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Conversion of biowaste leachate to valuable biomass and lipids in mixed cultures of *Euglena gracilis* and chlorophytes

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ABSTRACT

Microalgae are a sustainable alternative for production of valuable omega – 3 fatty acids (FAs), but high production costs limit commercialization. Utilization of waste as a nutrient source increases the economics of the cultivation process. Additionally, using mixed algal cultures instead of monocultures makes the cultivation process more flexible and can increase biomass and lipid production. Here, the growth and lipid production of microalgae *Euglena gracilis*, *Selenastrum* sp. and, *Chlorella sorokiniana* were studied in mono- and mixed cultures in small and pilot scale experiments in biowaste leachate. In pilot scale, also nutrient reduction and the number of bacteria were analyzed. Biomass production in the most productive mixed cultures was similar, but not higher than in most productive monocultures. The lipid production was highest in the small-scale monoculture of *Selenastrum* (10.4% DW) and in the pilot scale culture of *Selenastrum* with *E. gracilis* (11.1% DW). The content of alpha-linolenic acid (ALA) increased and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) remained stable during the cultivation period in all pilot scale cultures. However, increases in biomass and lipid production toward the end of the cultivation resulted in higher EPA and DHA yields in the well growing monoculture of *E. gracilis* and in the mixed culture of *E. gracilis* with *Selenastrum*. Co-cultivation of *E. gracilis* and *Selenastrum* also had a positive influence on nutrient uptake and resistance against bacteria. This type of mixed culture may be a good option for commercialization. However, as shown here, minor changes in cultivation conditions can rapidly result in dominance of a subdominant strain, and thus the stability of strain performance and production of desired FAs needs further investigation.

1. Introduction

Sustainable production practices and alternatives for fish oils as a source of essential long chain polyunsaturated fatty acids (LC-PUFAs) are needed to satisfy nutritional needs of a growing world population and to protect endangered aquatic ecosystems [1]. Concerns have been raised on pollution effects of growing fish farming and over-exploitation of wild fish populations as fish-feed ingredients [1,2]. Microalgae

produce several health promoting, essential omega – 3 FAs such as ALA (18:3n3), EPA (20:5n3) and DHA (22:6n3). The polyunsaturated fatty acid (PUFA) ALA is a common C₁₈ FA (18 carbon atoms in FA chain) in plant oils, while LC-PUFAs EPA and DHA, (C₂₀ and C₂₂) have traditionally been obtained from marine and fresh water food [3,4]. ALA is classified as an essential FA, since it is a precursor of LC-PUFAs in the omega – 3 family [5]. Most animals cannot synthesize precursor FAs de novo, whereas conversion efficiency to LC-PUFAs varies between

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organisms and taxa; for example among the zooplankton, conversion of precursor FAs has been found in freshwater copepods but not in cladocerans [6]. However, conversion of ALA to EPA and DHA in the human body is limited [7] and thus, it is necessary to have additional dietary sources. EPA and DHA have anti-inflammatory properties [8], and reduce the risk for cardiovascular diseases [9] while DHA is essential for the development and function of the brain, nervous system and retinas [5,10].

In comparison to fish oils, production costs of microalgae based LC-PUFAs are still high. In a techno-economic analysis (TEA) by Chauton et al. [11], costs of EPA and DHA production in flat-panel photobioreactor (PBR) in high irradiance conditions was estimated to be 39.10 USD per kg of EPA/DHA equivalents, whereas the current production costs of fish oil based EPA and DHA are 8 USD per kg of EPA/DHA equivalents. Biorefinery concepts utilizing waste for cost-effective and environmentally friendly production of lipid rich algal biomass mostly focus on biofuel production. However, converting waste nutrients to biomass makes algal cultivation more sustainable, reduce cultivation costs and converts waste to valuable biomass. Based on TEA by Chauton et al. [11], utilization of waste nutrients and CO₂ can reduce the EPA/DHA production costs by approximately 25%, whereas combining waste stream utilization, reduction of energy consumption, and doubling of EPA and DHA production from 6% to 12% (biomass DW) can decrease the total production cost to 11.90 USD per kg of EPA/DHA equivalents. Among the microalgae, euglenoids (Euglenophyta) have raised interest as producers of LC-PUFAs [12,13] whereas green algae (*Chlorophyceae*) are rich in ALA [14]. Furthermore, euglenoids and green algae have high capacity for production of biomass and removal of nutrients and dissolved organic matter from wastewaters [15–18].

Most studies so far have focused on algae cultivation in monocultures, although cultivation of algal strains in mixed cultures, in some circumstances, is known to enhance biomass [19,20] and lipid production [19,21]; transgressive overyielding refers to higher productivity in mixed cultures than in most productive single strain cultures [21]. Mixed culturing, where strains with differing growth requirements and potentials are combined, can also enhance nutrient uptake [21] and increase the biomass stability over time [22] and flexibility of the cultivation process [15]. In comparison to monocultures, mixed cultures can better tolerate potentially stressful changes in growth conditions, such as modifications in the growth medium, while maintaining continuous biomass production. They can also be more competitive and thus tolerant of invasive algal species [22] or other contamination [23]. Alternatively, the advantage of mixed cultures can be “multifunctionality” i.e. to have a good performance in several functions, instead of transgressive overyielding or superiority in some specific parameter [22]. On the other hand, variable algal characteristics in mixed cultures can make production of specific valuable compounds unpredictable [15].

Here we grew algae in diluted biowaste leachate and hypothesized that lipid production of algae and thus the yield of ALA, EPA and DHA is boosted in mixed cultures. Biomass production, nutrient removal capacity, and tolerance of bacterial contamination were hypothesized to be higher in mixed cultures than in monocultures. Chemical oxygen demand (COD) predicts both biodegradable and biologically inert organic matter in wastewater [24]. Heterotrophic bacteria as well as mixo- or heterotrophic microalgae can participate in COD removal and thus we assumed that COD removal by algae in mixed cultures is more efficient than in monocultures. Experiments with the euglenoid *E. gracilis*, and the green algae *Selenastrum* sp. and *Chlorella sorokiniana*, were carried out both in small laboratory scale and in pilot scale experiments using monocultures as well as mixed cultures. First, lipid and biomass production was tested in small scale units. Second, upscaling tests of the lipid and biomass production and nutrient removal capacity were carried out in pilot scale PBRs with a monoculture of *E. gracilis* and in mixed cultures of *E. gracilis* with *Selenastrum* sp. or *C. sorokiniana*.

