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Growth of *Chlorella vulgaris* and associated bacteria in photobioreactors

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Summary

The aim of this study was to test three flat plate photobioreactor configurations for growth of *Chlorella vulgaris* under non-axenic conditions and to characterize and quantify associated bacterial communities. The photobioreactor cultivations were conducted using tap water-based media to introduce background bacterial population. Growth of algae was monitored over time with three independent methods. Additionally, the quantity and quality of eukaryotes and bacteria were analysed using culture-independent molecular tools based on denaturing gradient gel electrophoresis (PCR-DGGE) and quantitative polymerase chain reaction (QPCR). Static mixers used in the flat plate photobioreactors did not generally enhance the growth at the low light intensities used. The maximum biomass concentration and maximum specific growth rate were 1.0 g l^{-1} and 2.0 day^{-1} respectively. Bacterial growth as determined by QPCR was associated with the growth of *C. vulgaris*. Based on PCR-DGGE, bacteria in the cultures mainly originated from the tap water. Bacterial community profiles were diverse but reproducible in all flat plate cultures. Most prominent bacteria in the *C. vulgaris* cultures belonged to the class Alphaproteobacteria and especially to the genus *Sphingomonas*. Analysis of the diversity of non-photosynthetic microorganisms in algal mass cultures can provide useful information on the public health aspects and unravel community interactions.

Introduction

Microalgae are a large and heterogeneous group of microorganisms carrying out oxygenic photosynthesis and converting CO_2 to biomass as a potential source of biofuels (Chisti, 2007; Wiley *et al*., 2011). Some algae produce large amounts of cellular lipids and their photosynthetic efficiencies are higher than those of traditional agricultural crops (Spolaore *et al*., 2006; Li *et al*., 2008). Microalgae have traditionally been produced for human nutrition and animal feed supplements (Chisti, 2006). Utilization of microalgal biomass in production of cosmetics, polyunsaturated fatty acids, pigments and other bioactive compounds as well as renewable biofuels has gained interest in recent years (Chisti, 2006; Spolaore *et al*., 2006; Li *et al*., 2008).

Current microalgal mass culture systems include batch-operated shallow open ponds (Belay, 1997), tubular photobioreactors (Carlozzi, 2000; Hai *et al*., 2000; Molina *et al*., 2001), flat plate photobioreactors (Degen *et al*., 2001), column photobioreactors (Zittelli *et al*., 2006; Hulatt and Thomas, 2010) and stirred tank reactors (Li *et al*., 2003; Eriksen *et al*., 2007). A major limitation of microalgal growth is the availability of light (Ogbonna and Tanaka, 2000; Eriksen, 2008). Ideally, light with saturation intensity characteristic for a given microalgal species should be distributed homogenously to the entire volume of the cultivation unit. In practice, this is impossible because of light absorption by microalgal pigments and light scattering due to cells and other particles in the culture solution (Ogbonna and Tanaka, 2000; Pottier *et al*., 2005). Thus, in practical cultivation systems with strong external illumination, light inhibition, light saturation, light limitation and dark zones coexist simultaneously in different parts of the culture vessel (Ogbonna and Tanaka, 2000). Fluctuations in the order of milliseconds between illuminated and dark zones have been shown to reduce light inhibition and increase biomass productivity (Ogbonna and Tanaka, 2000; Janssen *et al*., 2001). These fluctuations have been enhanced by swirling the culture with bends in the reactor design (Carlozzi, 2000) or by inserting static mixers inside the cultivation vessels (Degen *et al*., 2001; Muller-Feuga *et al*., 2003).

Many studies on microalgal cultivation in photobioreactors have used sterile culture media and aseptic culture conditions (Hai *et al*., 2000; Degen *et al*., 2001). However, sterilization of culture media and photobioreactors in large-scale biomass production for low-value products, such as biofuel or bioenergy, is neither economically nor practically feasible. Heterotrophic organisms in algal

