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Culturing of *Selenastrum* on diluted composting fluids; conversion of waste to valuable algal biomass in presence of bacteria

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HIGHLIGHTS

- Composting leachates supported growth of both algae and bacteria in co-cultures.
- Nutrients from composting leachates were efficiently converted to algal biomass.
- Prolonged cultivation time and CO_2 feed enhance the lipid production in co-cultures.
- In commercialization, co-culturing is a realistic alternative.

GRAPHICAL ABSTRACT

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ABSTRACT

Growth and fatty acid production of microalga *Selenastrum* sp. with associated bacteria was studied in lab-scale experiments in three composting leachate liquids. Nutrient reduction in cultures was measured at different initial substrate strengths. A small, pilot-scale photobioreactor (PBR) was used to verify lab-scale results. Similar growth conditions supported growth of both *Selenastrum* and bacteria. CO_2 feed enhanced the production of biomass and lipids in PBR (2.4 g L^{-1} and 17% DW) compared to lab-scale (0.1–1.6 g L^{-1} and 4.0–6.5% DW) experiments. Also prolonged cultivation time increased lipid content in PBR. At both scales, NH_4-N with an initial concentration of ca. 40 mg L^{-1} was completely removed from the biowaste leachate. In lab-scale, maximal COD reduction was over 2000 mg L^{-1}, indicating mixotrophic growth of *Selenastrum*. Co-cultures are efficient in composting leachate liquid treatment, and conversion of waste to biomass is a promising approach to improve the bioeconomy of composting plants.
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1. Introduction

Microalgae are promising as beneficial organisms in production of renewable energy or valuable metabolites such as fatty acids

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(FAs), pigments and vitamins for human or animal nutrition (Spolaore et al., 2006). In sustainable and cost-effective microalgal biomass production 'waste to valuable products' concepts have gained attention, since algae efficiently convert nutrients from effluents into biomass. In the modern bio economy waste should be seen as a potential source for novel and valuable products (O'Callaghan, 2016).

According to FAO, 1.3 billion tons of food is annually lost or wasted worldwide in the food supply chain from agricultural production to final consumption and dumped into landfill sites or treated as waste (Gustavsson et al., 2011). Waste from food industry processes and agriculture, such as brewery wastewater, food waste hydrolysate, dairy wastewater and dairy manure wastewaters have successfully been used as algal growth substrates (Faroog et al., 2013; Kothari et al., 2012; Pleissner et al., 2013; Wang et al., 2010). Integrating algal culturing units with existing industrial processes has clear benefits; for example utilization of waste heat in colder climate regions (Andersson et al., 2014), capturing CO_2 emissions in algal biomass (Andersson et al., 2014) and recycling of water (Yang et al., 2011) used in industrial processes will reduce ecological footprints. Synergies such as these can reduce culturing and other operating costs, and enhance operational efficiencies, thereby improving the overall economic feasibility of the concept.

In microalgal biomass cultivation, an alternative to pure microalgal cultures is to co-cultivate algae with bacteria. Co-cultivation may stimulate or inhibit growth as well as lipid metabolism of algae (de-Bashan et al., 2002; Guo and Tong, 2014; Zhang et al., 2012), depending on cultivation conditions and the combination of strains. Nitrogen limited growth often enhances algal lipid metabolism (Fields et al., 2014; Griffits et al., 2012) and the presence of bacteria in algal cultures can improve NH_4-N removal from wastewaters (Wang et al., 2015). While algae efficiently take up NH_4-N, bacteria take part in NH_4-N removal through nitrification (Wang et al., 2015). In addition to total lipid content, FA profile is important when assessing the suitability of algal lipids for applications such as biodiesel production or for nutritional needs (Spolaore et al., 2006; Liu et al., 2011).

Utilization of waste in algal cultivation allows recycling of carbon and nutrients by assimilation into biomass, but also the chemical properties of wastewater influence the biomass growth and biochemical composition of the produced biomass. When algae are capable of growing mixotrophically, high organic carbon content promote algal growth (Li et al., 2014) and significant COD removal indicating mixotrophic growth has been reported when algal cultures have been grown in wastewater (Wang et al., 2010, 2015). However, high organic carbon content may also inhibit algal growth (Lewitus and Kana, 1994). Growth inhibition of mixotrophs in the presence of organic substrates may result from reduction in pigmentation or photosynthetic activity or from problems in chloroplast development, but also enhanced growth and pigmentation has been reported (Lewitus and Kana, 1994).

Aerobic composting is an essential part of waste management wherein biomass is degraded and converted by micro-organisms into products that can be used as fertilizers or soil conditioners (Partanen et al., 2010). Alternatively, biowaste may be used as feedstock for biogas, bioethanol and biodiesel production (Pan et al., 2015). However, the leachates from different phases of the composting process, e.g. from stored biowaste or composting material, are still not fully utilized. The recovery of nutrients from composting wastewater into valuable biomass is an opportunity to improve the overall bioeconomy of a composting plant.

In this study, the viability of composting plant leachate liquids was evaluated as a substrate when growing our isolate *Selenastrum* sp. (now on code number SCCAP K-1877) (Chlorophyceae) in non-axenic conditions, i.e. in the presence of bacteria. *Selenastrum* was selected for the experiments because chlorophycean microalgae have successfully been cultured in wastewaters, and for example *Chlorella*, *Scenedesmus* and *Selenastrum minutissima* have a high capacity for nutrient removal and biomass production (Gentili, 2014; Zhang et al., 2012). This is the first time that composting leachate liquids were used as a substrate for algal cultivation. Besides the biomass and lipid production of the cultures, nutrient removal was measured. First, small laboratory scale experiments were conducted, after which pilot-scale cultivation was carried out in a PBR to verify the scalability of the results. Multiple types of wastewater were used in the small-scale experiments, and the one which appeared to be most suitable for the combined goals of high biomass production, rapid growth and efficient nutrient reduction, was used in the pilot-scale experiments. In the small-scale experiments also the influence of darkness on the growth of algae and bacteria was studied to test the resilience of the algae under conditions in which phototrophic organisms have no advantage and heterotrophic bacteria still thrive. This test simulated winter-time light conditions at northern latitudes, where cultivation of phototrophic algae is not possible around the year without artificial light. In these conditions mixotrophic algal strains can grow partly heterotrophically enabling year-round indoor cultivation which results in savings in energy consumption and cultivation costs.