Since the long time maintenance of axenic algal cultures in commercial scale is not cost-effective or even plausible, we used non-axenic algal cultures and determined the number of bacterial cells in pilot scale cultures. The overall aim of this research was to evaluate the potential of mixed cultivation for commercial production of omega – 3 FAs in combination with nutrient removal capacity.

2. Materials and methods

2.1. Strains and growth media

Before the experiments, the three microalgae, *E. gracilis* (CCAP 1224/5Z), *Selenastrum* sp. (SCCAP K-1877) and *C. sorokiniana* (UTEX 1230), were cultured for two months in mixed cultures to ensure co-existence of strains and to stabilize the relative abundance of strains in mixed cultures. Mixed cultures were prepared by combining equal amounts (based on cell numbers) of strains from EG-medium [25] grown stock cultures in the following combinations: *E. gracilis* with *Selenastrum*, *E. gracilis* with *C. sorokiniana*, *Selenastrum* with *C. sorokiniana* and all three strains together. These pre-cultures as well as monocultures were regularly transferred to fresh EG-medium and later used as inocula in small and pilot scale batch culture experiments carried out in composting leachate. In small scale experiments, monocultures were used as controls when evaluating influence of mixed cultivation on biomass and lipid production. The amounts of inoculated cell suspensions in experiments were 8 mL in small scale and 5 L in pilot scale (1.6 and 2.8% of cultivation volume). In small scale experiments, biomass DW was analyzed from pre-cultures before inoculation, and in pilot scale DW was determined after inoculation to the biowaste leachate, i.e. from the first sampling point for growth determination. Cell concentrations in mixed pre-cultures in small scale and in all pre-cultures were counted before inoculation for further evaluation of strain composition. Selection of strains was based on our earlier experiments showing, that monocultures of *E. gracilis*, *C. sorokiniana* (unpublished) and *Selenastrum* [26] grow well in composting leachate. For pilot scale testing, a monoculture of *E. gracilis* and mixed cultures of *E. gracilis* with *C. sorokiniana* and *E. gracilis* with *Selenastrum* were selected based on the results of the small scale experiments (see the Section 3.1).

At both experimental scales diluted (1%) leachate from a mixture of municipal organic waste and partly composted garden waste originating from a composting plant (Labio Oy, Lahti, Finland) (hereafter called “biowaste leachate”) was used for cultivation. Suitability of diluted biowaste leachate for algal cultivation was confirmed in an earlier study [26]. Prior to the experiments solid particles were removed from the biowaste leachate by centrifugation and filtering, and the supernatant was collected and sterilized by autoclaving. A detailed description of the pre-treatment process was published earlier [26]. In the small-scale experiments, biowaste leachate was diluted with distilled water and pH adjusted to 7 using NaOH, while tap water was used for dilution in the pilot scale and pH was kept below 7 by CO₂ feeding.

2.2. Growth conditions

The small scale experiments were carried out with five replicates in a growth chamber (SANYO growth cabinet MLR-350 H; 294 L) at 20 °C, in plastic tissue culture flasks containing 500 mL of the diluted (1%) biowaste leachate. In the pilot scale test (without replication) algae were grown at 25 ± 2 °C in 180 L of the same medium in transparent polycarbonate PBRs (99 × 24.5 × 84.5 cm, 200 L). The light and dark cycle was adjusted to 16:8 in both experiment types. At both scales light entered from the sides with a photon flux density of 150 μmol m⁻² s⁻¹ in the small scale and 230 μmol m⁻² s⁻¹ in the pilot scale cultivations. In the PBRs, where mixing was achieved by pumping air, pH was controlled continuously by feeding CO₂ (99.8%) to cultures when pH rose above 7. Installation and buildup of PBRs is described in detail by Tossavainen et al. [26]. The pH of the small-scale cultures was

Table 1
Biomass DW (g L⁻¹), cell densities (cells mL⁻¹) and relative abundance of strains (%) in inoculants¹ (n = 1) and at the end² of small (n = 5) and pilot scale (n = 1) experiments when algae were grown in mono- and mixed cultures. Results in small scale experiments at the end of the cultivation depict mean ± SD of experimental replicates (n = 5) and in pilot scale mean ± SD of replicate measurements (n = 3) for DW. Relative cell amounts (%) at the end of the cultivation are calculated from average cell concentrations in small scale cultures. (E = *E. gracilis*, S = *Selenastrum*, C = *C. sorokiniana*, (P) = pilot scale culture.) Inoculant DW is measured before inoculation in small scale and after inoculation in pilot scale. Statistically significant differences in biomass yields at the end of the cultivation in small scale cultures are shown.

Strain(s)	¹ Inoculant			² End of the cultivation					
	¹ E	¹ S	¹ C	¹ E + S + C	² Biomass	² E	² S	² C	² E + S + C
	cells mL ⁻¹	cells mL ⁻¹	cells mL ⁻¹	%	DW g L ⁻¹	cells mL ⁻¹	cells mL ⁻¹	cells mL ⁻¹	cells %
E	0.7			E100	0.97 ± 0.04 ^{a,†‡}	1.24 × 10 ⁶ ± 1.21 × 10 ⁵			E100
S	0.58			S100	0.68 ± 0.10 [#]		4.18 × 10 ⁶ ± 2.09 × 10 ⁶		S100
C	0.80			C100	1.20 ± 0.33 ^{a,†‡}			2.78 × 10 ⁷ ± 5.51 × 10 ⁶	C100
E + S	0.90	7.76 × 10 ⁵		E12 + S88	1.02 ± 0.11 ^{a,†,‡}	1.11 × 10 ⁶ ± 1.15 × 10 ⁵	2.30 × 10 ⁵ ± 1.15 × 10 ⁵		E77 + S23
E + C	0.92		3.24 × 10 ⁷	E ³ + C100	0.79 ± 0.07 [‡]	^a	^b		E ³ + C100
S + C	0.88	^a	1.74 × 10 ⁷	S ³ + C100	0.80 ± 0.12 [‡]		^b		S ³ + C100
E + S + C	0.96	5.95 × 10 ⁵	8.77 × 10 ⁶	E6 + S ³ + C94	1.04 ± 0.11 ^{†,‡}	2.02 × 10 ⁵ ± 1.70 × 10 ⁴	^b	2.56 × 10 ⁷ ± 4.05 × 10 ⁶	E1 + S ^b + C99
E (P)	0.12 ± 0.01	9.8 × 10 ⁵		E100	0.80 ± 0.05	9.29 × 10 ⁵			E100
E + S (P)	0.1 ± 0.0	5.91 × 10 ⁵		E9 + S91	0.72 ± 0.03	1.03 × 10 ⁶	^b		E100 + S ^b
E + C (P)	0.05 ± 0.01	6.29 × 10 ⁶	5.96 × 10 ⁷	E ³ + C100	0.49 ± 0.13	9.82 × 10 ⁵	^b	^b	E100 + C ^b

^a Cell concentration could not be reliably estimated.