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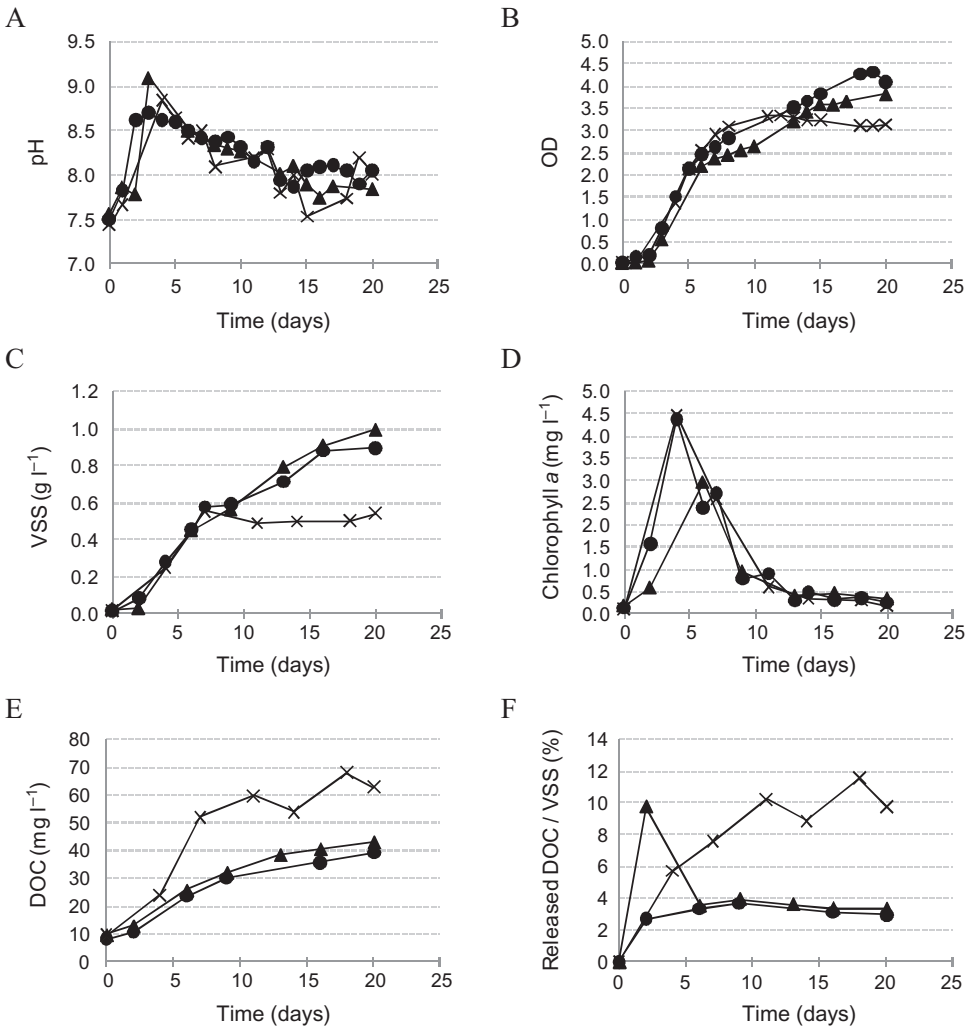

Fig. 1. Time courses of culture pH (A), OD (B), VSS (C), chlorophyll *a* (D), dissolved organic carbon (E) and released DOC as percentage of VSS in: ●, the flat plate with no mixer, ▲, the flat plate with the plain mixer and ×, the flat plate with the complex mixer.

cultures are generally considered as contaminants and harmful for algal growth in microalgal biomass production systems (Belay, 1997; Huntley and Redalje, 2007), although some reports suggest that certain bacteria and fungi may have positive effects on microalgal growth (Park *et al*., 2005; Watanabe *et al*., 2005). Algal–bacterial interactions have been widely studied in natural waters and artificial mesocosms (Cole, 1982; Riemann *et al*., 2000; Rooney-Varga *et al*., 2005). The occurrence of bacteria in algal mass cultures is generally recognized (Scragg *et al*., 2002; Hulatt and Thomas, 2010), but their enrichment and diversity have yet to be characterized.

The aim of this study was to test different flat plate photobioreactor configurations for biomass production of *Chlorella vulgaris* in non-axenic growth conditions and to characterize and quantify associated bacterial communities. The photobioreactor cultivations were conducted using tap water-based media in order to introduce background bacterial population. Growth of algae was monitored over time with three independent methods. Additionally, the quantity and quality of eukaryotes and bacteria in the non-axenic algal cultures were analysed using culture-independent molecular tools based on denaturing gradient gel electrophoresis (PCR-DGGE) and quantitative polymerase chain reaction (QPCR).

Results

Growth of C. vulgaris *in photobioreactors*

Three different flat plate photobioreactor configurations, a flat plate reactor with no mixer (NM), a flat plate reactor with a plain mixer (PM) and a flat plate reactor with a complex mixer (CM), were used to grow *C. vulgaris*. The results were as presented in Figs 1 and 2. The temperature varied between 23.6°C and 26.8°C and the dissolved O_2 (DO) between 7.6 and 8.8 mg l^{-1} (94–108% air