2. Material and methods

2.1. Characterization of leachates used as substrate

Composting and biowaste leachates from the Labio composting facility in Lahti (Finland) and the biowaste press leachate from NSR, Helsingborg (Sweden) were used in algal cultivation experiments. For characterization of the leachate liquids used in small-scale experiments, nutrients (PO_4^+, TN, and NH_4-N) and COD were analyzed using the Hach Lange Kits (Hach Lange, Germany), a DR 2800™ spectrophotometer (Hach Lange) and a HT 200S high temperature thermostat heating block (Hach Lange). BOD was measured with an Oxitop® Biological Oxygen Demand respirometer and the Oxitop® control system (WTW, Weilheim, Germany). For trace elements (Al, As, B, Hg, Cd, K, Ca, Co, Cr, Cu, Pb, Mg, Mn, Mo, Na, Ni, Fe, Se, Zn) and total P (TP) analysis, leachate liquids were pre-treated with 10% nitro-hydrochloric acid (molar ratio 1:3) and analyzed by ICP-MS (Agilent Technologies, Agilent 7500ce, Japan) according the standard SFS-EN ISO 17294-2. PBR experiments were carried out with biowaste leachate from the same composting plant collected at a later date. COD often predicts BOD in the same sample type (Bourgeois et al., 2001), and thus only COD was analyzed from the biowaste leachate used in the PBR. Trace element composition in the biowaste leachate used in the PBR was not analyzed.

2.2. Preparation of algal inocula

Algal cultures to be used as inocula in the lechate liquid experiments were prepared as follows: the *Selenastrum* stock culture, that contained a population of uncharacterized bacteria, was treated with antibiotics to reduce the amount of bacteria in the culture. Previously, bacteria had occasionally taken over the *Selenastrum* cultures. An antibiotic mixture of Penicillin (Penicillin G potassium salt), Streptomycin (Streptomycin sulfate salt), and Gentamicin (Gentamicin sulfate salt) was added to the final concentrations 50, 12.5 and 12.5 $\mu g\ mL^{-1}$, respectively, in COMBO-medium (Kilham et al., 1998). Antibiotics were purchased from Sigma-Aldrich. During the next four months, the algal culture

was inoculated once per month into fresh COMBO-medium without antibiotics. As intended, the cultures were non-axenic.

2.3. Cultivation conditions and biomass harvesting

In small scale, the microalga *Selenastrum* sp. with associated bacteria was cultivated in diluted composting leachate which is normally returned to the composting process by irrigation (i), biowaste leachate percolated from the municipal biowaste (fresh garden, household and industrial biowaste) mixed with similar waste after composting (ii), and biowaste press leachate generated by pressing municipal biowaste before composting (iii). Chu-10 medium was used as a control medium for algal growth (Chu, 1942). Prior to the experiments, particles from the leachate liquids were removed by centrifugation, the supernatant was filtered through glass fiber filters (GF/C, Ø47 mm, Whatman) and autoclaved (121 °C, 1 bar, 20 min). Since in the preliminary tests (results not shown) high bacterial concentrations in untreated leachates led to overgrowth of bacteria, these liquids were later autoclaved before use.

The liquids used in small scale experiments were diluted into Chu-10 medium and in PBR into tap water. The following concentrations were used in small scale: Composting leachate at 25% and 50%, biowaste leachate at 2.5% and 5%, and biowaste press leachate at 10% and 50%. To reach close to similar TN and TP concentrations in the different scale operations, a more diluted biowaste leachate was used in the PBR (1%) than in the small-scale (5% in the most optimal case) (see chapter 3.1). Before beginning the experiments, the pH in the diluted leachate liquids was adjusted to 7 using HCl or NaOH. Experimental units were inoculated with *Selenastrum* grown exponentially in COMBO-medium. In the small-scale experiments the starting cell density of *Selenastrum* was 7.74×10^4 mL^{-1} and in PBR the initial biomass concentration was 0.13 g L^{-1}.

Small scale experiments with four replicates were carried out in 250 mL plastic tissue culture bottles with a culture volume of 200 mL in a growth chamber (Sanyo MLR-350, Sanyo Electric Co. Ltd, Japan), a photon flux density 150 μmol m^{-2} s^{-1}, (Li-Cor 190R Quantum Sensor and LI-1400 Light Sensor Logger, Li-Cor, Lincoln, Nebraska, USA), and a temperature of 20 °C. The cultivation was up-scaled in PBR (100L) filled to a volume of 80 L. PBR was illuminated from two sides with eight fluorescent tubes (8 × 36 W, Osram Lumilux De Luxe Daylight L36W/954, Germany). Light intensity was 230 μmol m^{-2} s^{-1}. The PBR was placed in a temperature controlled room and the temperature inside the PBR was recorded as 24–27 °C.

The PBR was aerated and mixed with compressed air (Piston pump Hailea 318, 70 L min^{-1}) pumped through a perforated polyvinyl chloride (PVC) pipe. CO_2 (99.8%) was fed through a silicon tube (Ø 6 mm) connected to an aeration stone with a pore size of 0.5 μm. The CO_2 feeding rate was adjusted by a pH controller unit (Multi-Parameter System KM-3000, Sensortechnik Meinsberg GmbH, Ziegra-Knobelsdorf, Germany) connected to a pH electrode (SI Analytics GmbH, Mainz, Germany). Feeding was switched on automatically when the pH rose above 7 (pH 7.1 ± 0.1), since photosynthesis and CO_2 consumption by algae increases pH, while maintaining a pH less than 7 keeps the CO_2 concentration at a level that stimulates algal growth (Azov, 1982). Light and dark cycle in both scales was 16L:8D.

Cultivation times in different small scale cultures were 11–21 days (Fig. 1.) and in PBR cultivation was completed after 41 days, when the increase in biomass had ceased. To study the influence of heterotrophic growth conditions, well-growing small scale cultures were transferred to total darkness for three days before harvesting (Fig. 1.). Biomass was harvested by centrifugation (Multifuge 1 S-R, Kendro Laboratory Products) (2500 rpm, 15 min, 15 °C) for the bacterial DNA extraction from the small-scale cultures and for the lipid extractions from both culturing scales, whereas PBR biomass at the end of the experiment was harvested with a flow through centrifuge (Evodos type 10, Evodos Algae Technologies B.V.) (800 rpm, flow rate 750 L h^{-1}). Pellets for DNA extraction were stored at −20 °C and pellets for lipid extraction at -70 °C followed by freeze drying.

2.4. Microscopy, DW determination and nutrient analysis

In the small-scale cultures, sampling started one day after inoculation. Samples for microscopic cell counting of algal cell density (cells mL^{-1}) were taken three times a week, preserved with acidic Lugol's solution and counted microscopically (Leica, DM 1000, Germany; total magnification X200) using Lund chambers (Lund, 1959). Twenty random fields or at least one hundred cells (accepted limits for reliable counts) were counted from each sample. For calculation of specific growth rates (SGR, μ_{max} d^{-1}) cell abundances during the exponential growth phase were ln-transformed and SRGs were calculated as described in Issarapayup et al. (2009).