^b Not detected.

[†] Significantly higher DW than in other cultures with same amount of strains (P < 0.05).

[‡] Significantly higher DW than in other mixed cultures (P < 0.05).

[#] Significantly higher DW in mixed than in corresponding monocultures (P < 0.05).

^{‡‡} Significantly lower DW in mixed than in corresponding monocultures (P < 0.05).

measured at the end of the experiment.

2.3. Growth determination and biomass harvesting

Samples were taken thrice a week from all small and pilot scale cultures and examined microscopically to check survival and condition of algal strains and to determine biomass production (dry weight, DW). Determination of the abundance of algal strains was based on microscopical cell density counting (cells mL⁻¹) (Leica, DM 1000, Germany; total magnification ×200) using Lund chambers [27]. The limit for reliable counts was at least one hundred detected cells. DW was determined as described by Tredici and Zittelli [28], by filtering samples on pre-combusted (450 °C, 4 h) filters (GF/C, Ø 47 mm, Whatman) and drying the filters (120 °C, over night) before weighing.

In both experimental scales samples for FA analysis and for analysis of bacterial cell concentrations were taken from stationary phase cultures. In the small-scale cultures biomass for lipid extraction and analyses was collected after two weeks cultivation and in pilot scale on days 8, 10 and 13 for lipid extraction and on days 10 and 13 for qPCR analysis of bacterial cell concentrations by centrifugation at 2500 rpm, 15 min (Multifuge 1 S-R, Kendro Laboratory Products). From the pilot scale cultures bulk biomass for lipid extraction and qPCR analysis was collected on day 15 using a flow-through centrifuge (800 rpm, flow rate 750 L h⁻¹; Evodos type 10, Evodos Algae Technologies B.V.). Algal pellets were stored at -70 °C and lyophilized before analysis. Bacterial cell concentrations were analyzed from selected time points for evaluation of contamination tolerance in monocultures versus mixed cultures. The aim was also to confirm that bacteria did not dominate in any of the cultures and thus, did not have remarkable influence on biomass yield or FA profile.

2.4. Analytical methods

To study the nutrient (PO₄³⁻, TN, NH₄⁺, NO₃⁻) and COD reduction in pilot scale cultures, the filtrates from the three replicate filtrations for the DW determinations were combined, and analyzed using Hach Lange kits (Hach Lange, Germany), a DR 2800™ spectrophotometer (Hach Lange) and a HT 200S high temperature thermostat heating block (Hach Lange).

Three replicate DNA extractions for analysis of bacterial cell number were carried out from the biomass grown in the pilot scale PBRs. Cell concentrations were analyzed using qPCR determination of 16S rRNA gene copy numbers as previously described [26]. General primers for the 16S rRNA gene for bacteria pE and pF' (Oligomer Oy, Finland) [29,30], were used for qPCR. The PCR mixture was prepared as described by Yu et al. [31].

Three replicates from the small scale cultures and two replicate samples from each of the pilot scale cultures were used for lipid extractions and FA analysis. First, lipolytic enzymes were inactivated by adding isopropanol (1 mL) to the tube containing lyophilized biomass (ca. 10 mg), samples were centrifuged (2500 rpm, 1 min), and the isopropanol extract was transferred to a fresh test tube. Lipids were then extracted with chloroform:methanol:water as described by Parrish [32] with slight modifications [26]. Extracted lipids were combined with isopropanol extract, evaporated and stored at -20 °C in hexane until methylation. Blanks were included in each set of extractions.

Methylation was carried out as described by Christie and Han [33]. BHT (butylated hydroxytoluene) (20 µL, 1% in MeOH) was added before methylation to prevent FA oxidation. Samples were analyzed with GC-MS and fatty acid methyl esters (FAMES) identified and quantified using the internal standard method described by Tossavainen et al. [26]. Total lipid contents (% DW and mg L⁻¹) were calculated as the sum of identified and quantified FAMES.

2.5. Statistics

Differences in biomass production in the small-scale experiments were tested with the non-parametric Kruskal-Wallis test using the Mann-Whitney *U* test as post hoc test (significance level *P* < 0.05) (SPSS Statistics, version 22, IBM USA). Non-parametric tests were used because of the heterogeneity of variances (Levene statistic, homogeneity of variances test). One-way ANOVA and Tukey-tests were used to test total fatty acid (TFA) content in the small scale cultures (SPSS Statistics, version 22, IBM, USA).

3. Results and discussion

3.1. Survival of strains in different cultivation scales

After the transfer of inoculum from EG-medium into wastewater, the development of the relative abundance of strains in mixed cultures of *E. gracilis* with *Selenastrum* and *E. gracilis* with *C. sorokiniana* was drastically distinct at the two experimental scales despite the inocula being nearly identical with regards to the strain abundances (Table 1). In small scale mixed cultures of *E. gracilis* with *Selenastrum* both strains survived well (Table 1). However, *E. gracilis* and *Selenastrum* almost disappeared from the two-strain mixed cultures when they were cultured with *C. sorokiniana* in EG-medium and after inoculation to composting leachate *Selenastrum* did not survive in the two or three-strain culture (Table 1). Since *Selenastrum* was not able to survive in mixed cultures with *C. sorokiniana* in small scale wastewater grown cultures, the pilot scale experiments were carried out with *E. gracilis* in monoculture and with either *Selenastrum* or *C. sorokiniana* in two-strain mixed cultures. Cultivation of mixed cultures in pilot scale resulted in total dominance of *E. gracilis*. *E. gracilis* completely outcompeted the other strains (Table 1) and *Selenastrum* and *C. sorokiniana* were undetectable after six days.