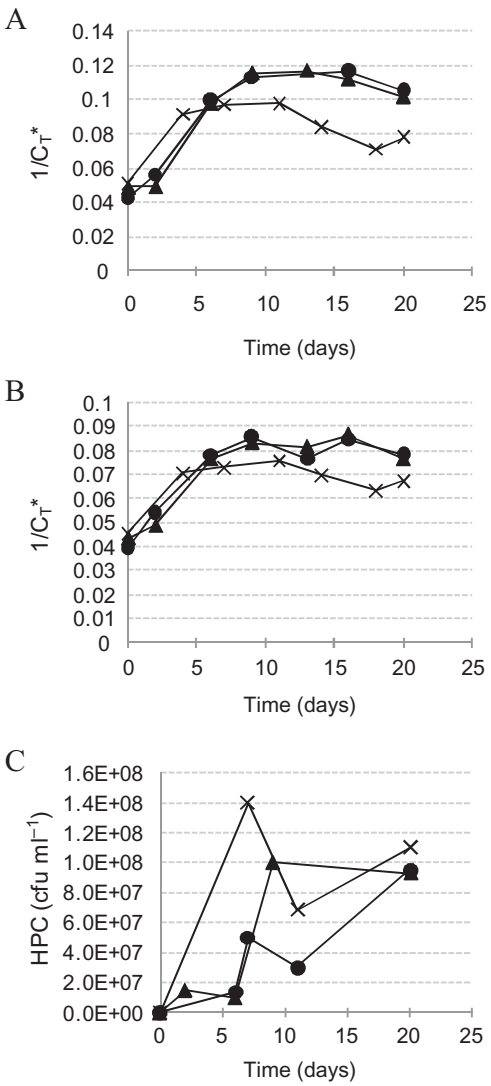

Fig. 2. Results from eukaryotic QPCR (A), bacterial QPCR (B) and HPC (C) in: ●, the flat plate with no mixer, ▲, the flat plate with the plain mixer and ×, the flat plate with the complex mixer. The asterisk in the $1/C_T$ value indicates that the QPCR values have been normalized to initial sample volume of 15 ml for DNA extraction.

saturation). The pH increased at the beginning from 7.5 to 8.6–9.1, started to decrease on days 4–5 and approached the initial pH by the end of all flat plate cultivations (Fig. 1A). The daily pH fluctuation was somewhat higher in the CM than in the other two reactor configurations.

Growth of *C. vulgaris* was monitored as changes in optical density (OD), volatile suspended solids (VSS), chlorophyll *a* and $1/C_T$ from QPCR with the eukaryotic primers. OD was measured at 600 and 680 nm, but as the results from the two wavelengths had a Pearson correlation coefficient of 1.00, only OD_{680} results are shown in Fig. 1B. *Chlorella vulgaris* grew similarly in the NM and in PM photobioreactors based on all growth indicators (Figs 1 and 2A). However, in the CM *C. vulgaris* only grew to 0.5 g VSS l^{-1} compared with 0.9 and 1.0 g VSS l^{-1} in the NM and PM respectively. The highest growth rates were achieved in all reactor configurations between days 0 and 4. Based on the OD measurements, the maximum specific growth rates in the NM, PM and CM photobioreactors were 2.0, 1.9 and 1.1 day^{-1} respectively.

The average Pearson correlation coefficient (r) of OD and VSS from all the reactor configurations was 0.98. Eukaryotic $1/C_T$ correlated moderately with OD (r 0.90) and VSS (r 0.85), while chlorophyll *a* correlated poorly with both OD (r 0.30) and VSS (r 0.40). In all photobioreactors, chlorophyll *a* concentrations increased during the first 5–7 days, followed by a decrease to near zero (Fig. 1D). However, this was not seen with the other growth parameters (Figs 1 and 2A).

Dissolved organic carbon (DOC) was released during the cultivation of *C. vulgaris* (Fig. 1E and F). The initial DOC concentration ranged between 8.3 and 9.9 mg l^{-1} and the highest DOC between 39 and 68 mg l^{-1}. The released DOC concentration stabilized to below 4% of the VSS in the NM and PM after day 7, while the portion of DOC of the VSS varied between 8.9% and 12% in the CM at the same time. Thus, the concentration of DOC and its relative proportion of VSS were higher in the CM than in the other two reactor configurations (Fig. 1E and F). DOC correlated with VSS in all photobioreactor cultivations, with r-values of 0.99, 0.99 and 0.94 in the NM, PM and CM respectively. DOC correlated well also with eukaryotic $1/C_T$ in the NM ($r = 0.93$) and in the PM ($r = 0.91$), but not in the CM ($r = 0.43$).

Growth of bacteria in the photobioreactors

Both heterotrophic plate counts (HPC) and $1/C_T$ from QPCR with bacterial primers showed enrichment of bacteria to the photobioreactors (Fig. 2B and C). Bacterial $1/C_T$ had an overall Pearson correlation coefficient of 0.98 with eukaryotic $1/C_T$ in all photobioreactor cultivations, showing that the bacterial numbers increased together with the algal numbers. The HPC results and bacterial $1/C_T$ values did not correlate well (overall $r = 0.54$). Total number of bacteria was slightly higher in the NM and the PM than in the CM based on QPCR. However, the HPC results were highest in the CM.

Microbial community profiles

Eukaryotic community profiles were identical in all cultivations in the three photobioreactor configurations (Fig. S1). PCR amplification with the eukaryotic primers yielded a single band on gel electrophoresis. This was resolved to two distinct bands on DGGE. The two DGGE bands were present in all flat plate photobioreactor cultures through-

out the incubation. Both bands had 100% similarity against each other and 100% similarity to *C. vulgaris* (accession number FM205885). Other much fainter bands were discernible in the eukaryotic DGGE profiles, but DNA from these sequences could not be retrieved for sequencing. Thus, *C. vulgaris* was the dominant and likely the only eukaryote present in the flat plate cultures.

Sequence analysis showed that chloroplast DNA from *C. vulgaris* was also amplified with the bacterial PCR primers used (Table 1). Community profiles obtained with

Table 1. Selected, PCR-DGGE-based 16S rDNA identities and affiliations of samples from the flat plate cultures.

