized to assess the potential utilizations. It is anticipated that selected filamentous algae can work as an effective tool for further purification of municipal tertiary wastewater to contribute to the circular economy development of Norwegian WWTPs.

Material And Methods

Algae screening

A total of 25 freshwater algae strains from 11 genera (Table 1) was selected from the Norwegian Culture Collection of Algae (NORCCA). Unicellular species, of genera commonly used in wastewater treatment (e.g., Oswald et al. 1957, Silkina et al. 2019), were included for a point of comparison. This study had a focus on the species with a filamentous macro-structure, compatible with cost-effective and simple harvesting by coarse filtration. As indicated from a local WWTP (VEAS, Vestfjorden Wastewater Company, Slemmestad, Norway), the temperature of municipal discharge has a mild fluctuation range of 10–15 ^{o}C over the year. This range was thereby taken as a criterion for the algae screening. Inclusion of algae selection was particularly based on the previous knowledge on local rivers ecosystem (e.g., Schneider and Lindstrøm 2009, Schneider and Lindstrøm 2011), to avoid the potential risk of biological contamination. The VEAS tertiary wastewater was freshly sampled and sterile filtered (0.2 µm) on the day for each individual laboratory test below.

Table 1. Algae selection for the experimental test (N.I.: not identified).

Nr	Phylum	Class	Genus	Species	Strain	Origin	Maintenance medium	Morphology
1	Chlorophyta	Chlorophyceae	Tetradesmus	obliquus	NIVA-CHL 6	Lake Årungen, Akershus, Norway, 1964-06-03	Z8	single cell
2	Chlorophyta	Chlorophyceae	Coelastrum	N.I.	NIVA-CHL 86	Lake Malawi, Malawi, 1991-03.08	Z8	single cell
3	Chlorophyta	Chlorophyceae	Chlamydomonas	reinhardtii	K-1016	Amherst, Massachusetts, USA, Unknown	CW15	single cell
4	Chlorophyta	Chlorophyceae	Chlamydomonas	reinhardtii	K-1017	Amherst, Massachusetts, USA, Unknown	CW15	single cell
5	Chlorophyta	Chlorophyceae	Oedogonium	vaucheri	K-0094	S. of Store Magleby, Amager, Denmark, Unknown	NF2	filamentous
6	Chlorophyta	Chlorophyceae	Oedogonium	cardiacum	K-1001	Small pond north of Dry Drayton, England, Unknown	20% Z8 + vitamins + soil extract	filamentous
7	Chlorophyta	Chlorophyceae	Oedogonium	cardiacum	K-1002	Small pond near Dry Drayton, England, Unknown	20% Z8 + vitamins + soil extract	filamentous
8	Chlorophyta	Chlorophyceae	Stigeoclonium	N.I.	K-0018	Avernakø, Denmark, Unknown	NF2	filamentous
9	Chlorophyta	Chlorophyceae	Stigeoclonium	N.I.	K-1030	Unknown	20% Z8 + vitamins + soil extract	filamentous
10	Chlorophyta	Chlorophyceae	Stigeoclonium	N.I.	K-1031	Unknown	20% Z8 + vitamins + soil extract	filamentous
11	Chlorophyta	Chlorophyceae	Stigeoclonium	N.I.	K-1032	Unknown	20% Z8 + vitamins + soil extract	filamentous
12	Chlorophyta	Chlorophyceae	Raphidocelis	subcapitata	NIVA-CHL1	River Nitelva, Akershus, Norway, 1959	Z8	filamentous
13	Chlorophyta	Trebouxiophyceae	Chlorella	vulgaris	K-1801	Revò (TN), garden soil, Italy, Unknown	Z8	single cell
14	Chlorophyta	Trebouxiophyceae	Chlorella	vulgaris	NIVA-CHL 108	Germany, Unknown	Z8	single cell
15	Chlorophyta	Trebouxiophyceae	Chlorella	sorokiniana	NIVA-CHL 176	Austin, Texas, USA, 1953	Z8	single cell
16	Chlorophyta	Conjugatophyceae	Spirogyra	singularis	K-1019	Utterslev Mose, Denmark, Unknown	20% Z8 + vitamins + soil extract	filamentous
17	Chlorophyta	Conjugatophyceae	Spirogyra	N.I.	K-1454	Samsø, Denmark, Unknown	20% Z8 + vitamins + soil extract	filamentous
18	Chlorophyta	Conjugatophyceae	Spirogyra	N.I.	CHL 189	Pond, Kindrogan, Scotland, 2013-06-14	20% Z8 + vitamins + soil extract	filamentous
19	Chlorophyta	Klebsormidiophyceae	Klebsormidium	N.I.	K-0148	Little Island, Cork, Eire, Unknown	NF2	filamentous
20	Chlorophyta	Klebsormidiophyceae	Klebsormidium	N.I.	NIVA-CHL 142	Dal, Akershus, Norway, 1993-06-16	Z8	filamentous
21	Chlorophyta	Klebsormidiophyceae	Klebsormidium	flaccidum	NIVA-CHL 80	Spruce nursery, 1990	Z8	filamentous
22	Cyanobacteria	Cyanophyceae	Arthrospira	platensis	NIVA-CYA 428	Lake Lonar, Maharashtra, India, Unknown	Spirulina	filamentous
23	Cyanobacteria	Cyanophyceae	Anabaena	subcylindrica	NIVA-CYA 323	Fuggdal, Rendalen, Hedmark, Norway, 1993-08-27	Z8	filamentous
24	Cyanobacteria	Cyanophyceae	Trichormus	variabilis	NIVA-CYA 19	Lake Mendota, Madison, Wisconsin, USA, 1948-09	Z8	filamentous
25	Cyanobacteria	Cyanophyceae	Trichormus	variabilis	NIVA-CYA 410	Mississippi, USA, 1964	Z8	filamentous

All 25 strains were inoculated in triplicates into the VEAS tertiary wastewater using 96-well microtiter plates following the NORCCA's standard protocol. Each well was filled with 150 µL of sterile filtered VEAS effluent and 50 µL of individual algal stock culture. As a reference, the algae strains were also inoculated into their specific standard media used to maintain growth and viability in the culture collection, while negative controls only with sterile growth medium were also prepared. Plates were covered and incubated at 15°C with 6 µmol photon m^{-2} s^{-1} of light intensity. The algal growth was detected by the chlorophyll fluorescent signal at a wavelength of 685 nm with excited emission at 450–550 nm on a microplate reader (Millipore CytoFluor 2300 Fluorescence Measurement System, Burlington, Massachusetts, USA). The cultures were measured every third day for 10 days. Based on the growth rate indication, the top filamentous strains were selected for subsequent co-culture studies.