Based on traditional competition theory [34], increased productivity in mixed cultures can be explained by more efficient resource allocation due to the niche differentiation [21,23]. In our small scale experiments *Selenastrum* was not able to survive with *C. sorokiniana* which indicates similar growth requirements of these related strains and resulted in dominance of the more productive strain. Dominance of *Chlorella vulgaris* over other *Chlorophyceae* and *E. gracilis* in a five strain consortium was earlier reported by Mustafa et al. [17]. Also the excretion of allelopathic chemicals in algal cultures as a response to resource competition is reported [23], but that was not plausible in our cultures, since the dominance of strains was different in small and pilot scale experiments. However, an important finding of the current study was that success and survival of strains in mixed cultures cannot be generalized by testing only one set of conditions. As shown here, despite using the same growth medium at both scales, the differences in growth conditions radically influenced the outcome, i.e. the final strain abundance and community composition. The results highlight how one round of batch cultivation is sufficient for an initially low abundance strain to become dominant when the conditions are favorable for that strain.

It is evident that all such switches in dominance result from variations in growth conditions. In small scale cultivations, pH in all cultures rose to 8.2–9.7. The optimal pH range for *E. gracilis* is acidic (2.5–7) [35], which was a likely reason for the better performance of *E. gracilis* in the pilot scale cultures with maximum pH adjusted to 7. However, the quality of wastewater is often variable and our results indicate that continuous algal biomass production is more robust in mixed cultures than monocultures; in unstable growth environments the strains better adapted to the new set of conditions will take over [15], and as long as a strain survives in the culture even a subordinate strain may rise to dominance if conditions change.

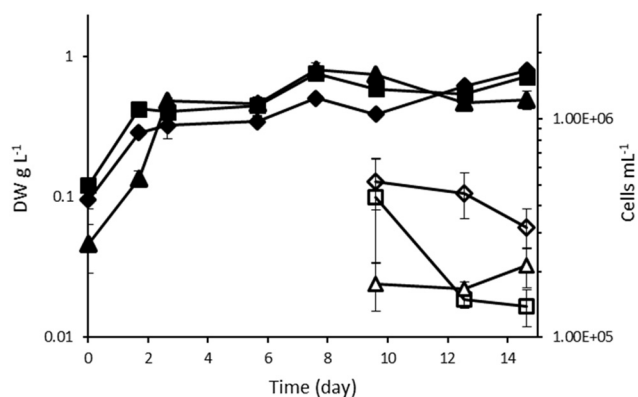


Fig. 1. Development of algal biomass (DW g L^{-1} , mean \pm SE of replicate measurements, $n = 3$) (closed markers) and bacterial density (cells mL^{-1} , mean \pm SE of replicate extractions, $n = 3$) (open markers) in pilot scale experiments with monoculture of *E. gracilis* (\blacklozenge , \diamond), mixed culture of *E. gracilis* with *Selenastrum* sp. (\blacksquare , \square) and mixed culture of *E. gracilis* and *C. sorokiniana* (\blacktriangle , \triangle). (N.B. Some error bars are smaller than the symbol.)

3.2. Growth in small and pilot scale cultures

In both cultivation scales, exponential growth ceased after two to three days. In small scale monocultures, *C. sorokiniana* and *E. gracilis* generated higher biomass yields (1.2 and 0.97 g L^{-1} respectively) than *Selenastrum* ($P < 0.05$) (0.68 g L^{-1}) (Table 1). In mixed cultures of *E. gracilis* with *Selenastrum* and in three-strain cultures the biomass yields were higher (1.02 and 1.04 g L^{-1}) than in other mixed cultures ($P < 0.05$) and comparable to the most productive monocultures (Table 1).

Generally, the biomass yield (g L^{-1}) in all pilot scale cultures was lower than that of the small scale cultures (Table 1). Slightly higher biomass yield was reached when *E. gracilis* was grown in monoculture (0.80 g L^{-1}) than in mixed culture with *Selenastrum* (0.72 g L^{-1}). The yield was lowest in mixed culture of *E. gracilis* with *C. sorokiniana* (0.49 g L^{-1}). When exponential growth ceased in all pilot scale cultures (on day 2 or 3) (Fig. 1), nutrients were still available (see Section 3.3), indicating that growth was probably light rather than nutrient limited.

Evidence for transgressive overyielding in biomass production was not found in this study. This is in accordance with earlier studies of cultures containing only green algae strains [15,22] and in algae communities with strains from diverse taxonomic groups [21]. Also opposite results are reported in mixed cultures consisting of five strains from *Dunaliella* and *Nannochloropsis* genera [19]. In a five strain culture consisting of green algae and the cyanobacteria *Anabaena*, the increased diversity usually resulted in higher average biomass than in monocultures, whereas the biomass production of the monoculture of *Selenastrum* was most efficient [36]. However, here the presence of *C. sorokiniana* in two strain cultures –whether dominating or not – lowered biomass production in both scales. In small scale cultures this can be explained by lower cell concentrations of *C. sorokiniana* in the mixed cultures (Table 1). Instead, in all pilot scale cultures dominated by *E. gracilis*, cell densities of *E. gracilis* were very similar, but there were clear differences in biomass yields (Table 1). Since the cell size of *E. gracilis* in different growth conditions varies [37], this indicates a smaller cell size or distinct cellular constitution in mixed cultures. The reason for a negative impact of *C. sorokiniana* on growth of other strains is unclear. Godwin et al. [22] indicated that the strong attenuation of light in *Selenastrum copricornutum* and *C. sorokiniana* cultures can result in more efficient light limitation in mixed than monocultures. Alternatively, bacteria from the non-axenic *C. sorokiniana* cultures hampered the growth or influenced the cellular structure of the other strains, but no direct evidence for this is available.

On day 10, when the first samples for qPCR analysis were taken

from the pilot scale experiments, bacterial densities (Fig. 1) were clearly lowest in the mixed culture of *E. gracilis* with *C. sorokiniana*. However, during the following days, the bacterial concentrations in the *E. gracilis* monoculture and especially in the mixed culture of *E. gracilis* with *Selenastrum* decreased, while in the mixed culture of *E. gracilis* with *C. sorokiniana* the bacterial density stayed the same. At every time point bacterial cell densities were higher in the monoculture than in the mixed cultures, indicating that bacterial contaminations are easier to control in mixed cultures. Preparation and maintenance of axenic cultures requires completely aseptic growth conditions [26,38], which is not realistic in commercial scale algal cultivation. In fact, naturally existing bacterial communities have their own niche in co-cultures, which can protect algae against invasive contaminations [23]. Also mutualistic or symbiotic interactions in algal-bacterial co-cultures can benefit both of the organisms [23].