Band label (acc)[a]	SL[b]	Sim (%)[c]	Affiliation (acc)[d]	Class/family	Origin of the sample with the closest match
A_1 (JF508903)	470	100	*Chlorella vulgaris*, chloroplast DNA (AB001684)	Trebouxiophyceae/ *Chlorellaceae*	*Chlorella vulgaris* C-27 grown in M-4NA medium
A_2 (JF508904)	454	99.1			
A_3 (JF508905)	450	98.0			
A_4[e]	320	76.6			
A_5 (JF508906)	481	99.8			
A_6 (JF508907)	389	80.7			
A_7[e]	413	77.0			
A_8 (JF508908)	449	99.3			
A_9 (JF508909)	371	82.2			
B (JF508910)	473	83.1	Uncultured bacterium (AY957921)	Unknown/unknown	Model potable water biofilm
C (JF508911)	354	84.7	*Brevundimonas* sp. (GQ454843)	Alphaproteobacteria/ *Caulobacteraceae*	Victoria Upper Glacier
D (JF508912)	445	94.8	Uncultured bacterium (AF314434)	Unknown/unknown	Sequencing batch reactor fed with synthetic wastewater
E (JF508913)	457	99.8	*Sphingomonas* sp. (GU596958)/ *Blastomonas* sp. (AB242676)	Alphaproteobacteria/ *Sphingomonadaceae*	Crude oil degrading consortium of cyanobacteria and bacteria/Tomato leaves
F (JF508914)	438	98.2	*Porphyrobacter* sp. (EU770257)	Alphaproteobacteria/ *Erythrobacteraceae*	Bacteria associated with *Microcystis* colonies
G (JF508915)	432	90.3	*Sphingobium* sp. (EU375371)	Alphaproteobacteria/ *Sphingomonadaceae*	Ultraoligotrophic alpine lake Puma Yumco
H_1 (JF508916)	436	98.6	*Sphingomonas xenophaga* (AM942552)	Alphaproteobacteria/ *Sphingomonadaceae*	Contaminated groundwater
H_2 (JF508917)	418	94.0			
H_3 (JF508918)	442	99.3			
H_4 (JF508919)	436	99.5			
I (JF508920)	469	87.8	Uncultured bacterium (HM326129)	Unknown/unknown	Environmental sample
J (JF508921)	457	99.7	*Sphingomonas* sp. (GU596958)	Alphaproteobacteria/ *Sphingomonadaceae*	Crude oil degrading consortium of cyanobacteria and bacteria
K (JF508922)	447	98.4	*Sphingomonas* sp. (GU074285)	Alphaproteobacteria/ *Sphingomonadaceae*	Water habitat
L (JF508923)	444	93.7	*Sphingobium* sp. (HM208431)	Alphaproteobacteria/ *Sphingomonadaceae*	Environmental sample
M (JF508924)	440	95.2	Uncultured bacterium (AB205842)	Unknow/unknown	Activated sludge under nitrate-reducing conditions
N (JF508925)	468	99.8	Uncultured Burkholderiales bacterium (EU642383)	Betaproteobacteria/ unknown	Lake Michigan
O (JF508926)	427	96.3	*Mesorhizobium* sp. (FJ493065)	Alphaproteobacteria/ *Phyllobacteriaceae*	Campos Basin, a petroleum rich area offshore Rio de Janeiro, Brazil
P[e]	325	81.8	Uncultured bacterium (GU206801)	Unknown/unknown	South China Sea
Q (JF508927)	490	97.8	Sphingobacteriales bacterium (EF636196)	Sphingobacteria/unknown	Freshwater pond sediment
R[e]	339	70.8	Uncultured *Blastomonas* sp. (EU632120)	Alphaproteobacteria/ *Sphingomonadaceae*	Showerhead swab sample
S[e]	356	75.0	Bacteroidetes bacterium (AF530981)	Bacteroidetes/unknown	Lake habitat
T (JF508928)	445	93.3	Uncultured bacterium (AF314434)	Unknown/unknown	Anaerobic–aerobic sequencing batch reactor treating wastewater
U (JF508929)	439	99.3	Alpha proteobacterium (AB002653)	Alphaproteobacteria/ unknown	Sea mud of the Mariana Trench, Pacific Ocean
V (JF508930)	443	98.4	*Sphingomonas* sp. (AY162145)	Alphaproteobacteria/ *Sphingomonadaceae*	Bottled water

a. Band label in Fig. S2 with a GenBank accession number.
b. Sequence length.
c. Similarity (%).
d. Closest species in GenBank database with an accession number.
e. Sequence quality was not high enough to obtain accession number from GenBank.

these primers from *C. vulgaris* stock cultures showed the presence of only *C. vulgaris* in the cultures grown in autoclaved medium. No other clear bands were present in the stock cultures (Fig. S2A). In contrast, all flat plate cultures of *C. vulgaris* grown in unsterilized tap water-based medium contained a variety of bacteria (Fig. S2, Table 1).

Bacterial community profiles were relatively stable during the flat plate cultivations of *C. vulgaris*. Some bands disappeared during the incubation, such as bands I and O, and some bands became intense only in the later phases of algal growth, such as bands H_2, H_3 and P (Fig. S2). Bacterial community profiles were similar in the NM and PM. Bacterial communities in the CM differed slightly from the others, while the same major bands were present. Among the major differences were the absence of the bands corresponding A_1 and A_2 in the CM (Fig. S2).