Co-culture combination

Three strains were selected and cultivated first in 250 mL of their respective medium (Table 1) until the cultures reached the stationary phase. Then the biomass was collected by filtration, washed and resuspended in sterile-filtered VEAS effluent, obtaining a similar fluorescence density between each other. As the co-cultures of selected filamentous strains was the priority in this study, the matrix of combinations had three levels for the inocula: 15 mL (monocultures as a reference), 7.5–7.5 mL (two species) and 5–5–5 mL (three species) with three replicates for each experimental unit. All the cultures were cultivated at 15 ^{o}C and shaken daily to prevent algal attachment. The algae growth was quantified by chlorophyll fluorescent measurement as above, using 200 µL of samples taken on days 0, 3, 6 and 9. Based on the growth performance, the top coculture group was treated as the prospective algal package for pilot test.

Indoor scale up cultivation

NIVA-CHL142 (*Klebsormidium* sp.) and NIVA K-1454 (*Spirogyra* sp.) were cultivated separately as the fresh inoculum for pilot coculture study. At the NIVA's Solbergstrand Algae R&D Facility, these algae were scaled up to 100 litres on each, using 0.15 g L^{-1} Cell-Hi WP medium (Varicon Aqua, Worcester, UK), in

vertical photobioreactors (PBR) made by 30 cm diameter of transparent polyethylene plastic column. Consistent aeration was supplied to the culture with 0.01–0.02 VVM, and pH was controlled at 7.5 with automatic CO_2 addition. The light radiation was maintained at 80–100 μE m^{-2} s^{-1} of light intensity. In order to facilitate these inoculums' growing, *Klebsormidium* sp. and *Spirogyra* sp. were exposed to 20°C and 25°C as suggested by the literatures (Graham et al. 1995, Kondo et al. 2016). When the inoculum biomass reached to the level of 2 g L^{-1}, all the algae cells were harvested by filtration (35 um plankton net), washed by clean water, and weighed (after 10 min of air-drying) for the pilot test below. In the meantime, a few grams of biomass were taken from each species and lyophilised for a benchmark study.

Outdoor phycoremediation pilot study

In this study, the pilot test was proposed on 1:15 (v/v) with each indoor inoculum in a raceway. The pilot test was performed in three consecutive cycles with about 1500 L of municipal tertiary effluent (non-filtered, provided by VEAS) on each. In the beginning, about 242 g wet *Klebsormidium* (equal to 22.3 g of dry weight, DW) and 547 g wet *Spirogyra* (about 39.9 g DW) were collected as coculture inoculum to the 1st batch test. With an attempt to continue algae cultivation, a similar amount (weight weight, WW) of coculture was taken from the final produced biomass and subsequently used as the new inoculum for 2nd batch, and so did on 3rd cycle. As the experimental period (28th Oct. – 18th Nov.) was during the fall season (Oslo, Norway), the outdoor pilot test was conducted with heat control (10–15°C) to simulate the expected conditions at VEAS, with 24 hr of LED light radiation (30–95 μE m^{-2} s^{-1}). Three batched tests were all monitored for 7 days, with 2 L of water sampling on each day. The water samples were filtered with pre-weighed GF/C membranes and stored frozen for subsequent water analyses, while the membranes were dried for biomass measurement at 105°C for overnight. As the pH value in the raceway increased from 7 to 9 within 24 hr in the first two cycles, the pH in the 3rd batch was controlled at 7.5 with CO_2 addition after day 2. The algae culture was checked daily by microscope (Nikon Eclipse E400, Tokyo, Japan). In the end of each batch, the entire coculture biomass in the raceway was harvested by filtering (80 um plankton net), rinsed with tap water and quantified for the yield measurement. Then a small proportion of produced biomass was freeze-dried for various analytical analyses.

Water analyses

The water samples were analyzed as: **(1)** *Total Kjeldal nitrogen.* 100 mL of water sample was digested with 12 mL 95% H_2SO_4 + Kjeltabs (7 g K_2SO_4 + 0.8 g $CuSO_4$) at 420°C for 1h on a Foss Digestor (2520). Most of the water content was evaporated at 200–230°C before ramping the temperature to 420°C. The digested samples were measured using a Kjeltec™ 8400 (FOSS, Hillerød, Denmark) using 0.01N HCL for titration. **(2)** *Total phosphorus*. A 20 mL of water sample was first oxidized with 0.2 g of $K_2S_2O_8$ at 120°C for 30 min. The digested sample was measured on a spectrophotometer (Shimadzu UV1800, Kyoto, Japan) as per the instruction of ammonium molybdate spectrometric method. **(3)** *Nitrite and nitrate*. Sum of nitrites and nitrates were measured on a Flow Solution 3700 Automated Chemistry Analyzer (OI-Analytical, College Station, TX, USA). **(4)** *Ammonium.* 5 mL water sample was added into LCK 304 ammonium test cuvette (Hach Lange), incubated 15 min at room temperature, and then measured on a DR3900 spectrophotometer (Hach Lange, Dusseldorf, Germany) at 685 nm.

Determination of heavy metals and other elements in biomass

The freeze-dried algal samples preserved above were used for the analyses of Al, Ca, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Zn, Hg, K and P at the VEAS analytical laboratory. Briefly, algae samples were digested with HNO_3 (50%) in a ventilated container at 120 ± 4°C for 30 min. The digested solutions were measured with the Optima 7000DV ICP-OES (inductively coupled plasma optical emission spectrometer, Perkin Elmer, Waltham, MA, USA). Hg was measured with a Millenium Merlin automated mercury analyzer with atomic fluorescence spectroscopy (AFS, P S Analytical, Kent, UK). Ca was measured with an Analyst 600 ET-AAS (electrothermal atomic absorption spectrometer, Perkin Elmer, Waltham, MA, USA).

