Algal Research 6 (2014) 152–159

Contents lists available at ScienceDirect

Algal Research

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

Production of algal biomass, chlorophyll, starch and lipids using aquaculture wastewater under axenic and non-axenic conditions

Trina Halfhide [a], Anette Åkerstrøm [b], Odd Ivar Lekang [c], Hans Ragnar Gislerød [b], Sarina J. Ergas [a,*]

[a] Department of Civil & Environmental Engineering, University of South Florida, 4202 E. Fowler Ave., ENB 118, Tampa, FL 33620, United States
[b] Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway
[c] Dept. of Mathematical Sciences and Technology, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway

ARTICLE INFO

Article history:
Received 21 February 2014
Received in revised form 18 October 2014
Accepted 29 October 2014
Available online xxxx

Keywords:
Algae
Microbial contamination
Intracellular end-products
Nitrogen limitation
Recirculating aquaculture systems (RAS)
System resilience

ABSTRACT

Although co-cultivation of algae with aquaculture products has the potential to reduce water use and pollutant discharges while producing energy feedstocks and other end-products, little research has been carried out in this area. Maintaining axenic conditions (algal monocultures without other microorganisms) in algal culturing systems using aquaculture wastewater as a nutrient source would not be practical. This study examined the effects of the use of aquaculture wastewater as a nutrient source on biomass development of three algae cultures (indigenous mixed species consortium, *Chlorella* sp. and *Scenedesmus*) under axenic and non-axenic conditions. Biomass development was assessed by cell growth, chlorophyll, starch and lipid production. The presence of aquaculture microorganisms decreased biomass productivity in *Chlorella* but not in the other algae cultures. Non-axenic conditions had no effect on overall starch and chlorophyll production; however, significantly higher lipid contents were achieved under non-axenic conditions for *Chlorella* and the indigenous culture. The higher algal lipid content for these cultures under non-axenic conditions may have been due to competition with bacteria for nutrients. The presence of bacteria was required for effective removal of organics, while effective nitrogen removal was observed in all systems containing algae. Results from this study also show that algae harvesting should be timed to coincide with the peak production of the desired target end-product (biomass, chlorophyll, starch or lipid).

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

The aquaculture industry has grown to meet increasing worldwide fish and protein demands [1]. As the scale and intensity of production increase, the volume and concentration of pollutants in the wastewater from aquaculture systems also increase. In addition, there is increasing emphasis on the need for aquaculture facilities to meet effluent standards for wastewater contaminants, such as solids organics, nitrogen (N) and phosphorus (P). However, conventional wastewater treatment processes have high capital, energy and chemicals costs and do not recover nutrients to produce useful or commercially viable end-products. Therefore using an integrated, biological approach that facilitates energy and cost savings and produces useful end-products, such as algal biomass, and intracellular products should be favored [2,3].

Aquaculture wastewater has been used previously to support symbiotic photoautotrophic growth using various co-cultivation approaches, such as aquaponics [3–6]. A potential alternative for integration of algae cultivation with aquaculture is shown in Fig. 1. Algal co-cultivation may be more advantageous than aquaponics because it has the potential to improve water quality and increase dissolved oxygen concentrations, which improves the target species' health, while producing a feedstock for onsite energy production and/or feed supplementation [3–5,7,8]. Drapcho and Brune [5] used algae in a partitioned aquaculture system to reduce ammonia concentrations and increase dissolved oxygen concentrations required for fish health. Haglund and Pedersén [7] used macrospecies algae, *Gracilaria tenuistipitata*, for wastewater treatment and epiphyte control in a rainbow trout system. Several prior studies produced algae for use as an onsite aquaculture feed supplement and found that algae grown on aquaculture wastewater had higher growth rates and protein contents and were more nutritious (containing a more complete amino acid profile) than non-leguminous plants such as oats, barley and rye [3,8–10]. Bioflocs technology (BFT) is an example of co-cultivation that takes advantage of the synergy between aquaculture, algae and microorganisms [6]. Bioflocs are an aggregate combination of heterotrophic bacteria, algae, colloidal particles and polymeric substances that can be used to supplement fish feed. The process also facilitates N immobilization and recovery [11].