Samples for biomass dry weight (DW) determination were taken at the end of all the successful, non-collapsed small-scale experiments and in the PBR, from samples taken two or three times a week. Samples were filtered through pre-dried (150 °C, over night) filters (GF/C, Ø 47 mm, pore size 1.2 μm Whatman) and DW was measured as described by Tredici and Zittelli (1998). Residual nutrients (TP, PO_4-P, TN and NH_4-N) in the leachate liquids used in both experimental scales and COD in the small-scale experiments were analyzed with Hach Lange kits (chapter 2.1.) using the supernatant after filtration for DW analysis. The supernatants derived from collecting biomass for DW analyses were stored at -20 °C until the nutrient analysis.

2.5. Determination of bacterial growth

For qPCR determination of bacterial cell numbers, samples were taken from the inoculant before inoculation to the growth media and twice a week from three of the four replicate cultures during the experiments. Since leachate liquids were diluted and handled in non-sterile conditions after autoclaving, the amount of bacteria was also checked from them. Determination of bacterial cell concentrations were based on qPCR analysis of 16S rRNA gene copy numbers. DNA was extracted from the pelleted cell samples using the Power Soil DNA isolation kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. General primers for the 16S rRNA gene for bacteria pE and pF (Ekman et al., 2007; Kanto Öqvist et al., 2008), purchased from Oligomer Oy (Finland), were used for qPCR. The reactions were executed in a LightCycler 96 (Roche, Germany). PCR mixture was prepared essentially as described by Yu et al. (2014). Cycling conditions were set at 10 min at 95 °C, followed by 30 cycles of 10 sec at 95 °C, 20 sec at 57 °C, 30 sec at 72 °C. *Cupriavidus necator* JMP134 (DSM 4058) was used for the standard curve to convert the copy number of 16S rRNA in qPCR to cell numbers. DNA concentrations were quantified using PicoGreen kit (Life Technologies). The limit of quantification (LOQ) for bacteria was 1.34×10^3 cells mL^{-1}.

2.6. Lipid extraction, methylation and FA analysis

Lipids were extracted and analyzed from all replicates of the three successfully grown laboratory cultures with highest cell concentrations and biomass DW at the end of the laboratory experiments. To investigate changes in total fatty acid (TFA) content and FA profile in different growth phases, extraction and analysis was conducted from samples collected from the PBR at exponential and late exponential growth phases as well as stationary phase on

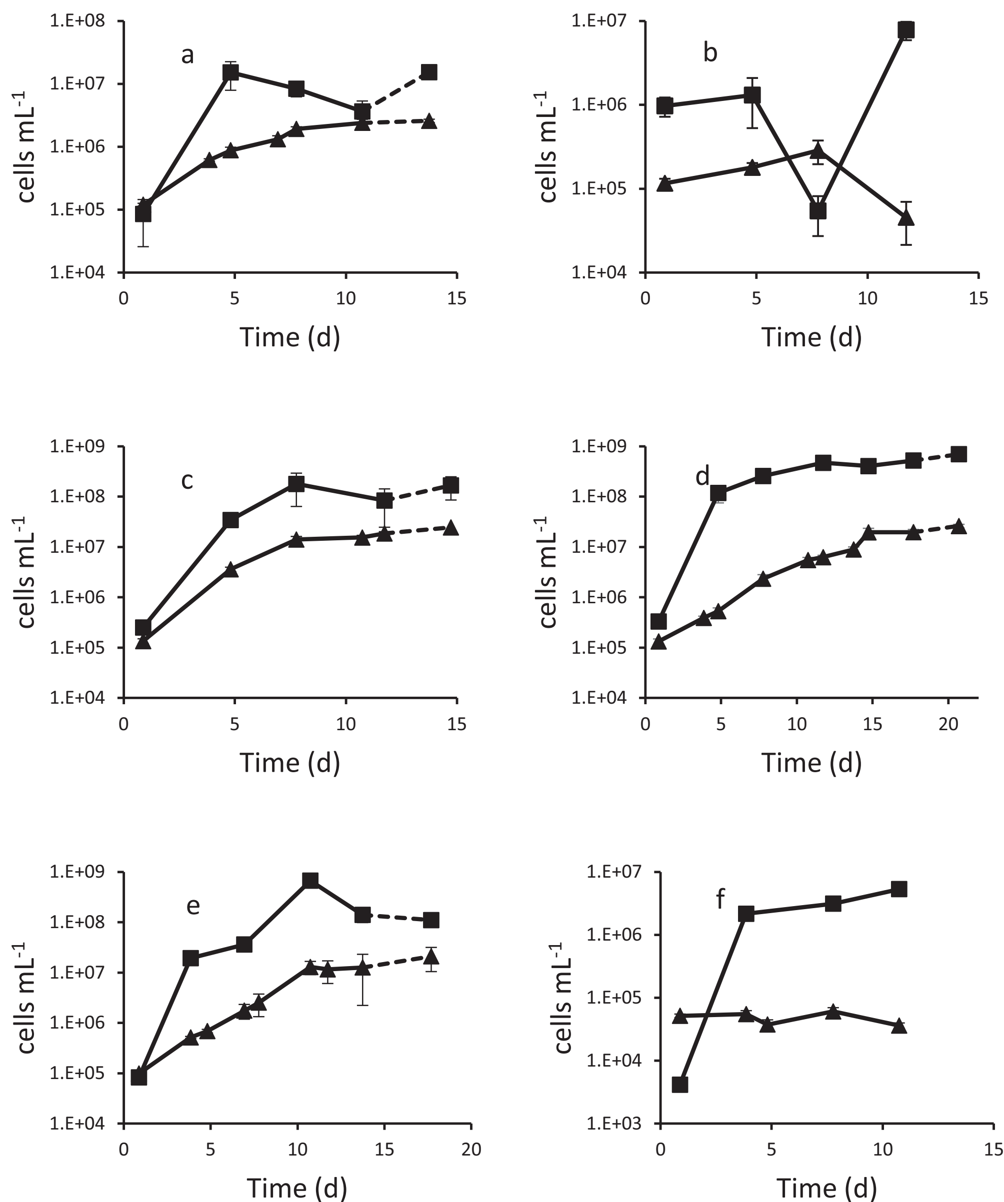

Fig. 1. a, b, c, d, e, f. Cell concentrations of *Selenastrum* (▲) (n = 4) and bacteria (■) (n = 3) in different cultivations in waste water (average ± SE). (a = composting leachate 25%, b = composting leachate 50%, c = biowaste leachate 2.5%, d = biowaste leachate 5%, e = biowaste press leachate 10% and f = biowaste press leachate 50%). Solid line means the cyclic light – dark cultivation and dashed line the dark incubation period.