Biomass chemical components analysis

The lyophilised algae samples were also preserved for a series of analytical analyses, which combined the qualitative and quantitative measurements. According to the previous analysis (Kosa et al. 2017), fourier-transform infrared spectroscopy (FTIR) analysis was performed for the qualitative identification. It was conducted on an Agilent 5500 Series FTIR Spectrometer (Agilent, Santa Clara, US) using a single-bounce type II A diamond crystal ATR (attenuated total reflectance) accessory with sample press. Approximately 10 mg freeze-dried algae samples were measured in the spectral range of 4000–650 cm^{-1} with a resolution of 8 cm^{-1} for 32 scans. Background spectra were measured on the empty crystal for data calibration, while each sample was measured for three times. The diamond crystal of the ATR accessory was cleaned with 70% ethanol between each reading.

The subsequent quantitative measurements included: **(1)** *Protein content (CNS elemental analyses)*. It followed the Pregl-Dumas method on a Vario El Cube element analyzer (Elementar, Langenselbold, Germany). The samples were combusted with oxygen in a combustion chamber (1150°C), and the combustion products were then passed through a reduction tube (850°C) of an inert carrier gas (helium). It was calculated by multiplying N from elemental analysis with 6.25. **(2)** *Starch content*. Basically, the starch in the biomass was hydrolyzed by α-amylase enzyme, then converted to glucose by amyloglucosidase enzyme. Finally, the concentration of glucose was determined with a spectrophotometer (RX Daytona +, Randox Laboratories, Crumlin, UK). **(3)** *Total lipid content*. The extraction took place by pumping petroleum ether into an extraction cell at 125°C and 1500 psi (ASE 200 accelerated solvent extractor, Waltham, Massachusetts, USA). After the solvent was evaporated by nitrogen gas, the extract was dried in a vacuum oven at 70°C for quantification. **(4)** *Ash content*. The ash content was determined after incineration at 550°C overnight in a muffle furnace (Nabertherm, Lilienthal, Germany).

Data Analysis

Principal component analysis (PCA) was performed for the qualitative analysis of biomass. The obtained raw FTIR spectra were first averaged and then subjected to EMSC (Extended Multiplicative Signal Correction). A Savitzky-Golay transformation (2nd derivative, polynomial order 2, smoothing points: 9) was applied for peak height readings. Processing of the spectra and PCA were performed with The Unscramble 11 (CAMO Software, Oslo, Norway).

Results And Discussion

Algae strains selection

Most of the selected algae species/strains grew when exposed to the VEAS effluent, indicated by the increase of fluorescence density (Fig. 1). The first three days were omitted to allow inoculated cells for acclimation and thereby the results were obtained between days 3 and 10 during their exponential growth phase. The relative increase in fluorescence was used for intra-strain comparison. In this study, there were two selection criteria; 1) being filamentous and 2) at least 100% increase in fluorescence, based on reproducible fluorescence readings. Only K-1454 (*Spirogyra* sp.) and NIVA-CHL142 (*Klebsormidium* sp.) passed the criteria. These two strains showed similar growth rates (109–137%) to those unicellular strains (129–195%). However, the filamentous cyanobacterial strain NIVA-CYA410 (*Trichormus* sp.) showed extensive growth with values relatively close to the 100% threshold limit (63%, 88% and 141%; average 97%). Therefore, this strain was also selected as a candidate for the subsequent coculture tests.

Preliminary coculture trial

When these three strains were exposed directly to the VEAS effluent, the growth of monoculture was only detected on *Spirogyra sp*. and *Klebsormidium sp*., rather than on *Trichormus sp.* (Fig. 2). As the preliminary screening test was conducted with diluted VEAS effluent with original stock medium, it is conceived that the fresh algal cells of *Trichormus sp.* were likely unsuitable to the direct effluent reclamation. However, the co-culture of *Klebsormidium sp*. and *Trichormus sp.* showed a quick growth on day 6. It could be either relevant to some elicitor molecules potentially released by *Trichormus sp.* as cyanobacteria can stimulate plant growing (e.g., Karthikeyan et al. 2007, Singh 2014), or just a coincidence as this phenomenon was not repeated in other cocultures. As the coculture of *Trichormus* and *Spirogyra* showed some growth inhibition compared to the monoculture of *Spirogyra*, it was confirmed that *Trichormus sp.* could have little contribute to the biomass yield, even in the mixture of three strains. So this strain was excluded from the coculture combinations. In the comparison, all the cocultures including *Spirogyra sp*. and *Klebsormidium sp*. showed a similar fluorescence increment in the end.

It is worth noting that the different sized algae cells have different affinities for the nutrient recovery (Edwards et al. 2012), so the algae mixture in different cell sizes would be better for the purpose of wastewater reclamation. In this regard, the coculture of *Klebsormidium sp*. and *Spirogyra sp.* was a matched combination, as the cell of *Spirogyra sp*. was much bigger than that of *Klebsormidium sp*. (approx. 10 time of difference). As they both showed good adaptability with positive growth performance in the VEAS effluent, it is deduced that their co-culture could effectively remove the nutrient residuals with uncompromised biomass productivity. In contrast to the monoculture, mixed culture would have a better resilience to the variable conditions and complex microbial consortia in the wastewater. Therefore, albeit the fluorescence increment of *Klebsormidium sp*. was more than that of *Spirogyra sp.* the coculture of *Klebsormidium sp*. and *Spirogyra sp.* was believed as an optimal filamentous combination to treat the VEAS effluent.