E. gracilis is large (length $32\text{--}55 \mu\text{m}$, width $8\text{--}9.6 \mu\text{m}$) [37] in comparison to aquatic bacteria (length $0.6\text{--}3 \mu\text{m}$, width $0.38\text{--}0.63 \mu\text{m}$) [39]. Despite bacterial cell numbers being in the same range as those of the algae, the bacterial biomass never exceeded 10% of total biomass and due to the small cell size of bacteria, differences in proportions in total biomass between cultures were small. However, further investigations are needed before conclusions on tolerance of algal monocultures versus mixed cultures against bacterial contamination can be drawn, or the findings on benefits versus disadvantages of bacteria for the cultures can be generalized.

3.3. Reduction of COD and nutrients

COD and nutrient concentrations in the autoclaved and diluted biowaste leachate used for cultivation in pilot scale experiments were as follows: COD, 865 mg L^{-1} ; TN, 148 mg L^{-1} ; NH_4^+ , 42.8 mg L^{-1} ; NO_3^- , 1.24 mg L^{-1} , and PO_4^{3-} , 2.78 mg L^{-1} . Reduction of COD was the most efficient during the first two days and on day five was over 70% in all pilot scale cultures (Fig. 2). In the *E. gracilis* monoculture, NH_4^+ was removed almost completely within ten days (97.5%) (Fig. 3a), and within eight days in the mixed culture of *E. gracilis* with *Selenastrum* (94.3%) (Fig. 3b). Over 90% removal was not reached until the last sampling day (day 15) in the mixed culture of *E. gracilis* with *C. sorokiniana* (Fig. 3c). TN reduction exceeded 90% in all cultures within five days (Fig. 3a, b, c). The organic form of nitrogen that the TN primarily consists of appears to be readily available for the algae and bacteria in the cultures. The removals of NO_3^- and PO_4^{3-} from initially low concentrations were 22.6–41.9% and 54.3–78.3%, respectively (Fig. 3a, b, c).

The higher removal of NH_4^+ in mixed algal cultures in comparison to monocultures was shown here in the mixed culture of *E. gracilis* with *Selenastrum* and also earlier by Stockenreiter et al. [21], who showed the increasing efficiency of nutrient utilization due to the increase of

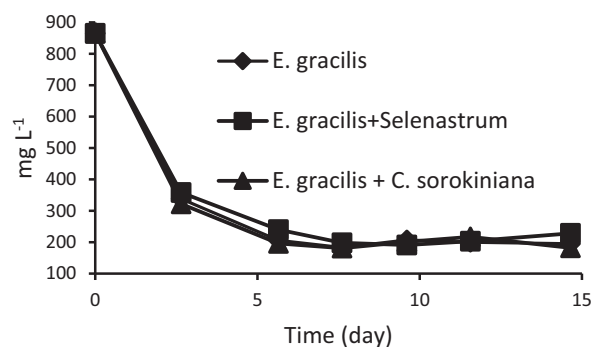


Fig. 2. COD (mg L^{-1}) reduction ($n = 1$) in the pilot scale experiments with the monoculture of *E. gracilis* and the mixed cultures of *E. gracilis* with *Selenastrum* sp. and *E. gracilis* with *C. sorokiniana*.

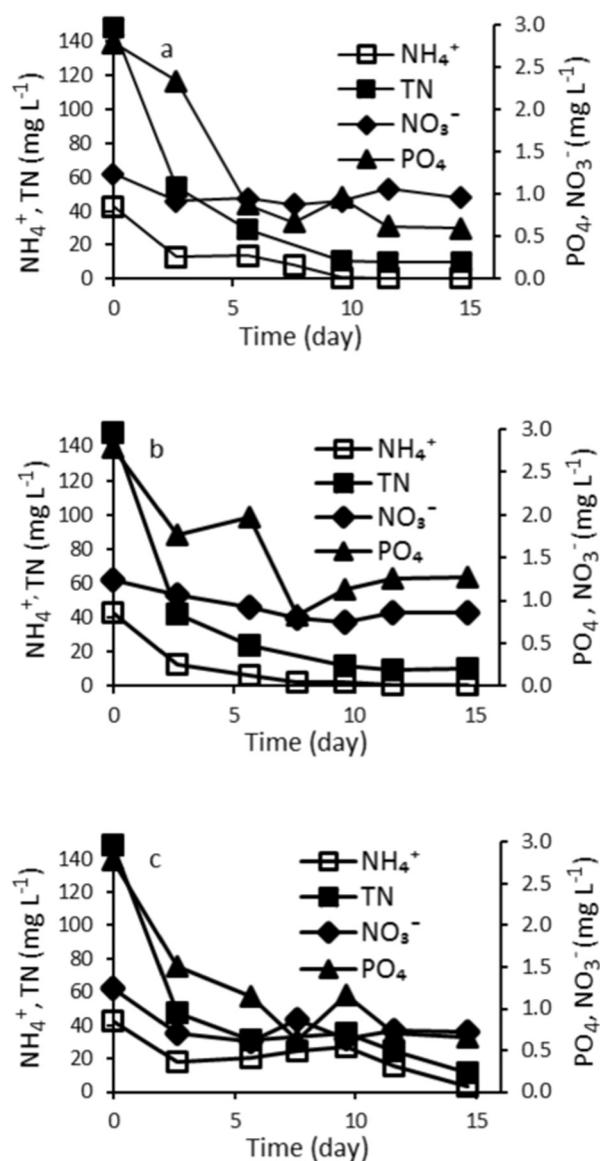


Fig. 3. Nutrient removal (mg L^{-1}) ($n = 1$) in the pilot scale experiments with monoculture of a) *E. gracilis*, and mixed cultures of b) *E. gracilis* with *Selenastrum* and c) *E. gracilis* with *C. sorokiniana*.

species diversity. Also COD removal in algal cultures due to mixotrophic growth has been reported [40], and the strains used in this study have been shown to grow mixotrophically [26,41,42]. However, different bacterial concentrations in our cultures did not have influence on COD removal efficiency, although in the non-axenic algal cultures, both mixotrophic algae and heterotrophic bacteria utilize organic carbon substrates [43]. Additionally, heterotrophic bacteria generate CO_2 for algal growth and participate in nutrient uptake, while algal photosynthesis generates oxygen for use by bacteria [43]. Coexistence may thus enhance nutrient uptake and COD reduction and benefit both groups of organisms. In this study, only 1% of total water volume was biowaste leachate. This type of concentrated wastewater is a good substitute of commercial fertilizers in algal cultivation. However, efficient nutrient reduction encourages using mixed algal cultures also for treatment of more diluted wastewaters.