The use of aquaculture wastewater as a nutrient feed for algae production increases the chances of contamination by microorganisms and non-target algal species. Many prior studies of algae photobioreactor systems have used axenic conditions (i.e. algal monocultures without other microorganisms) [12–15]. However, it would not be practical or economically

* Corresponding author at: Dept. Civil & Environmental Engineering, University of South Florida, 4202 E. Fowler Ave. ENB 118, Tampa, FL, United States.
E-mail address: sergas@usf.edu (S.J. Ergas).

http://dx.doi.org/10.1016/j.algal.2014.10.009
2211-9264/© 2014 Elsevier B.V. All rights reserved.

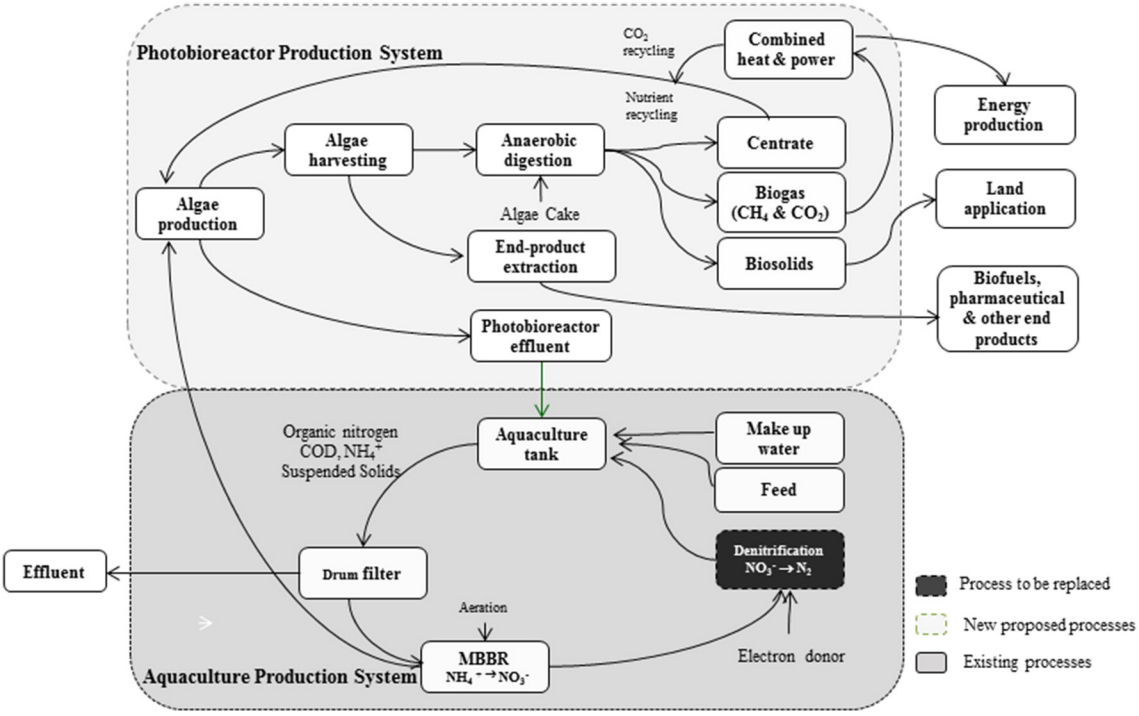

Fig. 1. Proposed integration of algae co-cultivation with aquaculture.

viable to maintain axenic conditions in large-scale open pond systems [13,14]. Non-target algae, bacteria or protozoans may compete with the target algal species for nutrients and light or may be toxic or predatory in nature [13,15–17]. However, some prior studies have shown that the presence of bacteria can improve algae production by making the system more resilient [7,17,18] (i.e., able to maintain its function although external stress and disturbances were present [18]). This increased resilience may be due to the ability of indigenous microorganisms to: 1) mineralize organic substrates to inorganic forms that are more bioavailable to algae [19,20]; 2) produce growth factors and micronutrients that support algal growth; and/or 3) convert toxic ammonia to nitrite and nitrate through nitrification [21–23]. In addition, the use of algae–bacteria consortia has the potential to reduce downstream processing costs. When cultures contain a mixture of algae and bacteria, algal cells have been shown to produce a matrix of carrageenan or alginate, which facilitates autoflocculation [24].

This is the first peer reviewed study to examine how indigenous microorganisms present in aquaculture wastewater affect algal biomass and end-product production. Three algal cultures were studied: a mixed indigenous consortium and pure cultures of *Chlorella* and *Scenedesmus*. The effects of axenic and non-axenic conditions on the ability of the system to maintain function and resilience were assessed. Two success criteria were used to examine system resilience: productivity of desirable end-products (biomass, chlorophyll, starch and lipids) and removal of nitrate and organic matter from the wastewater.