Nutrient depletion, CO2 supplement and biomass production in pilot tests

This study demonstrated that the N and P residues in the tertiary treated wastewater could be effectively removed by the inoculated coculture, even at the low level of mg L^{-1} (Fig. 3). Most of NH_4^+ and P in the 1st and 2nd batches were depleted by the algae in one day, whereas they became slower in 3rd batch. As P is a limiting factor for algae growth (Redfield 1934), the availability of P in the cultivation system can also indicate the growth rate of algae biomass, indicating that the algae biomass would have a slower proliferation in the beginning of 3rd batch. As the setting was a bit different in the 3rd batch, such a slow response will be elaborated below. Although the nutrient profile of VEAS effluent varied between three testing batches, the nutrient depletion followed a similar pattern. The depletion of NH_4^+ preceded the other inorganic N nutrients, even it was close to the detection limit in the 1st batch. The removal of nitrate and nitrite occurred once NH_4^+ was reduced close to zero. This is coincident with the previous literatures that algae have a preference with $NH_4^+ > NO_3^-$ on inorganic N nutrient (Van Den Hende 2014, Lopez-Serna et al. 2019). Regardless the difference in 3rd batch, it seems that the total N could be reduced by 1.5 mg L^{-1} in 3 days. As this rate was achieved by the experimental amount of inoculated coculture, it is conceivable that the nutrient depletion could be accelerated with more coculture inoculum.

In this study, three batches of pilot test all showed that the pH was increased from 7 to 9 within a day, and constantly maintained at about 9 if CO_2 control was not added (Fig. 4). As the treated wastewater used in this study was collected after the denitrification treatment, it could be CO_2 saturated. At this point, the filamentous coculture could also rapidly wipe out these carbon sources in the treated wastewater. However, this observation is contradictive to a previous phycoremediation study on *Klebsormidium* sp. that the algae growth led to a quick reduction of pH from alkaline to neutral (Stoyneva-Gärtner et al. 2019). Although it was lack of details to investigate this discrepancy, not many algae prefer growing in alkaline condition, including *Klebsormidium sp*. and *Spirogyra sp.* (Graham et al. 1995, Kondo et al. 2016). Moreover, CO_2 addition for pH control can benefit to the algae productivity in wastewater treatment (Uggetti et al. 2018). This study highlighted a great prospect that the selected filamentous coculture would need a large amount of CO_2 supplement along the wastewater purification process. It is meaningful for WWTPs to reduce the carbon footprint from the wastewater treatment.

Interestingly, although the P consumed by algae is largely dependent on the available C and N sources (Redfield 1934), it seemed that the CO_2 supplement on day 2 was not related to the slower P depletion in the 3rd batch (Fig. 3). After all, it was supposed to have a better nutrient balance of C, N and P in the 3rd batch for algae growth. As the cultivation conditions (temperature and light) were also similar between three batches, the plausible reason is associated with the difference of algae inoculum used in the 3rd batch. In this study, each batch used a similar amount of algae inoculum (Table 2), but their vitality was likely different. Due to the low level of nutrients (especially P) in the VEAS treated wastewater, the used biomass would confront prolonged P starvation in the latter batch. As reported before, initial P-starvation can be implemented on microalgae to maximize the P uptake in wastewater (Solovchenko et al. 2019). This could happen in the 2nd pilot test but was not occurred in the 3rd on the selected filamentous

coculture. Although it was attempted to recycle the produced algal biomass for continuous effluent purification, the results approved that the efficiency turned unsustainable after 14 days even with given CO_2 supplement. It is thereby deduced that the fresh algae inoculum is imperative to the rapid nutrient recovery in municipal tertiary wastewater. In another word, it is compulsory to employ two-stage cultivation strategy to accomplish better wastewater reclamation. At this point of view, the nutrient rich primary or secondary wastewater can be considered for the cultivation of fresh algae inoculum, whilst they will need proper pretreatment to meet the growth requirement of algae.

Table 2
The summary of algae production and nutrient consumed in the pilot tests.

Batches	1st	2nd	3rd
Initial biomass inoculum (g L^{-1}, DW)	0.04	0.04	0.05
Harvest biomass yield on day 7 (g L^{-1}, DW)	0.13	0.10	0.10
Algae productivity over 7 days (DW g m^2 d^{-1})	2.27	1.51	1.26
Biomass yield vs total N consumed in 7 days (DW g g^{-1})	55.59	19.93	19.13
Biomass yield vs total P consumed in 7 days (DW g g^{-1})	1668	1801	641
Initial N:P ratio in the wastewater	30.38	123.29	58.94

Overall, the biomass of coculture increased by 3.3, 2.5 and 2 times in three consecutive pilot tests (Table 2). The obtained algae productivity was consistent with the reported range of 0.8–50.0 g m^2 d^{-1} on filamentous algae (Liu et al. 2020). It is worth pointing out that the algae growth was doomed to slow down after day 3 in the first two pilot tests, as N and P became deficient afterward (Fig. 3). Hence, the real productivity of coculture could be much higher than the average values over 7 days. The harvest biomass yield was not attractive for the absolute production in this study as it was not the focus for the pilot tests. Certainly, the yield can be easily elevated by using optimal amount of inoculum. Furthermore, the efficiency of biomass yield (vs. both N and P consumed) was better in the first two batches, especially in the 1st cycle. This also underpins the suggestion above on fresh inoculum preparation for tertiary wastewater treatment. Therefore, how to optimize the algae inoculum for the pilot treatment will be a fruitful research focus for the future study, using two-stage cultivation strategy to target (semi-) continuous treatment process.

During the pilot test, both algae species grew well in the municipal treated wastewater. There was no sign of growth inhibition (dead cells or fade color) identified in the microscopy examinations. The good discovery was that two filamentous species clumped together. The coculture formed numerous small algae colonies (about 1 cm) in the raceway, making biomass harvested much easier and quicker via a simple filtration. Apparently, it is foreseen that this trait can be vital to the practicability and scalability of

selected coculture cultivation for wastewater treatment. Only a few ciliates *Vorticella* were visualized to attach to the filamentous algae colonies in the 3rd batch, and they were possibly the “carryover” with the consecutive cultivation. Due to the cell size, however, they were not the predator to the algae coculture. In this test, it was attempted to collect the biomass for growth monitoring measurement by filtering 2L of water samples. As big variations were obtained between sampling days (data not shown), the biomass quantification was only conducted on the entire algae production in the end of each batch. Furthermore, it was also impossible to monitor the change of combination ratio in the coculture, but the cell density of *Klebsormidium sp*. appeared gradually increase in the colonies along with the tests according to the microscope observation. Perhaps some delicate molecule techniques (e.g., QPCR) could be considered to fill in the knowledge gap for the future study.