days 20, 32 and 42. Lipids were extracted as described by Parrish (1999). Acid-catalyzed methylation of fatty acid methyl esters (FAMEs) was carried out as described by Christie and Han (2010) with some modifications. The internal standard (ISTD) C19:0 (non-adecanoic acid 72332-1 G-F/analytical standard, Sigma Aldrich) for quantification and 20 μL of BHT (butulated hydroxy toluene, 1% in methanol) to prevent oxidation were added prior to methylation. Samples were analyzed with GC–MS (GC/MS-QP 2010 Ultra SYSTEM, Shimadzu, USA) equipped with an autosampler (AOC-20 s, Shimadzu) and operating software (GCMS solution, Version 2.6) using the capillary column DB-23 (Agilent Technologies). Helium was used as the carrier gas (1 mL min^{-1}, splitless). The temperature program was as follows: injection temperature 250 °C, initial column temperature 60 °C at 1 min, the temperature increase to 110 °C at the rate of 20 °C min^{-1}, from 110 °C to 185 °C at the rate of 3 °C min^{-1}, from 185 °C to 195 °C at the rate of 0.5 °C min^{-1}, and from 195 °C to 250 °C at the rate of 25 °C min^{-1} where it was held for 2 min. FAME identification based on retention times in standard solution (Supelco™ 37 Component FAME Mix, Sigma) and mass spectra (http://lipidlibrary.aocs.org/). For quantification, ISTD was added to the standard mix solution prepared in five concentrations and FAs were quantified using the ratio of the peak area of known FA and ISTD on the chromatogram. Total TFA content (% of DW) was calculated as a sum of identified FAs and the volumetric yield as a unit mg L^{-1}. For individual FAs LOQ was 0.05–0.1 ng μL.

2.7. Statistics

Cell concentrations of *Selenastrum* in small-scale experiments were tested with Kruskal-Wallis test and pairwise comparison with Mann-Whitney U test because of heterogeneity of variances. Differences in growth rates, DW and lipid contents of *Selenastrum*

and bacterial cell concentrations in different waste waters were tested with ANOVA using a post hoc Tukey's test. All statistical analyses were performed with SPSS Statistics, version 22, IBM, USA.

3. Results and discussion

3.1. Characteristics of composting leachate liquids

Characteristics of the undiluted leachate liquids used in the small-scale experiments are shown in Table 1. Concentrations of macro- and micronutrients were initially high in all leachates. According to the Redfield ratio (molar N:P-ratio 16:1) the TN:TP ratios in the composting and the biowaste leachates (N:P-ratio 199:1 and 23:1) indicated P limitation, while in biowaste press leachate N availability (N:P-ratio 12:1) was lower (Redfield, 1958). Essential micronutrients were available in all undiluted composting leachates. In comparison to Miazek et al. (2015), Cu and Zn concentrations in undiluted composting and biowaste press leachates and Al concentration in biowaste leachate were so high that without dilution, they could have inhibited growth. Concentrations of toxic heavy metals (Hg, Cd and Pb) were under detection limit. Because the leachate liquid composition at composting plants varies with the quality of the composted waste, there were some differences between the nutrient concentrations in the small-scale and the PBR experiments. The TN (14,800 mg L^{-1}), NH_4-N (4280 mg L^{-1}), TP (912 mg L^{-1}) and PO_4-P (278 mg L^{-1}) contents in the undiluted biowaste leachates were higher and COD considerably lower (6400 mg L^{-1}) in the PBR than in the small-scale experiments.

3.2. Growth of microalgae and bacteria in small-scale experiments

The pre-testing of different types of leachate liquids indicated that in general, the same growth conditions supported the growth of both eukaryotes and prokaryotes. When the growth of *Selenastrum* was successful, bacteria also grew well and vice versa. Despite the pretreatment of algae with antibiotics, bacteria were present in the algal inoculum (5.4×10^5 cells) and also in the diluted (25% and 50%) composting leachates (1.8×10^4 mL^{-1} and 1.3×10^5 mL^{-1}, respectively), but in the rest of the diluted leachate liquids, the bacterial cell concentrations were below LOQ (see 2.7). However, after one day of cultivation, bacteria were found in every culture.

Initially growth of *Selenastrum* was efficient in all cultures (Fig. 1a-f), but in two cultures with media composed of 50% composting leachate and 50% biowaste press leachate, algal cultures collapsed after 12 and 11 days, respectively, and the cultivation was stopped without dark incubation (Fig. 1b and f). In those experiments with collapsing algal cultures, bacteria survived, but bacterial concentrations during the whole cultivation period were lower than in the others. SGR of *Selenastrum* was higher in 2.5% biowaste leachate ($P < 0.05$) than in the other cultures (Table 2.). In all non-collapsed cultures SGR was higher than in the control ($P < 0.05$). On the last culturing day, the cell concentrations of *Selenastrum* and bacteria (Figs 1a-f) as well as concentrations of DW (Table 2) were the highest when cultivated in biowaste leachates (2.5 and 5%) and composting leachate 10% ($P < 0.05$). In comparison to control, algal cell concentrations were higher ($P < 0.05$) in all composting liquids, except in 25% composting leachate. However, biomass DW was always lowest ($P < 0.05$) in control medium and very small algal cell sizes were observed microscopically. Although the cell concentrations of bacteria were higher than the concentrations of *Selenastrum* in all the cultures, the biomass mainly consisted of algal cells because of the considerably larger cell size of

Table 1
Nutrient, BOD, COD, trace element concentrations (mg L^{-1}) and pH in undiluted composting leachate, biowaste leachate and biowaste press leachate used in lab scale experiments. Values under the lowest quantification limit are given (<).

Compound	Composting leachate	Biowaste leachate	Biowaste press leachate
TN	486	2540	1960
NH_4-N	333	1650	238
TP	5.4	240	357
PO_4-P	3.4	149	308
BOD_7	124	46,000	13,556
COD	643	80,000	40,640
Al	1.8×10^{-1}	78	6.9
As	4.8×10^{-3}	6.0×10^{-2}	8.0×10^{-2}
B	1.5×10^{-1}	9.2	1.7×10^{-1}
Hg	<	<	<
Cd	<	<	<
K	150	2400	880
Ca	17	1680	1400
Co	4.4×10^{-3}	3.0×10^{-1}	1.8×10^{-2}
Cr	7.6×10^{-3}	1.2	1.0×10^{-1}
Cu	2.1×10^{-2}	<	1.1×10^{-1}
Pb	<	<	<
Mg	21	820	160
Mn	4.2×10^{-2}	17	2.3×10^{-2}
Mo	1.4×10^{-2}	4.2×10^{-1}	2.6×10^{-2}
Na	110	6400	890
Ni	2.1×10^{-2}	2.2	8.4×10^{-2}
Fe	5.0	580	36
Se	<	<	<
Zn	3.5	0.62	3.6
pH	9.1	4.3	4.3

Table 2
Specific growth rates (SGR, d^{-1}) of *Selenastrum* and dry weights (DW, g L^{-1}) of co-culture in small scale cultures.