3.4. Lipid production

In the small scale cultures total lipid content (Table 2) was highest in the monoculture of *Selenastrum* (10.4% DW) ($P = 0.000$). Among the

mixed cultures the highest lipid content was reached when *E. gracilis* was grown with *Selenastrum* ($P = 0.000$) (6.5% DW), which was comparable to the FA content in *E. gracilis* and *C. sorokiniana* monocultures. FA content was always lowest in the mixed cultures when *C. sorokiniana* was present ($P = 0.000$). However, higher biomass yield (see Section 3.2) in monocultures of *C. sorokiniana* than *Selenastrum*, resulted in similar lipid yields (79.9 and 75.1 mg L^{-1}) in these cultures (Table 2). This underlines the fact that both high biomass lipid content and high biomass yield is needed to maximize lipid productivity.

In the pilot scale cultures, the total lipid content and the lipid yield (Tables 2 and 3) were highest in the mixed culture of *E. gracilis* with *Selenastrum* (11.1% DW and 79.9 mg L^{-1} , respectively). In the monoculture of *E. gracilis*, the lipid content and lipid yield were 7.6% DW and 61.1 mg L^{-1} respectively, while, similarly to small scale cultures, they were the lowest in the mixed culture of *E. gracilis* with *C. sorokiniana* (5.2% DW and 25.8 mg L^{-1} , respectively). Already by day 8 culture-specific differences in the lipid contents were clear, and these differences remained until the end of cultivation (Table 3). Lipid accumulation to biomass (% DW) continued in all cultures during the whole recorded period (days 8–15) (Table 3). FA accumulation was especially rapid near the end of cultivation in the mixed culture of *E. gracilis* with *Selenastrum* where the lipid content of the biomass and the yield (mg L^{-1}) doubled during the two last cultivation days (days 13–15). In the mixed culture of *E. gracilis* with *C. sorokiniana*, the lipid yield remained fairly stable during the cultivation as a consequence of lowering biomass growth (see Section 3.2).

Contrary to our hypothesis, we did not find clear evidence for the transgressive overyielding of lipid production in mixed cultures, which is in accordance with findings in an earlier study using mixed cultures of chlorophytes *Scenedesmus* sp. and *Haematococcus pluvialis* [20]. Similar observations were done by Godwin et al. [22], i.e. no positive influence of mixed cultivation for biocrude (raw material for transportation fuels) yields was seen in two or four strain green algae cultures [22]. Also opposite results are reported; increasing species diversity in multispecies mixed cultures clearly enhanced the lipid production in communities of randomly selected strains [21], but this happened only in cultures with at least five strains. Algal diversity can partly explain the differences in results, but also the strain composition has importance; among the four green algae strains, lipid content of *Scenedesmus* in monoculture was usually higher than in four strain mixed culture or other monocultures [15]. However, in pilot scale cultures, lipid accumulation in the mixed culture of *E. gracilis* with *Selenastrum* was more efficient than in monoculture of *E. gracilis*, indicating that the presence of the minor strain may influence the lipid content of the major strain. Alternatively, this could be a response to more efficient N consumption (see Section 3.3) in mixed culture of *E. gracilis* with *Selenastrum* relative to other pilot scale cultures. Enhanced lipid production of *E. gracilis* [44] and other microalgae species under N limitation has been shown in several studies [45]. In agreement with this, we found that the slow removal of NH_4^+ in the mixed culture of *E. gracilis* with *C. sorokiniana* resulted in a lower cellular lipid content. In future studies, it will be important to clarify when overyielding takes place and whether this is a consequence of species richness or simply a result of nutrient deficiency as a response to more efficient resource allocation.

3.5. FA profiles

In small scale monocultures of *Selenastrum* over 40% of total FAs (TFAs) consisted of oleic acid (C18:1n9c) (Table 2). The main FAs in all other cultures at both cultivation scales (Table 2) were palmitic acid (C16:0) and α -linolenic acid (ALA, C18:3n3). In two strain mixed cultures dominated by *C. sorokiniana* half of the FAs were ALA and the content of linoleic acid (LA 18:2n6) was almost 20% of the total FAs. In comparison, corresponding values for ALA and LA in *C. sorokiniana* monocultures were lower, 33.1 and 19.4%. High content of ALA and LA

Table 2

Proportion of FAMES (% of TFAs), total lipid content in biomass (TFAs % DW), total lipid yields (TFAs mg L⁻¹) and yields of 18:3n3 (ALA), 20:5n3 (EPA) and 22:6n3 (DHA) at the end of the cultivation in the small (mean ± SD of experimental replicates, n = 3) and pilot scale (PBR) (mean ± SD of replicate extractions, n = 2). FAs with a proportion < 1% in TFAs in all cultures are not shown. E = *E. gracilis*, S = *Selenastrum*, C = *C. sorokiniana*. < 1% = under 1% in TFAs, nd = not detected. Statistically significant differences in TFA contents in small scale cultures are shown.