Accumulation of mineral chemicals and heavy metals in the biomass

There was a total of 14 elements detected in this study, but only the mercury (Hg) was undetectable on all the experimental samples (Table 3). As a benchmark level, the content of these elements was analyzed on the indoor monocultures (inoculum) which was influenced by the cultivation medium. In terms of the differences between indoor inoculums, it was indicated that the *Klebsormidium sp*. and *Spirogyra sp.* had variable affinities to these elements. The difference from the outdoor cocultures represented the accumulation/absorption of these chemicals and heavy metals from the wastewater treatment. It is conceivable of less P in the biomass produced in pilot test. The levels of Mg and K were close between indoor and outdoor cultures, while Ca content was overwhelming in the outdoor biomass. Apparently, these mineral chemicals in the municipal treated wastewater were also eliminated by the tested coculture to constitute the produced algae biomass (g kg^{-1}).

Table 3
Chemical elements analysis in the biomass (Mean ± SE). (Note: Spirogyra is the mean of 2 replicates because of an accident with one of the replicates; Cd is the result of only one replicate.)

Parameter	*Unit*	*Spirogyra*	*Klebsormidium*	Batch #1	Batch #2	Batch #3
Ca	g kg^{-1}	9.14	2.74 ± 0.07	84 ± 1	77.3 ± 0.4	41.3 ± 0.6
K	g kg^{-1}	6.10	11.2 ± 0.1	7.00 ± 0.09	6.50 ± 0.04	6.10 ± 0.05
Mg	g kg^{-1}	1.45	2.35 ± 0.02	2.82 ± 0.04	3.21 ± 0.02	1.70 ± 0.01
Tot-P	g kg^{-1}	3.24	8.31 ± 0.05	2.31 ± 0.03	1.46 ± 0.00	1.79 ± 0.01
Fe	g kg^{-1}	0.13	0.34 ± 1.69	0.97 ± 0.04	1.29 ± 0.02	2.65 ± 0.03
Al	g kg^{-1}	< 0.07	< 0.07	0.82 ± 0.00	1.34 ± 0.01	2.08 ± 0.03
Cr	mg kg^{-1}	< 2	< 2	6.4 ± 0.2	5.14 ± 0.05	7.79 ± 0.02
Cu	mg kg^{-1}	< 17	23.1 ± 1.69	19.2 ± 0.3	18.02 ± 0.09	45 ± 1
Mn	mg kg^{-1}	187	34.0 ± 0.18	176 ± 4	334 ± 3	357 ± 5
Ni	mg kg^{-1}	< 2	< 2	5.1 ± 0.3	3.5 ± 0.1	3.1 ± 0.1
Pb	mg kg^{-1}	< 2	< 2	2.6 ± 0.3	2.50 ± 0.06	3.6 ± 0.1
Zn	mg kg^{-1}	102	56.9 ± 0.06	153 ± 1	121.8 ± 0.4	194 ± 2
Cd	mg kg^{-1}	< 0.06	< 0.06	0.07	0.07	0.08
Hg	mg kg^{-1}	< 0.04	< 0.04	< 0.04	< 0.04	< 0.04

A special attention was paid on the accumulation of Al and Fe residues in the outdoor samples, which reached to a level of g kg^{-1} as well. These two metal ions were used commonly in the algal cultivation medium, but the content of indoor samples was much less than that of outdoor samples. This indicated that there were still notable amounts of Al^{+} and Fe^{+} ions existing in the treated wastewater. They were quite likely produced from the chemical treatment process at the WWTP, where a large amount of cationic coagulants/ flocculants (e.g., ferric chloride, aluminum chloride and polymers) were commonly used for P precipitation during the secondary treatment of municipal wastewater. So this study would shed lights on a new approach to recycle and reduce these cationic residues in the final discharge of municipal wastewater, minimizing the footprint of the chemical treatment for WWTP.

It is well known that heavy metals are part of hazardous substances and persistent pollutants existing in municipal wastewater (Liu et al. 2020), but they are close to undetectable levels at ug L^{-1} or ng L^{-1}

(Kõrgmaa et al. 2020). In this study, these trace elements were concentrated from tons of municipal wastewater and eventually encapsulated in the algae biomass at a level of mg kg^{-1}. This process is accomplished through a combination of non-active biosorption and active metabolism dependent mechanisms (Matagi et al. 1998, Chekroun and Baghour 2013), due to algae's high binding affinity, abundance of binding sites and large surface area (Cameron et al. 2018). Given the different affinities between two species, the coculture would have a better ability to uptake these metal ions from the municipal wastewater. Nowadays, numerous studies in recent years approved the existence of heavy metals and emerging contaminants in aquatic system and pointed out wastewater discharge as one of the main pollution sources (Caracciolo et al. 2015, Villar-Navarro et al. 2018). Therefore, using filamentous algae coculture represents an effective green tool to remove these problematic elements to purify wastewater discharge. However, the appearance of these metal residues will be one of the prominent factors for produced algae biomass in the evaluation of any potential application.

Quality evaluation of algae biomass

The purpose of using FTIR in this study was to ensure the quality of algae samples, as a good reference for the quantitative evaluation on produced biomass. Without destructive and extraction impacts, FTIR represents a rapid, simple and reproducible method to identify the different compositions in the different biomass (Stehfest et al. 2005). The FTIR spectroscopy showed that there were several differences among samples in certain range of wavenumbers (Fig. 5A). The moisture condition was similar between different algae samples (3400 – 3200 cm^{-1}). As the variation between 3000 – 2500 cm^{-1} was not correlated to the changes in biochemical composition (Mayers et al. 2013), the major differences between 1800 – 800 cm^{-1} were used for assessment. In comparison, *Klebsormidium* contained more lipid, protein and phospholipid than *Spirogyra*, whereas the latter had more carbohydrates (Fig. 5B). In the coculture, the biochemical profile of biomass was relatively consistent across three batches and the proportion of those compounds seemed to "neutralized" between two species. The PCA analysis showed that the *Klebsormidium* gradually became dominant over *Spirogyra* along with the consecutive pilot tests (Fig. 5C). This conclusion was congruent with the microscopy observation above. It could be because *Spirogyra* prefers growing in warm temperature (Graham et al. 1995). Also, on account of the smaller size, *Klebsormidium* would have a competitive edge for limiting nutrients over *Spirogyra* with wider filaments as elucidated in a phytoplankton study (Edwards et al. 2012).