A total of 24 sequences for bacteria were determined in the flat plate reactor cultures, when the chloroplast sequences are excluded; 15 were assigned to Alphaproteobacteria, one to Betaproteobacteria, one to Bacteroidetes, one to Sphingobacteria and six were not matched with known classes (Table 1). There were cases where a sequence of similar bacterial species was detected in two different bands. Several bands, such as B, C, I, P, Q, R and S, had very low matches in the database and may represent novel species (Fig. S2, Table 1).

Discussion

This study characterized growth of *C. vulgaris* under non-axenic growth conditions in three different batch-operated flat plate photobioreactor configurations. All cultures were accompanied with heterotrophic bacteria introduced with tap water and possibly also from air borne organisms. The municipal tap water used in this study for media preparation is known for constant quality and does not account for the chemical and biological differences in the performance between the three photobioreactors.

The DO in all flat plate cultures remained near saturation value. The lack of supersaturation is attributed to the relatively low light intensity used in the illumination of the photobioreactors and exit of O_2 via ports in the lids. Heterotrophic organisms enriched in the photobioreactor cultures also consumed oxygen to alleviate supersaturation. The reported DO levels were optimal for microalgal growth. DO levels significantly higher or lower than air saturation value have been reported to reduce microalgal biomass productivity (Molina *et al*., 2001). The transient change in the pH to 8.6–9.1 did not appear to suppress phototrophic growth and bacterial growth also ensued regardless of the pH. This is consistent with observations that *C. vulgaris* grows at a relatively wide pH range, including pH 9.5 in outdoor ponds (Mohan *et al*., 2009). Possible carbon limitation is not clear from these data. Preliminary shake flask experiments (data not shown) with controlled air supply demonstrated that 2% and 5% CO_2 enrichment in aeration lowered the pH and thereby suppressed *C. vulgaris* growth. Therefore, CO_2 enrichment was not used in flat plate photobioreactors. Toward the end of the incubation, growth may have been N- and P-limited as in comparable growth experiments soluble N and P were consumed in *C. vulgaris* cultures by day 4 (results not shown), but a complete nutrient mass balance is not available to confirm the nature of the limitation.

The specific growth rates of *C. vulgaris* were generally somewhat higher and the biomass concentrations lower than those previously reported (Table S1). Biomass yields achieved in this study were comparable with previously reported values (Illman *et al*., 2000; Scragg *et al*., 2002; Hulatt and Thomas, 2010). Yields reported by Degen and colleagues (2001) were much higher but that study involved a completely different reactor configuration and mode of light supply (Table S1). The mixers in the PM and the CM did not increase the growth of *C. vulgaris*. In fact, growth was significantly lower in the CM photobioreactor than in the other two configurations. There are reports of enhancing growth of microalgae with static mixers that increase fast transfer of microalgae from more illuminated areas to darker areas of the photobioreactor and back again (Degen *et al*., 2001; Muller-Feuga *et al*., 2003). The light intensity used in this study may have been too low to manifest this effect, although the *C. vulgaris* cells were adapted to low light intensities before the experiments.

The concentration of chlorophyll *a* increased initially in the flat plate cultivations, but after the exponential growth phase it decreased nearly to zero. A similar pattern was not seen with OD, VSS or eukaryotic QPCR. While it is possible that the exact timing of the peak of chlorophyll *a* was missed in the sampling schedule, these data emphasize that chlorophyll *a* is not a useful measure of *C. vulgaris* biomass concentration. The decrease in chlorophyll *a* may reflect algal nutrient limitation as in comparable growth experiments N and P were consumed in *C. vulgaris* cultures by day 4 (data not shown). Trace metal limitation is also possible. Similar decreases in chlorophyll *a* content of *C. vulgaris* have been reported by Eriksen and colleagues (2007) and Perner-Nochta and colleagues (2007).

Plate counts require a relatively long time course and underestimate the numbers of bacteria because not all heterotrophs are able to grow on solid media. In contrast, QPCR enables processing of multiple samples in a relatively short time but it also has some limitations. Some of these can be alleviated with proper controls and standards. The copy number of genes coding rRNA can vary between 1 and more than 12 000 among phytoplankton (Zhu *et al*., 2005) and between 1 and 15 among bacteria

(Schmidt, 1997). This problem has not been resolved satisfactorily in environmental studies and normalization, if needed, would require additional measurements of biomass components.

The eukaryotic DGGE results indicated that the culture was unialgal and thus, bias caused by species differences in rDNA copy number was negligible. The eukaryotic QPCR results had a positive correlation with OD and VSS values. Fowler and Wade (2006) have also reported a linear association between *C. vulgaris* cell numbers and threshold of SYBR Green fluorescence in real-time PCR. Based on bacterial DGGE, bacterial communities in the flat plate cultures were diverse and therefore, some differences among species in 16S rDNA copy numbers were possible. *Sphingomonas* spp. were the most prominent bacteria in the flat plate cultures and rDNA copy numbers of 1 for a *Sphingomonas* strain RB2256 (Fegatella *et al*., 1998) and 2 for *Sphingomonas wittichii* (Lee *et al*., 2009) have been reported. In addition, Klappenbach and colleagues (2000) showed that the number of rDNA copies in bacteria correlates with the response rate to growth substrates, indicating that fast-growing bacteria have higher rDNA copy number than slow-growing ones. Bacteria in oligotrophic environments such as in phototrophic algal cultures grow slowly, as observed also in this study based on both HPC (first colonies appeared on days 4–5) and bacterial QPCR results.