FAME	E	S	C	E + S	E + C	S + C	E + S + C	E (PBR)	E + S(PBR)	E + C (PBR)
C13:0	3.0 ± 0.5	nd	nd	3.9 ± 0.7	nd	nd	1.1 ± 0.2	2.4 ± 0.4	1.8 ± 0.0	1.1 ± 0.0
C14:0	8.5 ± 0.7	< 1	< 1	9.2 ± 0.6	nd	nd	2.8 ± 0.4	7.3 ± 0.5	6.8 ± 0.1	6.4 ± 1.1
C15:0	3.7 ± 0.5	< 1	< 1	3.3 ± 0.5	< 1	< 1	1.6 ± 0.1	2.2 ± 0.0	2.1 ± 0.1	1.8 ± 0.2
C16:0	20.5 ± 2.5	35.2 ± 3.3	33.6 ± 0.5	23.5 ± 2.0	24.3 ± 0.2	24.9 ± 1.4	25.7 ± 0.2	18.6 ± 0.1	18.3 ± 0.5	15.4 ± 1.2
C16:1	2.9 ± 0.2	< 1	4.1 ± 0.9	2.5 ± 0.3	< 1	< 1	1.2 ± 0.2	2.7 ± 0.0	2.7 ± 0.0	2.8 ± 0.1
C17:0	< 1	< 1	< 1	< 1	1.2 ± 0.2	1.0 ± 0.2	< 1	1.1 ± 0.0	1.0 ± 0.2	1.9 ± 0.4
C18:0	< 1	3.3 ± 0.4	2.0 ± 0.2	< 1	< 1	< 1	< 1	2.3 ± 0.0	2.0 ± 0.3	3.8 ± 0.6
C18:1n9c	2.8 ± 0.2	40.6 ± 3.8	5.8 ± 0.3	4.6 ± 0.8	3.1 ± 0.3	3.0 ± 0.1	4.8 ± 0.5	3.5 ± 0.1	3.3 ± 0.2	3.9 ± 0.5
C18:2n6c	7.8 ± 0.4	2.9 ± 0.4	33.1 ± 2.4	5.9 ± 0.5	19.6 ± 0.6	20.3 ± 1.2	19.7 ± 1.0	11.1 ± 0.2	10.6 ± 0.3	6.2 ± 0.0
C18:3n3	24.8 ± 1.1	12.6 ± 1.4	19.4 ± 0.9	22.5 ± 0.5	50.1 ± 0.4	49.2 ± 0.6	35.2 ± 1.5	25.4 ± 0.6	28.6 ± 0.9	30.2 ± 1.8
C 20:0	nd	< 1	< 1	nd	nd	nd	< 1	1.6 ± 0.0	1.3 ± 0.2	2.7 ± 0.6
C20:2	3.0 ± 0.1	nd	nd	3.0 ± 0.0	nd	nd	< 1	2.7 ± 0.0	2.8 ± 0.1	3.0 ± 0.3
C 20:3n6	< 1	nd	nd	< 1	nd	nd	< 1	1.6 ± 0.0	1.4 ± 0.2	2.6 ± 0.5
C20:4n6 ARA	5.7 ± 0.3	nd	nd	4.9 ± 0.1	nd	nd	1.4 ± 0.2	4.4 ± 0.0	5.0 ± 0.1	4.2 ± 0.2
C20:3n3	2.3 ± 0.1	nd	nd	2.0 ± 0.2	nd	nd	< 1	2.3 ± 0.0	2.2 ± 0.2	2.6 ± 0.4
C20:5n3 EPA	9.4 ± 0.5	nd	nd	8.9 ± 0.3	nd	nd	2.5 ± 0.3	5.7 ± 0.2	5.9 ± 0.1	5.5 ± 0.2
C22:6n3 DHA	3.4 ± 0.3	nd	nd	3.1 ± 0.1	nd	nd	< 1	4.4 ± 0.2	3.8 ± 0.1	5.2 ± 0.2
C24:0	nd	1.3 ± 0.2	< 1	< 1	nd	nd	nd	nd	nd	nd
TFAs % DW	6.6 ± 0.2	10.4 ± 1.3 ^{*,#}	5.6 ± 0.4	6.5 ± 0.5 ^{*,#S}	3.4 ± 0.2 ^{*,E,C}	3.3 ± 0.1 ^{*,S,C}	4.0 ± 0.3 ^{*,E,S,C}	7.6 ± 0.0	11.1 ± 2.1	5.2 ± 0.7
TFAs (mg L ⁻¹)	64.7 ± 5.2	75.1 ± 6.2	79.9 ± 10.9	66.9 ± 15.1	27.0 ± 3.7	28.3 ± 0.9	40.1 ± 7.6	61.1 ± 0.1	79.9 ± 15.1	25.8 ± 3.2
C18:3n3 (mg L ⁻¹)	16.0 ± 1.1	9.5 ± 1.8	15.4 ± 1.4	15.1 ± 3.7	13.5 ± 1.8	13.9 ± 0.6	14.1 ± 2.2	15.5 ± 0.3	22.9 ± 5.0	7.8 ± 0.4
C20:5n3 (mg L ⁻¹)	6.1 ± 0.8	nd	nd	6.0 ± 1.4	nd	nd	1.0 ± 0.3	3.5 ± 0.1	4.7 ± 0.8	1.4 ± 0.1
C22:6n3 (mg L ⁻¹)	2.2 ± 0.3	nd	nd	2.1 ± 0.5	nd	nd	0.4 ± 0.1	2.7 ± 0.1	3.1 ± 0.4	1.3 ± 0.0

* Significantly higher TFA content than in other monocultures (P = 0.000).

+ Significantly higher TFA content than in other mixed cultures (P = 0.000).

Significantly lower TFA content in mixed than in corresponding monocultures (E, S or C) (P < 0.05).

Table 3

Yields of C18:3n3 (ALA), 20:5 n3 (EPA) and 22:6n3 (DHA) and TFAs (mg L⁻¹) and the percentage of SAFAs, MUFAs, PUFAs and LC-PUFAs (% of TFAs) and TFA content (% DW) in biomass from the day 8 to 15 during the pilot scale cultivation. Results are means ± SD of the replicate extractions, n = 2.

		Day 8	Day 10	Day 13	Day 15
<i>E. gracilis</i>					
Yield (mg L ⁻¹)	C18:3n3	4.9 ± 0.8	4.3 ± 1.8	6.5 ± 0.5	15.5 ± 0.3
	C20:5n3	1.1 ± 0.1	1.0 ± 0.3	1.6 ± 0.1	3.5 ± 0.1
	C22:6n3	1.0 ± 0.0	1.0 ± 0.2	1.5 ± 0.1	2.7 ± 0.1
% of TFAs	TFAs	21.9 ± 1.5	19.0 ± 4.6	31.8 ± 1.6	61.1 ± 0.1
	SAFA	36 ± 0.8	38.7 ± 1.9	40.4 ± 0.7	36.2 ± 1.3
	MUFA	11.3 ± 0.5	8.1 ± 0.9	7.9 ± 0.1	6.2 ± 0.1
	PUFA	27.8 ± 2.3	28.4 ± 0.5	29.4 ± 0.8	36.5 ± 0.8
	LC-PUFA	24.9 ± 1.1	24.8 ± 1.6	22.2 ± 0.0	21.1 ± 0.4
% of DW	TFAs	3.8 ± 0.4	4.1 ± 0.9	4.4 ± 0.1	7.6 ± 0.0
<i>E. gracilis</i> & <i>Selenastrum</i>					
Yield (mg L ⁻¹)	C18:3n3	8.6 ± 0.7	8.0 ± 1.2	7.9 ± 0.6	22.9 ± 5.0
	C20:5n3	1.9 ± 0.1	1.7 ± 0.1	1.7 ± 0.0	4.7 ± 0.8
	C22:6n3	1.6 ± 0.0	1.4 ± 0.0	1.3 ± 0.0	3.1 ± 0.4
% of TFAs	TFAs	39.7 ± 0.9	33.4 ± 1.3	31.4 ± 0.3	79.9 ± 15.1
	SAFA	41.8 ± 0.7	40.7 ± 1.0	36.8 ± 1.2	33.7 ± 0.1
	MUFA	7.9 ± 0.2	7.3 ± 0.6	7.1 ± 0.4	5.9 ± 0.2
	PUFA	26.3 ± 1.5	29.2 ± 2.8	33.8 ± 2.0	39.1 ± 1.1
	LC-PUFA	24 ± 0.6	22.8 ± 1.1	22.2 ± 0.3	21.2 ± 0.9
% of DW	TFAs	4.9 ± 0.4	6.1 ± 1.0	6.4 ± 0.8	11.1 ± 2.2
<i>E. gracilis</i> & <i>C. sorokiniana</i>					
Yield (mg L ⁻¹)	C18:3n3	2.8 ± 0.2	3.7 ± 0.0	4.8 ± 0.0	7.8 ± 1.4
	C20:5n3	1.3 ± 0.1	1.2 ± 0.0	1.1 ± 0.1	1.4 ± 0.1
	C22:6n3	1.6 ± 0.2	1.5 ± 0.1	1.1 ± 0.1	1.3 ± 0.0
% of TFAs	TFAs	26.4 ± 2.3	22.2 ± 1.4	20.8 ± 1.5	25.8 ± 3.2
	SAFA	51.5 ± 1.1	44.4 ± 0.1	39.7 ± 0.5	33.8 ± 0.8
	MUFA	10.2 ± 0.4	10.8 ± 3.0	8.5 ± 0.1	6.7 ± 0.5
	PUFA	15.3 ± 1.4	22.1 ± 1.3	28.1 ± 0.2	36.3 ± 1.8
	LC-PUFA	23.0 ± 0.8	22.7 ± 1.8	23.7 ± 0.2	23.2 ± 2.1
% of DW	TFAs	2.7 ± 0.0	3.2 ± 0.3	4.1 ± 0.2	5.2 ± 0.7