In the quantitative analysis, *Klebsormidium* biomass was rich in protein close to 50% of DW, while *Spirogyra* biomass had only 21% (Fig. 6). In contrast, *Spirogyra* contained more starch (7%) than *Klebsormidium* (3.3%). These results were corresponding to the qualitive observation in FTIR analysis. In the pilot test, the protein content was increased gradually from 1st to 3rd batch (20–30%), while the starch content decreased from 5–4%. These tendencies also demonstrated that the proportion of *Klebsormidium* in the coculture became more in the later stage. As discussed above, the coculture of *Klebsormidium* and *Spirogyra* still needs further optimizing, including the possibility of adding more filamentous algae like *Klebsormidium* as polyculture. However, it is undeniable that any new screening laboratory results will need further validation via pilot study in the future study.

The lipid content was detected below 8% of DW with a small variation across different samples (Fig. 6). As microalgae could increase lipid content in a condition of nutrient starvation (Amer et al. 2011), it was supposed to have more lipid content in the outdoor algae samples. However, it seems that the experimental filamentous algae did not comply with this biological response on microalgae. Perhaps this is the reason that filamentous algae was not compelling to the research attention for typical algae-economy values (e.g., biofuel and omega oil) in past years. The ash content was high in the first two WWT batches (26.6 % – 23.3 %), lower in 3rd batch (13.8%), but still significantly higher than those for the monoculture controls (*Klebsormidium* 6.4% and *Spirogyra* 9.4%). As the ash content was related to the amount of total minerals in a biomass (Liu 2019), this result was coincident with the content of mineral chemicals and heavy metals derived from the wastewater purification.

Apart from those potential hazardous substances (e.g., heavy metals) in the biomass, high ash content will also affect the algae inclusion level for food and feed utilization (Austic et al. 2013), and increase problems in combustion for energy conversion (Hupa 2012). With the notable protein content, the potential impact on anaerobic digestion (AD) process shall be investigated if the biomass is used for AD biogas production. There is a great potential to utilise the produced biomass for biofertilizer, soil ameliorator or new material development. For example, the heavy metals content in the produced biomass was below the maximum limit for permissible content in different classes of organic fertilizers, according to the Norwegian regulations on organic fertilizers (FOR-2003-07-04-951). Certainly, this initiative will need comprehensive evaluations to match other restrictions on such as pesticides, hygiene conditions and requirements for soil mixtures, as well as public perception. In terms of potential usage on the produced biomass, this is an inclusive question to both technical and economic perspectives. However, with the progress of scalable filamentous algae cultivation and defined bioproducts' investigation, it is envisioned that all of these endeavours will provide a new impulse for phycoremediation development.

Conclusions

This study reveals that the mixed filamentous algae *Klebsormidium* and *Spirogyra* can act as an effective tool to further purify the municipal tertiary wastewater in Norway. The co-culture successfully recovered macronutrients, mineral elements and heavy metals, and thereby this new purification process could significantly reduce or eliminate the environment footprint of municipal wastewater discharge in nature. In order to facilitate the green transition of wastewater management, future research will likely focus on the remaining questions that require proper optimizations in areas of algae inoculum density and combination, the effect of nutrient and carbon loading, hydraulic retention time reduction and proposed two-stage cultivation strategy for continuous process. The produced algae biomass might be not favorable for typical algae-economy values, whereas the derived nutrient profiles and the easy scalable production would bring unprecedented levels to other different value-added applications. However, it is important in positioning this new phycoremediation approach in the circular economy concept for WWTPs, which can also shape future investment consideration. Moreover, the associated techno-

economic analysis and environmental impact assessment will need to be performed before deciding on full-scale implementation.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

Designed the experiments: YL, EW, GK, BM, CV. Methodology: EW, GK, BM, CV, YL, RH. Performed the experiments: EW, GK, BM, CV, YL, RH. Discussed the results: YL, EW, GK, BM, CV. Analyzed the data: YL, EW, GK, CV. Wrote the manuscript: YL, EW, GK. Discussed and revised the manuscript: YL, EW, GK, BM, CV, RH. All authors read and approved the final manuscript.

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Figures

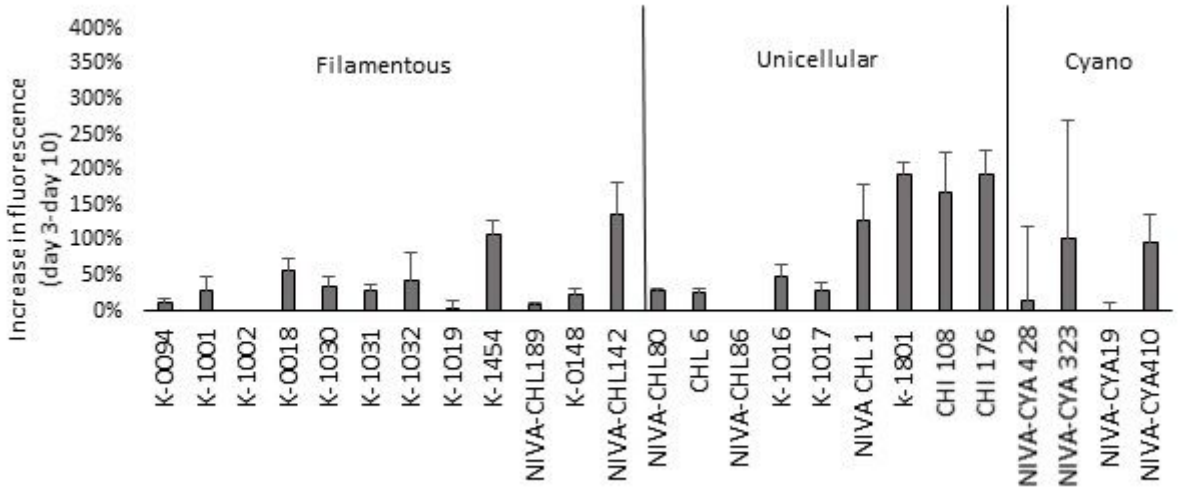

Figure 1

The comparison of fluorescence increments among different selected algae in the screening test (mean ± SD).