2. Materials and methods

Experiments were conducted at the Norwegian University of Life Sciences (UMB), Ås, Norway. Algae biomass, chlorophyll, starch and lipid production were investigated using wastewater from a recirculating aquaculture system (RAS). Algal system performance was compared under axenic and non-axenic conditions for an indigenous consortium and two pure algae cultures.

2.1. Aquaculture wastewater feed

Approximately 10 L of wastewater was collected from a UMB campus tilapia RAS, which has a total volume of 4200 L. The flow rate in the RAS was approximately 150 L/min, with 98–99% recirculation. The RAS included a drum filter with a 40 micron screen mesh size (Hydrotech HDF 501) and a moving bed bioreactor (MBBR) containing extruded plastic media for nitrification. The mean annual tilapia biomass produced was 300 kg/year. Tilapia are fed Aller 37/10 FLOAT daily, which has a protein content of 37%. For the axenic treatments, aquaculture wastewater was filter sterilized using a 0.2 μm glass fiber filter (AP 1504700). In order to

Table 1
Aquaculture wastewater feed characteristics.

Mean concentrations	Axenic	Non-axenic
TN (mg/L)	17.9	18.5
NO_3^- (mg/L as N)	17.6	18.1
COD (mg/L)	238	253
TP* (mg/L)	17.0	17.5
PO_4^{3-}-P* (mg/L)	16.9	17.1
pH	6.94	6.97
Transmissivity (%)	99.0	97.8
HPC (CFU/100 mL)	0	183
Potassium (K) (mg/L)	66	65
Calcium (Ca) (mg/L)	62	64
Sodium (Na) (mg/L)	21	21
Sulfur (S) (mg/L)	15	16
Magnesium (Mg) (mg/L)	10	11
Iron (Fe) (mg/L)	0.016	0.069
Zinc (Zn) (mg/L)	0.011	0.022
Copper (Cu) (mg/L)	0.006	0.007
Manganese (Mn) (mg/L)	0.002	0.003
Aluminum (Al) (mg/L)	<MDL	0.006

*TP and PO_4^{3-}-P concentrations given after supplementation with 15 mg/L of TP.
MDL = method detection limit.

Table 2
Heterotroph bacterial population viability under non-axenic conditions (HPCs were <30 CFU/100 mL for all samples under axenic conditions).

Time (hours)	Viability under non-axenic conditions			
	Indigenous	*Chlorella*	*Scenedesmus*	No algae
0	+	+	+	++
14	++	++	++	++
25	++	++	++	++
38	++	++	++	++
49	–	+	+	++
72	–	–	–	+

−, HPC < 30 CFU/100 mL; +, HPC > 30 CFU/100 mL; ++, HPC > 10^3 CFU/100 mL.

maintain N rather than P limited conditions (discussed below), 15 mg/L of phosphorous was added to the feed in the form of K_2HPO_4.

2.2. Algal cultures

Three different algae cultures used in this study were an indigenous mixed species consortium [25], *Chlorella sp* (NIVA CHL-137) and *Scenedesmus quadricauda* (NIVA-CHL 7). The indigenous algae were harvested from the surface of a secondary clarifier at the Howard F. Curren Advanced Wastewater Treatment Facility in Tampa, Florida. The consortium was identified and enumerated by the Environmental Biotechnology Laboratory in the Department of Soil & Water Science at the University of Florida. The primary genera within the consortium identified included: *Chlorella* (95.2%), *Chlamydomonas* (3.1%), and *Stichococcus* (1.1%). Pure cultures of *Chlorella* and *Scenedesmus* were acquired from the Norwegian Institute for Water Research (NIVA) culture collection. All three algae cultures were initially grown using an aseptically prepared synthetic medium, a continuous light irradiance of 153.3 ± 18.8 μmol/m^2/s and a temperature of 25 °C (controlled using a water bath). The medium consisted of 1000 mg of a balanced agricultural fertilizer (Superex gronnsak) in tap water, resulting in the following approximate composition (mg/L): NO_3^--N (90), Ca (30), P (50), K (310), Mg (20), S (30), Mn (0.90), B (0.30), Zn (0.25), Cu (0.12), Mo (0.05), and Co (0.01). The algae were grown under aseptic conditions in a 300 mL photobioreactor (described below) for 4 days. A 10.0 mL aliquot of the algae stock culture was centrifuged using an Eppendorf Model # 5810 (Horsholm, Denmark) centrifuge. The supernatant was decanted and 5.0 mL of phosphate buffered dilution water was added to the centrifuge tubes to gently resuspend the algae. This process of washing to remove residual nutrients from the growth medium was repeated. Phosphate buffered dilution water was prepared by adding the following to 1.0 L of deionized water (mg/L): KH_2PO_4 (3500), $KHPO_4$ (4300) and NaCl (8500). The pH of the dilution water was measured and adjusted to 7.2 ± 0.5 using 1N sodium hydroxide, if needed, and the water was autoclaved at a pressure and temperature of 103.4 kPa and 115 °C.