The initial DOC values were in the similar range in all flat plate cultivations and DOC mainly originated from the tap water and algal inoculum. Final DOC concentrations were much higher in the CM than in the NM and PM. Microalgae excrete organic material, and bacteria respire and grow with this pool of organic material (Watanabe *et al*., 2008; Hulatt and Thomas, 2010). In the NM and PM photobioreactors the concentration of released DOC, algal growth and bacterial growth all correlated with each other. In the CM, however, the concentration of DOC did not correlate well with algal or bacterial growth. Bacterial numbers were lower than in NM and PM, which may be the reason for high levels of DOC. Thus, with fewer heterotrophic consumers, the concentration of DOC increased more than in NM and PM and may even have reached levels inhibitory to *C. vulgaris*.

Based on eukaryotic DGGE profiles and microscopic examination, *C. vulgaris* was likely the only eukaryote present in the photobioreactor cultures. *Chlorella vulgaris* (SAG 211-11b) was originally obtained from the culture collection as an axenic culture. Despite serial batch incubations in shake flasks to maintain the culture for more than 2 years in the laboratory, no distinct DGGE bands of bacterial origin were detected in the samples from *C. vulgaris* stock cultures. Thus, the bacteria detected in the flat plate photobioreactor cultures originated from the tap water used in the culture medium. *Sphingomonas* spp. were the most dominant bacteria in the *C. vulgaris* cultures. The same major bacterial bands were detected in all flat plate photobioreactor cultures, demonstrating that bacterial enrichment was a reproducible event. Even closely related microalgae have shown to harbour distinct bacterial communities and the bacterial diversity with a certain algal species has shown to be generally reproducible (Schäfer *et al*., 2002; Grossart *et al*., 2005). Sequence analysis indicated that bacteria of public health concern were absent, as expected because the medium was prepared with municipal tap water. Public health aspects may, however, be a concern if untreated surface water or groundwater is used for large-scale algal cultivation.

Sphingomonas spp. have been repeatedly isolated from non-axenic *Chlorella* cultures, such as from *Chlorella* spp. isolated from soil (Otsuka *et al*., 2008), from *Chlorella saccharophila* (Ueda *et al*., 2009) and from *Chlorella sorokiniana* (Watanabe *et al*., 2005). *Sphingomonas* spp. are also common in Finnish drinking water distribution systems (Koskinen *et al*., 2000; Vuoriranta *et al*., 2003) such as the one used as source water in this study. Watanabe and colleagues (2005) reported that the presence of *Sphingomonas* spp. had no effect on growth of *C. sorokiniana*. However, algal and bacterial interactions are generally very complex and not well understood (Cole, 1982). Bacteria may, for example, compete with microalgae for the available nutrients such as N and P, produce metabolites that are inhibitory to microalgal growth (Joint *et al*., 2002), supply vitamin B_{12} for the algae that are not able to synthesize B_{12} themselves (Croft *et al*., 2005), reduce oxygen tension (Mouget *et al*., 1995) and increase solubility of nutrients and trace elements and make them more bioavailable for algae (Keshtacher-Liebson *et al*., 1995). Ueda and colleagues (2010) showed that soil borne bacteria can be grown in algal association. Their results demonstrated heterotrophic bacterial utilization of algal exudates, which are complex and variable mixtures of organic nutrients that may support otherwise non-cultivable heterotrophs (Watanabe *et al*., 2008).

One of the underlying premises for this work was to test bench-scale production of algal biomass under conditions that exclude sustenance of pure cultures. Non-axenic cultivation is inevitable in large-scale open or closed algal biomass production intended for biofuel systems. Phototrophic, biomass-based biofuel systems are still under development because of the lack of knowledge, for example, on algal cultivation systems to produce biomass with desired stable properties under specific reactor or open bioprocess conditions. For production of biofuels especially, whether the heterotrophic bacteria are beneficial by contributing to the overall lipid pool remains to be established.