Waste water	Conc.%	SGR	DW
Composting leachate	25	0.38 ± 0.01	0.10 ± 0.01
Biowaste leachate	2.5	0.68 ± 0.02	1.14 ± 0.03
Biowaste leachate	5	0.34 ± 0.01	1.63 ± 0.08
Biowaste press leachate	10	0.42 ± 0.07	0.9 ± 0.38

Selenastrum (length 20–25 µm, width 2.5 µm) in comparison to the average cell size of bacteria (length 2 µm, width 1 µm). Dark incubation did not affect the growth of *Selenastrum* or bacteria, i.e. cell concentrations of algae as well as bacteria remained constant or grew only slightly during the dark period.

Concentrations of TP as well as PO_4-P were low in the 25% composting leachate (Table 3), which probably limited the *Selenastrum* as well as bacterial growth. Also a high NH_4-N concentration together with high pH can lead to inhibition of algal growth, since as pH increases, non-toxic NH_4-N dissociates to toxic NH_3-N, inhibiting photosynthesis (Azov and Goldman, 1982). In this study pH was not measured, but it is well known, that due to photosynthesis pH increases during cultivation. This may explain the weak growth in 50% composting leachate and 50% biowaste press leachate with the NH_4-N concentrations after dilution of 166.5 and 122.0 mg L^{-1}, respectively. Zn can be toxic for microalgae at concentrations as low as 10 nmol L^{-1} (Reynolds, 2006) which was clearly exceed in the collapsed cultures and can be the reason for poor growth. In 50% composting leachate, the amount of bacteria was high which possibly inhibited growth of *Selenastrum* in the early phase of the cultivation.

As shown here and elsewhere (Lee et al., 2015), without completely aseptic experimental conditions the algal cultures do contain bacterial contaminants. When considering algal cultivation in composting liquids, the presence of bacteria is difficult to avoid, in which case co-culturing is viable and realistic alternative for commercial production. Limiting the bacterial amounts at the

Table 3
Initial nutrient and COD concentrations (mg L^{-1}) in diluted composting liquids and total reduction (%) in small scale cultures.

Compound	Composting leachate 25%		Biowaste leachate 2.5%		Biowaste leachate 5%		Biowaste press leachate 10%	
	mg L^{-1}	Reduction (%)	mg L^{-1}	Reduction (%)	mg L^{-1}	Reduction (%)	mg L^{-1}	Reduction (%)
TN	121.5	16.9	63.5	47.7	127	50.5	196.0	73.9
NH_4-N	83.3	18.6	41.3	100.0	82.5	81.5	23.8	100.0
TP	1.4	22.2	6.0	60.5	12	28.7	35.7	74.6
PO_4-P	0.9	16.1	3.7	80.3	7.5	8.2	30.8	80.0
COD	160.8	19.8	2000	49.7	4000	54.3	4064	38.9

onset of the cultivation may, however, be crucial for initial survival and performance of the algae despite increased processing costs. Growth and survival of both organisms in co-cultures in light as well as dark indicates that year-round indoor cultivation is possibly also at northern latitudes.

3.3. Nutrient reduction in small scale experiments

Good algal-bacterial growth correlated well with efficient removal of nutrients, especially TN and NH_4-N (Table 3). When comparing the most efficiently growing co-cultures, the NH_4-N was completely consumed in the cultures in 2.5% biowaste leachate and 10% biowaste press leachate, but was still available in 5% biowaste leachate at the end of the cultivation. The fact that the algal biomass yield reached much higher values in the PBR than in the small-scale cultivations despite almost similar TN and TP and lower NH_4-N and PO_4-P concentrations in the PBR (see chapter 3.3.) shows that the availability of these nutrients was not a growth-limiting factor in the small-scale cultures. Most probably, the shortage of inorganic carbon limited the biomass production in the small-scale cultures because the CO_2 level in air is not sufficient for maximum algal growth (Cho et al., 2016) and even in optimal light conditions photosynthesis is restricted. Actually, the light conditions in the small-scale culture bottles were likely better than in the PBR due to the thin structure of the culture bottles and the lower biomass concentration.

Growth as well as nutrient removal was lower in composting leachate than in other cultures. The most prominent differences between the composting leachate and the other liquids used as media were the relatively low TP, PO_4-P concentrations and COD (Table 3). Low P concentration presumably influenced to the growth, but as seen in PBR experiments (see chapter 3.3) algal-bacterial co-culture can grow efficiently for a long time under P limitation. High concentration of organic matter undoubtedly supported the growth of bacteria and in co-cultures, efficient bacterial growth provide CO_2 and other beneficial substances for algal growth and the algal photosynthesis generates the oxygen for use of bacteria (Han et al., 2016). This makes algal-bacterial co-culturing as a practical option for efficient biomass production, a positive finding, given that bacterial presence is inevitable.

However, algae are often capable of mixotrophic growth, and high COD removal by mixotrophic *Chlorella* cultures grown in wastewater has been reported (Wang et al., 2010). In the experiments presented here, the relative role of bacteria and algae in COD removal was not possible to distinguish, but high COD removal in efficiently growing algal-bacterial cultures and good algal growth during the dark incubation, indicates that *Selenastrum* also grew mixotrophically and participated in the removal of organic substrates.

3.4. Growth and nutrient reduction in PBR

Better availability of CO_2 in PBR (see chapter 3.2.) favored the growth of *Selenastrum* with associated bacteria, and on the last culturing day a biomass of 2.4 g L^{-1} DW was reached (Fig. 2) – roughly twice the concentrations in the best growing small-scale culture. In the PBR, NH_4-N was removed almost completely (98%) in six days, while TN reduction was 70%, and over 90% TN reduction was reached in eighteen days (Fig. 2). TP as well as PO_4-P concentrations in biowaste leachate (1%) were initially low and stayed low until the end of the incubation (day 42) when the total reduction was 92% and 84%, respectively (Fig. 2).

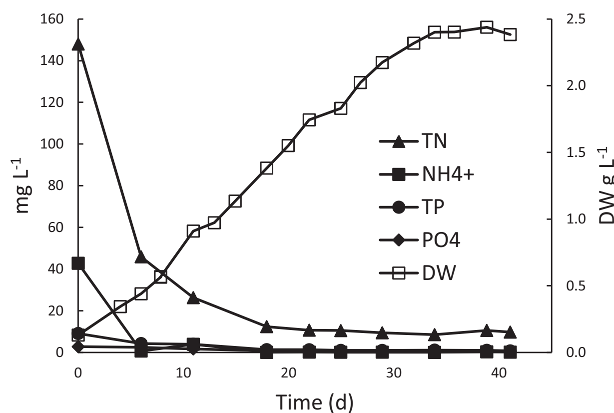

Fig. 2. Growth of biomass (DW) and nutrient removal (TN, NH_4-N, TP, PO_4-P) in co-culturing of *Selenastrum* and bacteria in PBR in diluted (1%) biowaste leachate.