2.3. Reactor setup and operation

Photobioreactors consisted of cylindrical glass tubes with tapered bottoms, with an internal diameter of 4.12 cm, a height of 31.2 cm and an overall volume of 300 mL. A 280.0 mL aliquot of wastewater, filtered or unfiltered, was added to each photobioreactor. Washed algae (described above) were added to the respective photobioreactor. Unfiltered RAS wastewater without added algae was used as an uninoculated control. Growth experiments were performed using three replicates, for each algal culture (indigenous, *Chlorella*, and *Scenedesmus*) and the two treatment types (axenic and non-axenic) and one non-axenic control. The experiment had a total of 21 photobioreactors. Algal growth conditions for all treatments included: continuous light irradiance of 153.3 ± 18.8 μmol/m^2/s (using daylight fluorescent tubes), a temperature of 25 °C (controlled using a water bath) and a filtered 1% CO_2–air mixture (provided using gas diffusers). Based on visual observations, air bubbling was sufficient in maintaining samples well mixed and algal cultures continuously suspended. A continuous lighting regime was used, as the maximum biomass production during the growth period was achieved (during preliminary experiments), when an irradiance of 153.3 ± 18.8 μmol/m^2/s was used. A 10.0 mL sample was collected from each photobioreactor every 6–8 h for the duration of the experiment and tests were conducted as described below to determine biomass, end-product productivity, and nutrient and organic compound removal. Samples were taken under a biological hood using 25 mL sterile pipettes.

2.4. Analytical methods

The optical transmissivity of the RAS wastewater was determined at 256 nm. Samples were analyzed in accordance with *Standard Methods* [26] for the following parameters: pH (4500H^+-B), total nitrogen (TN) (4500-N), nitrate (NO_3^--N) (4500-NO_3 B), Chemical Oxygen Demand (COD) (5220 D), phosphate (PO_4^{3-}) (4500-$KMnO_4$), heterotrophic plate counts (HPC) (9215) and total suspended solids (TSS) (2540B). Measured TSS values were used to calculate the time averaged biomass productivity during the growth phase (P; mg/L/h):

$$P = \frac{B_{max} - B_i}{\Delta t}$$

where B_i and B_{max} are the initial and maximum biomass concentration and Δt is the time when maximum productivity was achieved.

The starch content of the algae biomass was measured using a Megazyme total starch (AA/AMG) kit (catalog # K-TSTA), which follows Association of Official Agricultural Chemists (AOAC) Method 996.11. The method was modified to allow for smaller sample volumes. The final lipid content (%) was determined using the method of Bligh and Dyer [23] and calculated on an ash free dry weight basis. Total chlorophyll was determined using the method described by Franco et al.

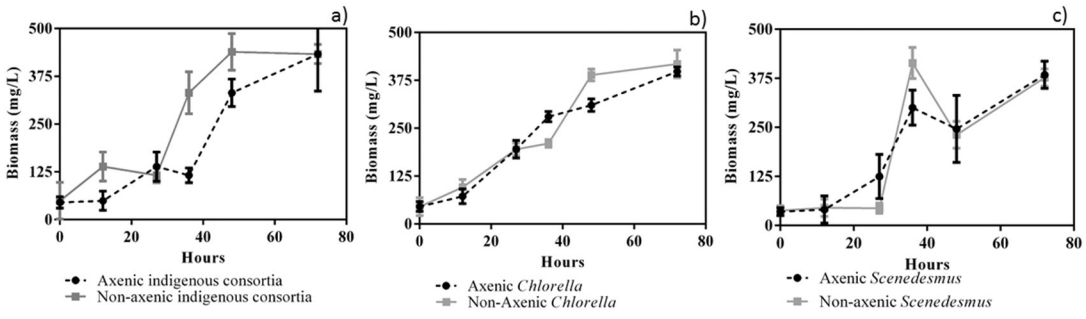

Fig. 2. Biomass (a) over time for indigenous consortium culture, (b) over time for *Chlorella*, and (c) over time for *Scenedesmus*.