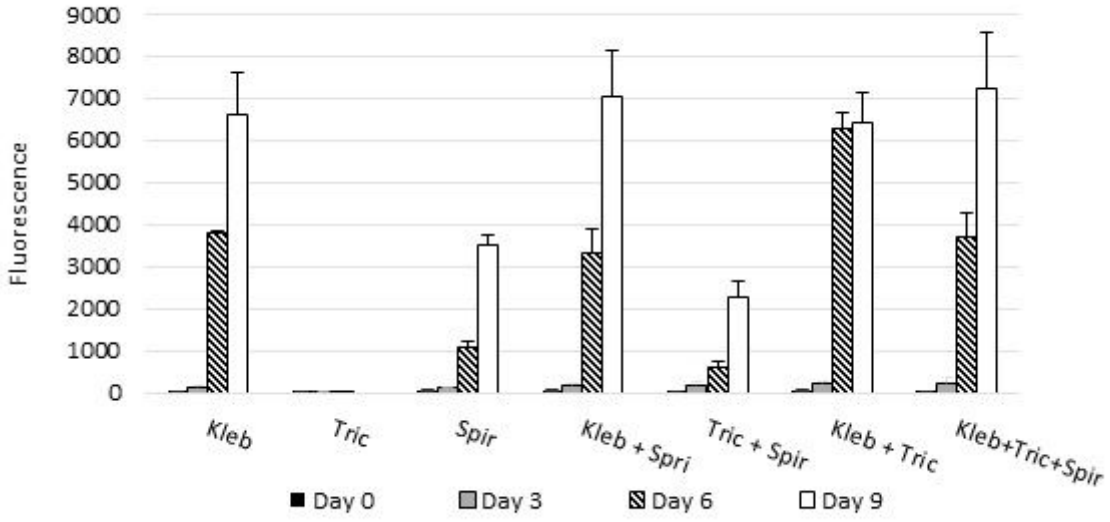

Figure 2

Monoculture and co-culture flask experiments (mean ± SD). (Kleb: Klebsormidium sp. Tric: Trichormus sp. Spir: Spirogyra sp.)

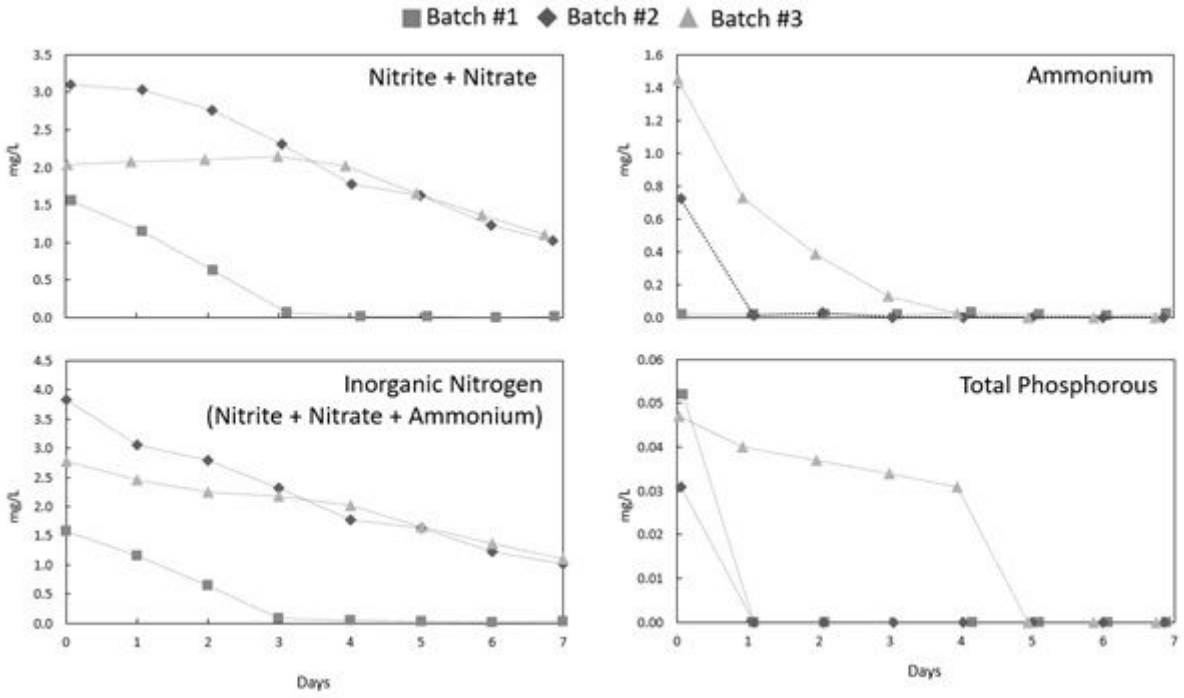

Figure 3

Macronutrients during WWTP effluent treatment with algal co-culture.

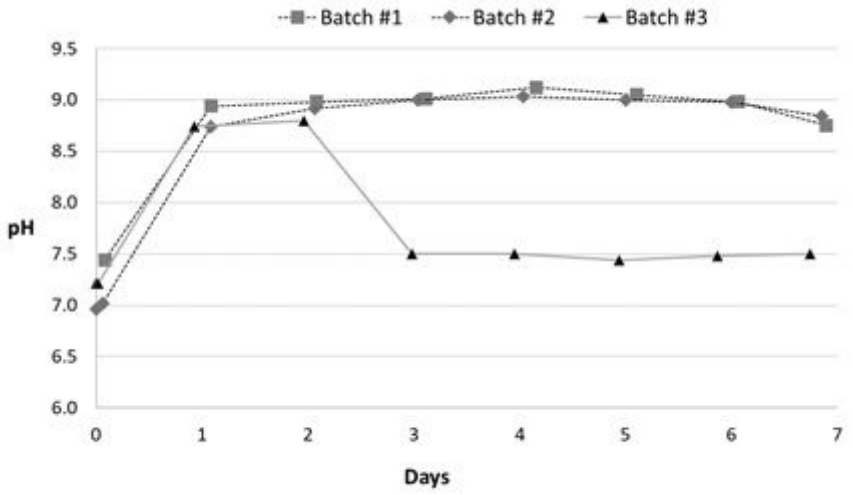

Figure 4

The pH value monitored over three pilot testing batches (note: CO2 injection was added to #3 on day 2).