Biomass yield in PBR was high since e.g. Miranda et al. (2012) reported a biomass production 0.8 g L^{-1} for *Scenedesmus obliquus* grown in tubular PBR (volume 50 L) in conventional medium. Zhu et al. (2014) estimated that the biomass yield of *Chlorella zofingiensis* grown in outdoor PBR (volume 40 L) in artificial wastewater was 1 g L^{-1}. In small scale cultures high biomass yields are typical; *Chlorella sorokiniana* grown in conventional medium reached a biomass of 5 g L^{-1} (Li et al., 2014). In the present study, growth continued long after the nutrients were exhausted from the growth media, indicating the use of intracellularly stored nutrients. The cultivation in PBR lasted for more than 40 days, and the actual growth lasted for 34 days, while the nutrient removal took place rapidly, i.e. mainly during the first two weeks. This shows that algae accumulate nutrients faster than they need for growth. This is an important observation, since in continuous cultivation algae efficiently take up the nutrients from the medium and the wastewater feed and simultaneously flow-through or circulation of purified wastewater needed for dilution of composting liquids can be increased. Algal yield may potentially then be maximized during a separate phototrophic post-cultivation phase where harvested algal cells exhaust their nutrient reserves.

3.5. Lipid content

Straightforward comparison of lipid production between scales is not plausible due to the differences in cultivation conditions. Main FAs in small scale cultures were palmitic acid (16:0), stearic

Table 4
Main fatty acids, TFA content (% DW) and proportion (% of TFAs) of SAFAs, MUFAs and PUFAs in small scale (mean ± stdv) (n = 4) and PBR (mean of two replicate extractions) cultures. nd = not detected, <LOQ = under the lowest quantitation level.

	Biowaste leachate[1] 2.5%	Biowaste leachate[1] 5%	Biowaste press leachate[1] 10%	Biowaste leachate[2] 1%
C16:0	23.1 ± 2.2	21.9 ± 2.9	24.4 ± 0.9	24.3
C18:0	4.9 ± 0.1	8.1 ± 2.1	3.7 ± 1.1	2.3
C18:1n9	35.1 ± 11.6	39.9 ± 4.4	40.7 ± 1.4	56.8
C18:2n6	4.8 ± 0.9	4.6 ± 0.7	4.2 ± 0.3	8.4
C18:3n3	23.1 ± 6.0	12.0 ± 1.2	17.7 ± 0.7	5.7
C22:0	2.4 ± 0.7	3.9 ± 1.6	2.6 ± 0.2	nd
C24:0	6.6 ± 1.9	9.5 ± 3.7	3.4 ± 0.1	<LOQ
TFA% DW	4.7 ± 0.6	4.0 ± 0.7	6.2 ± 0.7	17.0
SAFA	37.0 ± 4.7	42.6 ± 4.5	35.3 ± 1.5	26.6
MUFA	35.1 ± 11.6	41.3 ± 4.4	42.1 ± 1.5	57.9
PUFA	27.9 ± 6.9	16.1 ± 1.0	22.6 ± 0.6	14.1

[1] Small scale experiments.
[2] PBR experiment.

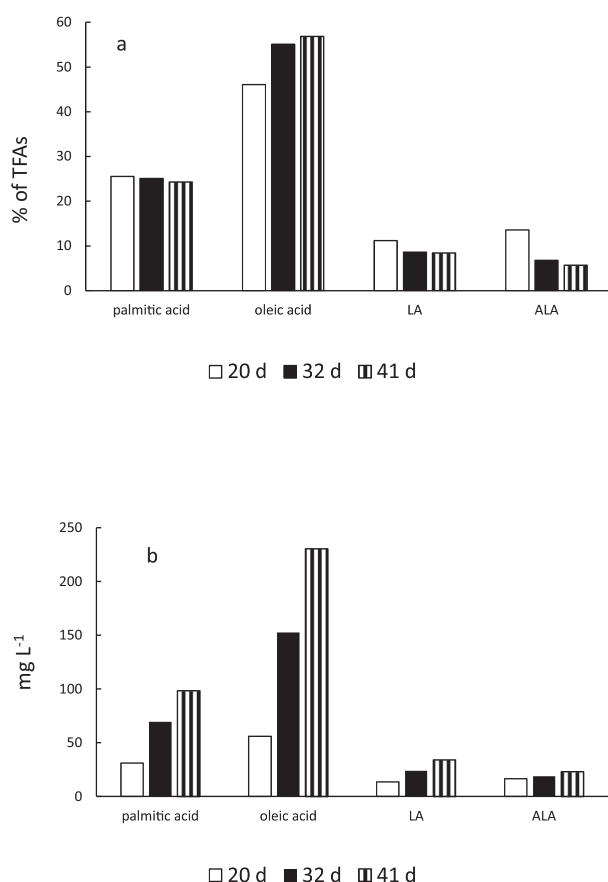

Fig. 3. a, b. a) Proportions (% of TFAs) of palmitic acid C16:0, oleic acid C18:1n9, LA C18:2n6 and ALA C18:3n3 and b) yields (mg L^{-1}) of palmitic acid C16:0, oleic acid C18:1n9, LA C18:2n6 and ALA C18:3n3 in the PBR culture grown in biowaste leachate (1%) (mean of two replicate extractions) in sampling days (d) 20, 32 and 41.

acid (18:0), oleic acid (18:1n9), linoleic acid (18:2n6, LA), linolenic acid (18:3n3, ALA), behenic acid (22:0) and lignoceric acid (24:0) (Table 4). Among the small-scale cultures the FA content was highest in cultures grown in 10% biowaste press leachate (6.5% DW) ($P < 0.05$) (Table 4). Main differences in FA profiles were found in relative proportions of oleic acid and ALA (Table 4).

In the PBR culture, the TFA content (% DW) doubled between days 20–41 from 8% DW to 17% DW, while during the same time period, the lipid yield (mg L^{-1}) showed a more than threefold increase from 120 mg L^{-1} and 406 mg L^{-1}. Monounsaturated FAs (MUFAs) were more abundant than SAFAs and PUFAs and (Table 4). Prolonged cultivation time increased the yields (mg L^{-1}) of all main FAs. Also the proportion (% of TFAs) of oleic acid (Fig. 3a, 3b) in TFAs was increased, while the contents of LA and ALA were slightly decreased (Fig. 3a, b).