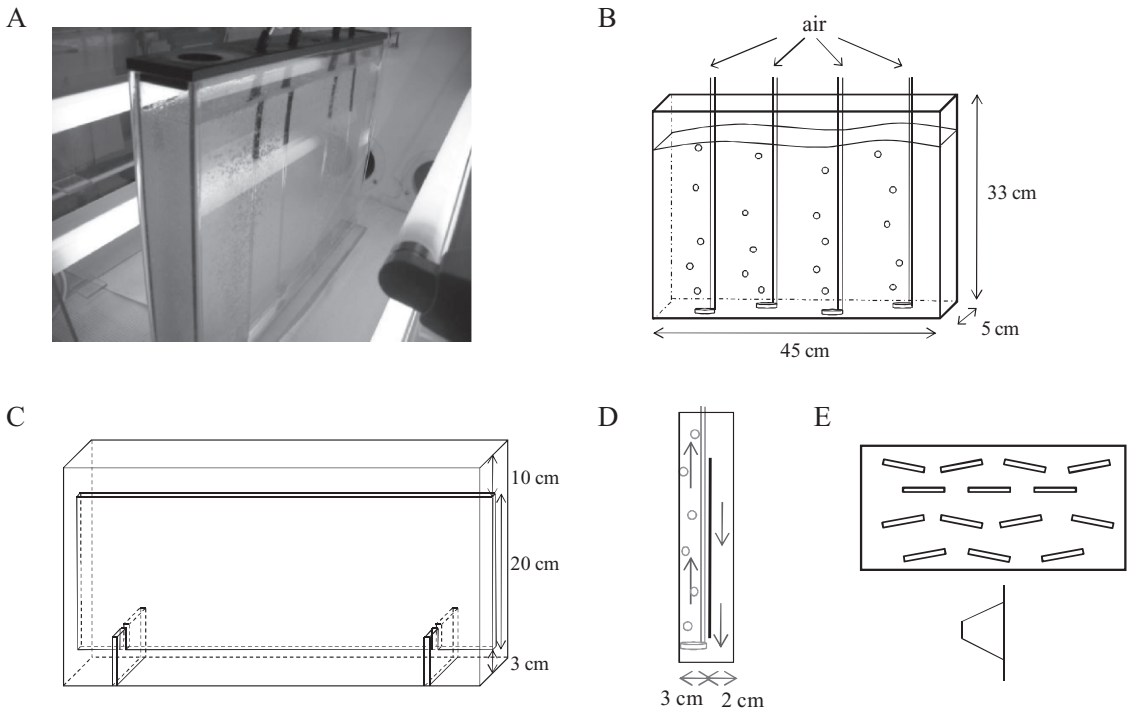

Fig. 3. Photograph (A) and schematic diagram (B) of the flat plate photobioreactor with no mixer (NM). The flat plate reactor with the plain mixer (PM) and the flat plate reactor with the complex mixer (CM) had the same dimensions but contained also a removable mixing element parallel to the illuminated surfaces to enhance the mixing (C, D). The plain mixer was a smooth sheet (C, D), whereas the complex mixer was a sheet with baffles towards the aerated (upflow) side of the mixer (E).

Experimental procedures

Test organisms and culture media

Chlorella vulgaris (SAG 211-11b) was obtained from the Culture Collection of Algae (SAG) at the University of Göttingen, Germany and grown in Jaworski's medium (Nichols, 1973). *Chlorella vulgaris* was adapted to low light intensities and maintained in shake flasks at 22 ± 2°C at 120 r.p.m. under continuous 20 μmol photons m^{-2} s^{-1} illumination (Sylvania GRO-LUX F36W/GRO-T8 fluorescent lamps). Stock cultures were transferred into sterile media at 2–4 week intervals.

Photobioreactors

Chlorella vulgaris was grown in tap water-based media in three different flat plate photobioreactors; a flat plate reactor with no mixer (designated as NM), a flat plate reactor with a plain mixer (PM) and a flat plate reactor with a complex mixer (CM). The flat plate reactors were thin rectangular glass vessels with light path of 5 cm, illuminated area of 29.7 dm^2, total volume of 7.4 dm^3 and effective volume of 7.0 dm^3. The reactors had polyoxymethylene lids with ports for oxygen outlet and sampling (Fig. 3). The PM and CM designs also contained a removable Plexiglas mixing element mounted with Plexiglas brackets. The purpose of the static mixers was to enhance the mixing by dividing the reactor into upward flow region (aerated region) and downward flow region (unaerated region). The mixers were placed in parallel to the illuminated surfaces. The plain mixer consisted of a smooth sheet and the complex mixer of a sheet with baffles towards the upward flow side (Fig. 3). The flat plates were continuously illuminated with four daylight white fluorescent lamps (Osram L 18W/965 biolux) with light intensity of 50 μmol photons m^{-2} s^{-1}. The intensity of light was measured with a Delta OHM, HM9221 lux meter. Air was introduced from the bottom of the reactors at 1.6 dm^3 min^{-1} and the air flows were controlled with multitube flow meters (Kytola Instruments E4K-LK04). The photobioreactors were inoculated with stock cultures previously grown in 1 l Erlenmeyer flasks on an orbital shaker (145 r.p.m.) under three fluorescent lamps (Osram L 18W/965 biolux) for 2 weeks. The volumes of inocula per working volume of the reactor were 5.3% (vol/vol). The reactors were acid washed (1 M HCl) between the batch cultivations.

Analytical methods

Optical density was determined by measuring absorbance at 600 and 680 nm with a Shimadzu UV-1700 Pharmaspec spectrophotometer. If necessary, the samples were diluted to give OD below 1. Growth rate was calculated as $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 is the OD at time t_1 and N_2 is OD at t_2. The maximum specific growth rate calculations were based on the following: NM (reactor A), OD increased from 0.031 to 0.240 between days 0 and 1; PM, OD increased from 0.084 to 0.565 between days 2 and 3; CM, OD increased from 0.047 to 1.38 between days 1 and 4.