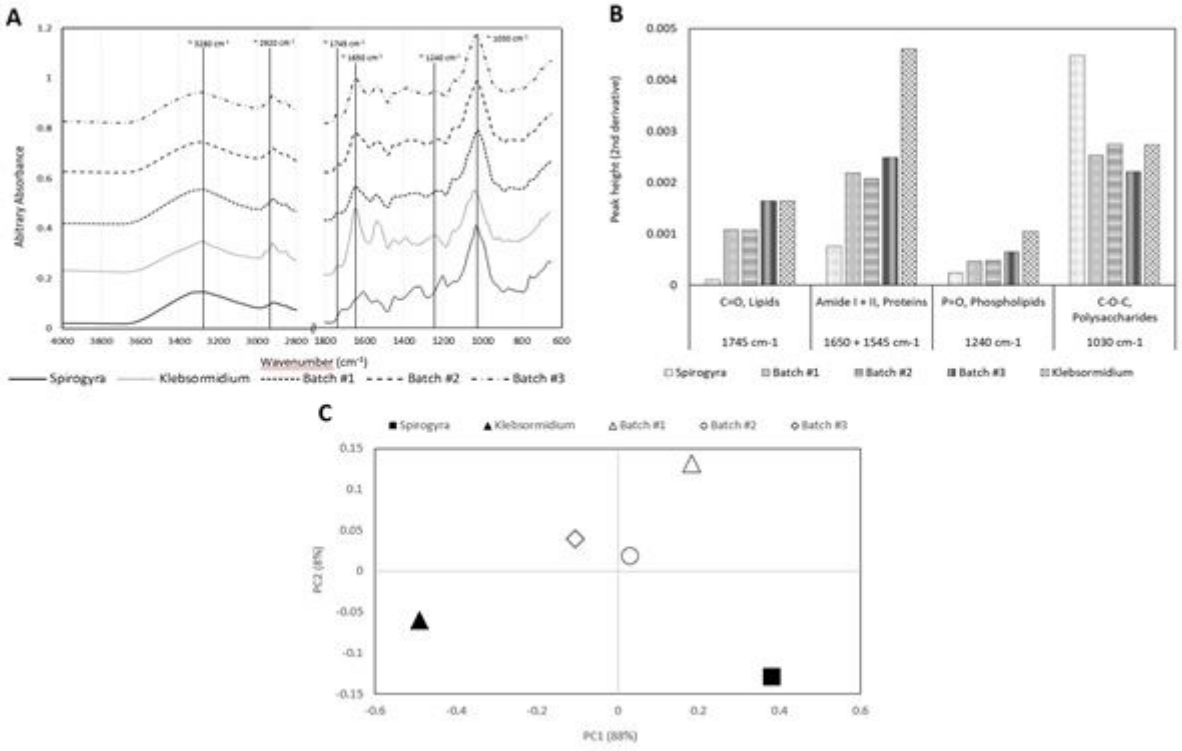

Figure 5

FTIR analysis of microalgae biomass. A) FTIR spectra with characteristic bands noted, B) Peak height of characteristic bands, C) Scores plot of Principal Component Analysis (PCA).

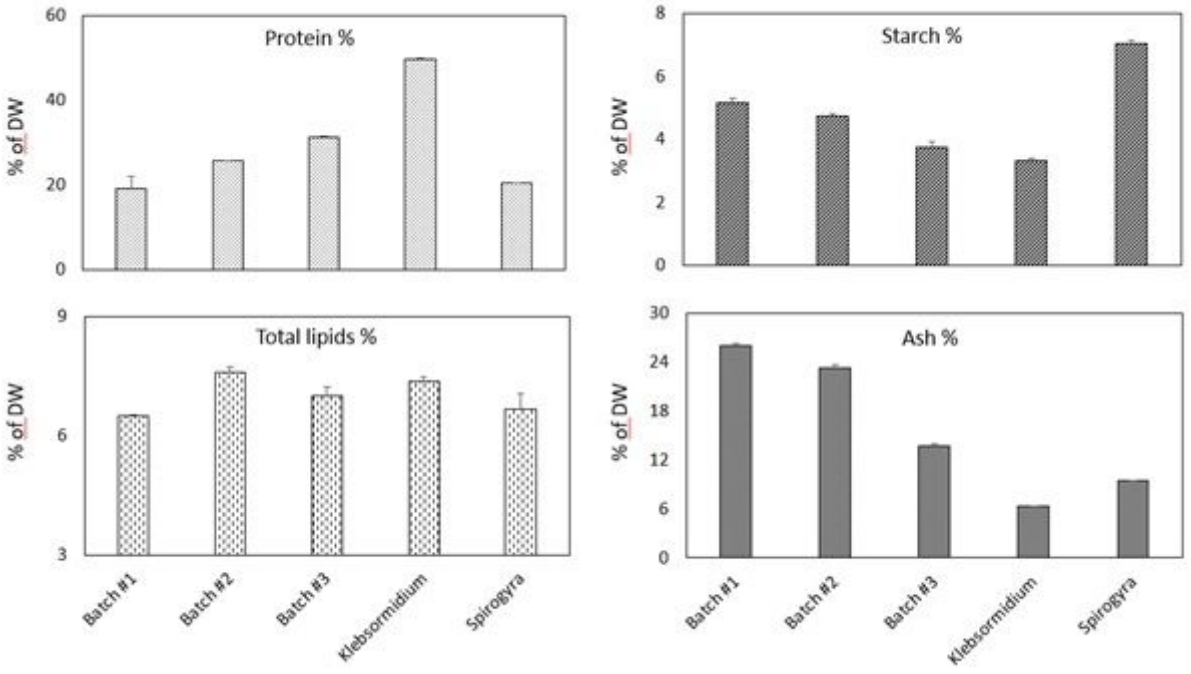

Figure 6

Proximate biochemical analyses of microalgae biomass from indoor monocultures and outdoor co-cultures on wastewater.

Supplementary Files

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- Graphicalabstract.jpg