are typical of *Chlorella* strains [46] and our results indicate that mixed cultivation can enhance production of essential PUFAs in *C. sorokiniana*. However, also growth phase and culture age influence proportions of algal FAs as earlier shown for *Selenastrum* [26] and *E. gracilis* [13]. In our small scale cultures, samples for FA analysis were taken only once from the late stationary phase, and the influence of cultivation phase or culture age on FA profile could be evaluated only from the stationary phase pilot scale cultures (see below). EPA content in the small scale monoculture of *E. gracilis* and in the mixed culture of *E. gracilis* with *Selenastrum* was high (9.4 and 8.9%) and the DHA content was over 3% of total lipids in both cultures. FA profiles reflected strain composition in cultures; the relative amount of *E. gracilis* specific LC-PUFAs was lower in small scale, three strain mixed cultures than in other small scale cultures or in pilot scale cultures with *E. gracilis*, which can be explained by lower cell concentrations of *E. gracilis*.

Generally, in all pilot scale cultures, the proportions (% of TFAs) of PUFAs increased, LC-PUFAs stayed stable or slightly decreased, and saturated fatty acids (SAFAs) and monounsaturated fatty acids (MUFAs) decreased toward the end of the cultivation in ageing stationary phase cultures (Table 3). However, the efficient biomass growth in monoculture of *E. gracilis* and in mixed culture of *E. gracilis* with *Selenastrum* increased also the yield of EPA and DHA (Table 3). Constancy of LC-PUFAs in biomass during the stationary phase indicates, that those were mainly located in membrane lipids, which is typical of microalgae [45]. Instead, SAFAs and MUFAs are generally high in triacylglycerols (TAGs) commonly acting as storage lipids in algae [45,47]. Contrary to most of the microalgae, typical storage product of *E. gracilis* is paramylon, which is converted to wax esters in anoxic or hypoxic conditions [48]. Additionally, the N limitation in organic carbon rich growth medium increases TAGs, but also the wax ester synthesis of *E. gracilis*. Increase of ALA as a main PUFA, can indicate the increase of galactolipids, which have been shown earlier in *E. gracilis* cultures under C and N deficiency [44]. Lipid class composition has importance when evaluating algal lipids for nutritional use. Since the TAGs are most desirable for nutritional oils, further investigation of lipid classes produced by *E. gracilis* in different growth conditions is needed. Alternatively, adjusting growth conditions favorable to co-existence of green algae and *E. gracilis* in mixed cultures could be a good option for optimal oil constitution.

3.6. Criteria for selection of strains

For consistent production of desired products, control of strain abundance and performance is a key issue. As shown here, the existence of strains in mixed cultures is sensitive to growth conditions. Since microalgae were here grown in batch cultures and the duration of experiments was only two weeks, changes in community composition or diversity effects for overyielding are only indicative, and cannot be generalized to continuous long term cultures at commercial scale. Additionally, in continuous and semi-continuous cultures, the influence of changing conditions due to the regular harvesting and growth substrate addition would be difficult to separate from strain diversity effects. However, for consistent production of target products, the growth of the appropriate strains should be ensured. When wastewater is used as a growth medium, the presumed variability in composition of cultivation medium makes the succession and interplay of strains especially unpredictable [15] and the metabolism of strains is difficult to control. However, in the case of the chlorophytes and euglenoids, differences in optimal growth pH can be used to regulate strain performance; the presence of LC-PUFA producing *E. gracilis* in mixed cultures can be ensured by keeping pH low enough to prevent dominance of chlorophytes. As discussed also by Stockenreiter et al. [21], one of the most important issues is to pinpoint the factors determining relevant strain performance. For that purpose, the selection of strains with some clear differences in growth requirements could be a good option. Additionally, careful characterization of growth substrate, biomass and extracts is needed for acceptance of wastewater grown microalgae in

nutritional products. Alternatively, biomass or extracts can be utilized in feed or cosmetics. Biowaste leachate is comparable to compost that traditionally is used as an agricultural fertilizer. Thus, algal biomass produced on biowaste leachate may be more an “image” rather than a safety problem.

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Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent, human or animal rights

No conflicts, informed consent, human or animal rights applicable.

Declaration of authors agreement

The authors agree to authorship and submission of the manuscript for peer review.

Author contributions

All the authors contributed to conception and design of the study and drafting the article. Anne Ojala and Martin Romantschuk critically revised the manuscript for important intellectual content. Data were collected by Marika Tossavainen, Neha Katyal Chopra, Kalle Valkonen and Suvigya Sharma and analyzed and interpreted by Marika Tossavainen and Neha Katyal Chopra. Marika Tossavainen actualized statistics. Martin Romantschuk obtained funding for the study. All the authors gave their final approval for the submitted article. Marika Tossavainen takes responsibility for the integrity of the work as a whole.

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