The temperature, pH and DO were measured with HACH HQ40d portable multi meter; temperature and pH with a

HACH PHC101-03 IntelliCAL probe and DO with a HACH LDO101-03 IntelliCAL probe. Total suspended solids (TSS) and VSS were determined by filtering a measured volume of culture solution through a glass fibre filter (Whatman GF/A), followed by drying the filter at 105°C for 20 h and igniting it at 550°C for 2 h. For chlorophyll *a* analysis (modified from Hsu and Hsu, 1998) biomass was centrifuged at 2600 *g* for 10 min and the pellet was resuspended in 90% methanol followed by extraction of the pigments at 22 ± 2°C for 24 h. The absorbance was measured at 650 and 665 nm. The starting points of VSS were 0.018, 0.028 and 0.020 g l^{-1}, and starting points of chlorophyll *a* were 0.14, 0.18 and 0.13 mg l^{-1} for NM, PM and CM respectively (Fig. 1C and D).

Dissolved organic carbon was measured from samples filtered through 0.45 μm polyester syringe filters (Chromafil, Macherey-Nagel) with a TOC-5000 Analyzer (Shimadzu) according to the Finnish Standard SFS-ISO 8245 (SFS-ISO 8245, 1989). The amount of released DOC was calculated by subtracting the initial DOC from the measured DOC values. HPC were conducted by diluting culture samples with 0.9% NaCl solutions and by spreading 0.1 ml aliquots of appropriate dilutions on R2A-agar plates (a low nutrient medium developed for the study of potable water bacteria). The plates were incubated at 26°C for 7 days.

Microbial community analyses

Duplicate samples of 8–20 ml were taken from *C. vulgaris* cultivations and stock cultures and stored at −20°C. Before DNA extraction, biomass was harvested by centrifugation (10 000 *g*, 10 min) followed by DNA extraction with PowerSoil DNA isolation kit (Mo Bio laboratories, Carlsbad, CA, USA).

DNA samples were used as templates for polymerase chain reaction (PCR). Partial eukaryotic 18S rRNA genes of the community were amplified by using primer pair Euk1A and Euk516r-GC (Díez *et al*., 2001) and partial bacterial 16S rRNA genes by using primer pair GC-BacV3f (Muyzer *et al*., 1993) and 907r (Muyzer *et al*., 1996) as described by Koskinen and colleagues (2007). Denaturing gradient gel electrophoresis (DGGE) was performed with INGENYphorU2×2-system (Ingeny International, Goes, The Netherlands) using 8% polyacrylamide gels with denaturing gradient from 30% to 70% for both eukaryotic and bacterial DNA (100% denaturing solution contains 7 M of urea and 40% formamide). Gels were run at 60°C in 1 × TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) with 100 V for 22 h and stained with SYBR Gold (Molecular Probes Invitrogen). The dominant bands were excised from the gels, eluted in 20 μl of sterile water at +4°C overnight, stored in −20°C and reamplified for sequencing. Sequencing was conducted at Macrogen (Korea). Sequence data were analysed with BioEdit-software and compared with sequences in GenBank. The accession numbers of the 16S rRNA gene sequences submitted to GenBank were JF508903-JF508930 and the 18S rRNA gene sequence JF508931.

Primers specific for the nuclear 18S rRNA gene of eukaryotes, EUK345f and EUK499r (Zhu *et al*., 2005) and primers specific for 16S rRNA gene of bacteria, 27F (Lane, 1991) and 518R (Muyzer *et al*., 1993) were used for quantitative PCR (QPCR). Reactions were performed separately for the bacterial QPCR and eukaryotic QPCR in final volume of 20 μl with 10 μl Maxima SYBR Green/ROX QPCR master mix (2×) (Fermentas Life Sciences), 0.4 μl of forward and reverse primers and 9.2 μl of diluted DNA extract. Thermocycling and monitoring of SYBR Green fluorescence were conducted with StepOne Plus Real Time PCR machine (Applied Biosystems) using the following PCR program: 95°C for 10 min, 30 cycles of 95°C for 30 s, 55°C for 30 s and 60°C for 1 min followed by melting point analysis with 95°C for 15 s, 60°C for 1 min and 95°C for 15 s to demonstrate amplification of a discrete DNA fragment. The number of gene copies in the sample is inversely related to cycle threshold (C_T), i.e. the number of PCR cycles required to cross a certain fluorescence threshold (Zhu *et al*., 2005). Thus, the value of C_T was used to estimate the amount of algae and bacteria in the samples.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Eukaryotic community profiles from the flat plate with no mixer (A), the flat plate with the plain mixer (B) and the flat plate with the complex mixer (C) with sampling days shown in the top. All the bands labelled with A (accession number JF508931) had 100% similarity to *Chlorella vulgaris*, accession number FM205855.

Fig. S2. Bacterial community profiles from the *C. vulgaris* stock culture (A), the flat plate with no mixer (B), the flat plate with the plain mixer (C) and the flat plate with the complex mixer (D) with sampling days shown in the top. See Table 1 for the labelled bands.

Table S1. Maximum specific growth rates and biomass yields of *C. vulgaris*.

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