Results here were in accordance with earlier study (Taipale et al., 2013) revealing palmitic and oleic acids, LA and ALA as a dominant FAs in chlorophyceae and oleic acid as the most abundant FA in *Selenastrum* biomass. Growth conditions, stage in biosynthesis (Guschina and Harwood, 2006) and culture age (Gong et al., 2013) influence the FA profile in algal lipids. ALA is an important FA in nutrition, but in biodiesel, the proportion of ALA is limited to below 12% (Griffits et al., 2012). Since time was the dominant factor regulating the relative amounts of FAs, the harvesting time is a key factor influencing the quality of extracted oil.

Nitrogen limitation has often been shown to increase FA content of algae (Fields et al., 2014; Griffits et al., 2012), and in N-starved cultures P starvation can regulate the carbon flow from starch synthesis to lipid synthesis (Zhu et al., 2015). In the experiments presented here, however, the nutrient availability as such was not the factor explaining the differences in lipid biosynthesis. Instead, the observed time dependent increase in lipid content in PBR experiment and earlier study (Gong et al., 2013), reveal that longer period under nutrient starvation is required for enhanced lipid production.

Secondly, carbon availability is essential for lipid synthesis (Fields et al., 2014), and the addition of inorganic carbon in the form of CO_2 gas in the PBR culture boosted lipid biosynthesis. As discussed earlier, (chapter 3.2) the efficient removal of COD was probably a consequence of mixotrophic growth of *Selenastrum* in the small-scale cultures. Research has shown that through mixotrophy (Li et al., 2014) or heterotrophy (Liu et al., 2011) some algae can accumulate more lipids than what is possible in purely autotrophic growth. However, small scale results in this study indicate, that in co-culture nutrient uptake and COD removal is efficient also in inorganic carbon-limited conditions and that the growth and lipid production can be improved further by boosting autotrophic growth with CO_2. This observation is encouraging for combined biomass and lipid production purposes; for producing 1 kg of algal biomass, 1.8 kg of CO_2 is needed (Chisti, 2007) and thus, the mixotrophic co-culturing has economic advantages. Ideally all the needed CO_2 should originate from other processes present at the waste management plant.

In summary, as shown here, a maximized biomass production clearly out-weighted the possible benefits of an optimization of the FA composition. Secondly, the increased biomass production

and extended cultivation time under nutrient starvation generally increased the total lipid yield and yield of main FAs. Conversion of 'no value' waste liquid to algal biomass allows recycling of carbon and nutrients to valuable FAs and other metabolites, e.g. proteins and pigments. However, before using the algal-bacterial biomass in human nutrition, stability and quality (incl. toxicity) of the growth substrate should be analyzed. Detailed analysis of produced biomass or extracts is also a necessity.

4. Conclusions

In the co-cultures studied, similar conditions supported vigorous growth of both the algal strain *Selenastrum* and the bacteria present in the culture. When algal growth was rapid, bacteria also grew well, but the bacteria did not inhibit algal growth. CO_2 feeding boosted the algal biomass and lipid production and prolonged cultivation time increased the total lipid content in the PBR culture. In well growing small scale cultures in diluted biowaste leachates (2.5 and 5%) and diluted biowaste press leachate (10%) and in PBR culture, the nutrient uptake was efficient, which might be utilized when converting waste to valuable biomass.

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References

Andersson, V., Viklund, S.B., Hackl, R., Karlsson, M., Berntsson, T., 2014. Algae-based biofuel production as part of an industrial cluster. Biomass Bioenergy 71, 113–124.
Azov, Y., 1982. Effect of pH on inorganic carbon uptake in algal cultures. Appl. Environ. Microbiol. 43, 1300–1306.
Azov, Y., Goldman, J.C., 1982. Free ammonia inhibition of algal photosynthesis in intensive cultures. Appl. Environ. Microbiol. 43, 735–739.
Bourgeois, W., Burgess, J.E., Stuetz, R.M., 2001. On-line monitoring of wastewater quality: a review. J. Chem. Technol. Biotechnol. 76, 337–348.
Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.
Cho, D.-H., Ramanan, R., Heo, J., Shin, D.-K., Oh, H.-M., Kim, H.-S., 2016. Influence of limiting factors on biomass and lipid productivities of axenic *Chlorella vulgaris* in photobioreactor under chemostat cultivation. Bioresour. Technol. 211, 367–373.
Christie, W.W., Han, X., 2010. Preparation of derivates of fatty acids. In: Christie, W. W., Han, X. (Eds.), Lipid Analysis, Isolation, Separation, Identification and Lipidomic Analysis. Woodhead Publishing, New Delhi, pp. 145–158.
Chu, S.P., 1942. The influence of the mineral composition of the medium on the growth of planktonic algae. Part I. Methods and culture media. J. Ecol. 30, 284–325.
de-Bashan, L.E., Bashan, Y., Moreno, M., Lebsky, V.K., Bustillos, J.J., 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillium brasilience*. Can. J. Microbiol. 48, 514–521.
Ekman, J., Kosonen, M., Jokela, S., Kolari, M., Korhonen, P., Salkinoja-Salonen, M., 2007. Detection and quantitation of colored deposit forming *Meiothermus* spp. in paper industry processes and end products. J. Ind. Microbiol. Biotechnol. 34, 203–211.
Faroog, W., Lee, Y.-C., Ryu, B.G., Kim, B.-H., Choi, H.-S., Kim, Y.-E., Yang, J.-W., 2013. Two-stage cultivation of two Chlorella sp. strains by simultaneous treatment of brewery wastewater and maximizing lipid productivity. Bioresour. Technol. 132, 230–238.
Fields, M.W., Hise, A., Lohman, E.J., Bell, T., Gardner, R.D., Carredor, L., Moll, K., Peyton, B.M., Characklis, G.W., Gerlach, R., 2014. Sources and resources: importance of nutrients, resource allocation, and ecology in microalgal cultivation for lipid accumulation. Appl. Microbial. Biotechnol. 98, 4805–4816.
Gentili, F.G., 2014. Microalgal biomass and lipid production in mixed municipal, dairy, pulp and paper wastewater together with added flue gases. Bioresour. Technol. 169, 27–32.
Gong, Y., Guo, X., Wan, X., Liang, Z., Jiang, M., 2013. Triacylglycerol accumulation and change in fatty acid content of four marine oleaginous microalgae under nutrient limitation and at different culture ages. J. Basic Microbiol. 53, 29–36.
Griffits, M.J., Hille, R.P., Harrison, S.T.L., 2012. Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions. J. Appl. Phycol. 24, 989–1001.
Guo, Z., Tong, Y.V., 2014. The interactions between *Chlorella vulgaris* and algal symbiotic bacteria under photoautotrophic and photoheterotrophic conditions. J. Appl. Phycol. 26, 1483–1492.
Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog. Lipid Res. 45, 160–186.
Gustavsson, J., Cederberg, C., Sonesson, U., Otterdijk, R.V., Meybeck, A., 2011. Global Food Losses and Food Waste – Extent, Causes and Prevention. FAO, Rome.
Han, J., Zhang, L., Wang, S., Yang, G., Zhao, L., Pan, K., 2016. Co-culturing bacteria and microalgae in organic carbon containing medium. J. Biol. Res. (Thessalon) 23, 1–9.
http://lipidlibrary.aocs.org/ Last accessed Apr 4, 2017. Christie, W.W.
Issarapayup, K., Powtongsook, S., Pavasant, P., 2009. Flat panel airlift photobioreactors for cultivation of vegetative cells of microalga *Haematococcus pluvialis*. J. Biotechnol. 142, 227–232.
Kanto Öqvist, C., Kurola, J., Pakarinen, J., Ekman, J., Ikävalko, S., Simell, J., Salkinoja-Salonen, M., 2008. Prokaryotic microbiota of recycled paper mills with low or zero effluent. J. Ind. Microbiol. Biotechnol. 35, 1165–1173.
Kilham, S.S., Kreeger, D.A., Lynn, S.G., Goulden, C.E., Herrera, L., 1998. COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiology 377, 147–159.
Kothari, R., Pathak, V.V., Kumar, V., Singh, D.P., 2012. Experimental study for growth potential of unicellular *Chlorella pyrenoidosa* on dairy wastewater: an integrated approach for treatment and biofuel production. Bioresour. Technol. 116, 466–470.
Lee, H.-G., Shin, S.-Y., Jin, L., Yoo, C., Srivastava, A., La, H.-J., Ahn, C.-Y., Kim, H.-S., Oh, H.-M., 2015. Establishment and maintenance of an axenic culture of *Ettlia* sp. using a species-specific approach. Biotechnol. Bioprocess. Eng. 20, 1056–1063.
Lewitus, A.J., Kana, T.M., 1994. Responses of estuarine phytoplankton to exogenous glucose: stimulation versus inhibition of photosynthesis and respiration. Limnol. Oceanogr. 39, 182–189.
Li, T., Zheng, Y., Yu, L., Chen, S., 2014. Mixotrophic cultivation of *Chlorella sorokiniana* strain for enhanced biomass and lipid production. Biomass Bioenergy 66, 204–213.
Liu, J., Huang, J., Sun, Z., Zhong, Y., Jiang, Y., Chen, F., 2011. Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic *Chlorella zofingiensis*: assessment of algal oils for biodiesel production. Bioresour. Technol. 102 (106–110), 24.
Lund, J.W.G., 1959. A simple counting chamber for nannoplankton. Limnol. Oceanogr. 4, 57–65.
Miazek, K., Iwanek, W., Remacle, C., Richel, A., Goffin, D., 2015. Effect of metals, metalloids and metallic nanoparticles on microalgae growth and industrial product biosynthesis: a review. Int. J. Mol. Sci. 16, 23929–23969.
Miranda, J.R., Passarinho, P.C., Gouveia, L., 2012. Bioethanol production from *Scenedesmus obliquus* sugars: the influence of photobioreactors and culture conditions on biomass production. Bioenergy Biofuels 96, 555–564.
O'callaghan, K., 2016. Technologies for the utilisation of biogenic waste in the bioeconomy. Food Chem. 198, 2–11.
Pan, S.-Y., Du, M.A., Huang, I.-T., Liu, I.-H., Chang, E.-E., Chiang, P.-C., 2015. Strategies on implementation of waste-to-energy (WTE) supply chain for circular economy system: a review. J. Clean. Prod. 108, 409–421.
Parrish, C.C., 1999. Determination of total lipid, lipid classes, and fatty acids in aquatic samples. In: Arts, M.T., Wainman, B.C. (Eds.), Lipids in Freshwater Ecosystems. Springer, New York, pp. 4–20.
Partanen, P., Hultman, J., Paulin, L., Auvinen, P., Romantschuk, M., 2010. Bacterial diversity at different stages of the composting process. BMC Microbial. 10, 1–11.
Pleissner, D., Lam, W.C., Sun, Z., Lin, C.S.L., 2013. Food waste as nutrient source in heterotrophic microalgae cultivation. Bioresour. Technol. 137, 139–146.
Redfield, A.C., 1958. The biochemical control of chemical factors in the environment. Am. Sci. 46, 2015–2221.
Reynolds, C.S., 2006. Ecology of Phytoplankton. Cambridge University Press, Cambridge.
Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101, 87–96.
Taipale, S., Strandberg, U., Peltomaa, E., Galloway, A.W.E., Ojala, A., Brett, M., 2013. Fatty acid composition as biomarkers of freshwater microalgae: analysis of 37 strains of microalgae in 22 genera and in seven classes. Aquat. Microb. Ecol. 71, 165–178.
Tredici, M.R., Zittelli, G.C., 1998. Efficiency of sunlight utilization: tubular versus flat photobioreactors. Biotechnol. Bioeng. 57, 187–197.
Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., Ruan, R.R., 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour. Technol. 101, 2623–2628.
Wang, M., Yang, H., Ergas, S.J., Steen, P., 2015. A novel shortcut nitrogen removal process using an algal bacterial consortium in a photo-sequencing batch reactor (PSBR). Water Res. 87, 38–48.
Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfield, M., Chen, Y., 2011. Life-Cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. Bioresour. Technol. 102, 159–165.
Yu, D., Kurola, J.M., Lähde, K., Kymäläinen, M., Sinkkonen, A., Romantschuk, M., 2014. Biogas production and methanogenic archaeal community in mesophilic

and thermophilic anaerobic co-digestion processes. J. Environ. Manag. 143, 54–60.

Zhang, Y., Su, H., Zhong, Y., Zhang, C., Shen, Z., Sang, W., Yan, G., Zhou, X., 2012. The effect of bacterial contamination on the heterotrophic cultivation of *Chlorella pyrenoidosa* in wastewater from the production of soybean products. Water Res. 46, 5509–5516.

Zhu, L., Hiltunen, E., Shu, Q., Zhou, W., Li, Z., Wang, Z., 2014. Biodiesel production from algae cultivated in winter with artificial wastewater trough pH regulation by acetic acid. Appl. Energy 128, 103–110.

Zhu, S.N., Wang, Y.J., Xu, J., Shang, C.H., Wang, Z.M., Xu, J.L., Yuan, Z.H., 2015. Luxury uptake of phosphorus changes the accumulation of starch and lipid in *Chlorella* sp under nitrogen depletion. Bioresour. Technol. 198, 165–171.