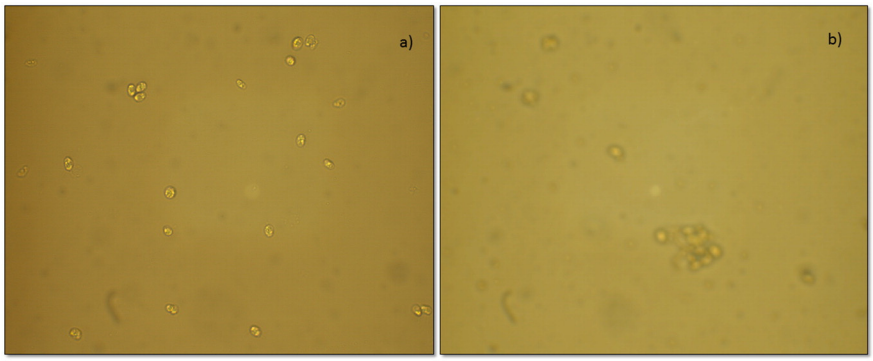

Fig. 3. Microscopic observations (100× magnification) for *Scenedesmus* under axenic (a) and non-axenic (b) conditions.

[27]. Total chlorophyll was calculated using Liechtenthaler equations [28]. Elemental analyses of algal biomass and aqueous samples were carried out using a Perkin Elmer (Waltham, Massachusetts) Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES; Optima 5300 DV) for: total phosphorous (TP), K, Ca, Na, S, Mg, Fe, Zn, Cu, Mn and Al. Samples were decomposed by adding HNO_3 at 10% (v/v) before oxidation with peroxydisulfate during autoclaving at 250 °C for 1.5 h. A light microscope (Leica DM 5000B) equipped with a camera (Leica DFC 425) was used to periodically monitor algae growth and physiological changes. Different filters and magnifications (10, 40,100×) were used to obtain the best visual analysis.

2.5. Statistical analyses

One-way analysis of variance was used to determine whether differences in means for different algal cultures were significant. T-tests were used to determine whether the differences between axenic and non-axenic conditions within a given algal species were significant. These tests were done in Microsoft Excel. A p value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Aquaculture wastewater as a feed

A summary of the initial aquaculture wastewater feed characteristics for both axenic and non-axenic treatments is shown in Table 1. The observed TN values (17.9 and 18.5 mg/L) were slightly lower than values reported by other authors (between 20 and 40 mg/L) for a RAS with a denitrification process [29]. The observed TN concentrations should be able to support an algal biomass concentration of approximately 285 mg/L in a batch reactor, assuming algal biomass has a chemical formula of $C_{106}H_{263}O_{110}N_{16}P$ [30]. In this study, experiments were conducted under batch conditions to maintain axenic algal treatments; however, higher biomass densities are possible if cultures are grown using the proposed process (Fig. 1), where nitrified effluent from the MBBR and recovered nutrients from anaerobic digestion are continuously circulated through the photobioreactor, which replaces the denitrification process. Most (>97%) of the initial TN was in the form of NO_3^- (Table 1). Although algae utilize ammonia in preference to NO_3^- as a growth substrate [31], high ammonia concentrations (>34 mg/L), such as those found in many municipal and agricultural waste streams are a toxicity concern, as free (unionized) ammonia dissipates transmembrane proton gradients in algae [32–35]. Therefore utilizing RAS wastewater with NO_3^- concentrations such as those observed in this study is favorable as a feed.

The observed TP concentrations (2.0 and 2.5 mg/L prior to supplementation) were lower than typical values seen in RAS, which have been shown to range between 6.2 and 37 mg/L [36]. The observed N/P ratio of approximately 9 was within the range (7 to 10 gN/gP) that has been shown to be optimal for algal growth [37]. Additional P was provided (15 mg/L added), however, to ensure that the algal system in this study was N rather than P limited to favor lipid accumulation [14,38–41].

Light transmissivity at 256 nm was 99.0% and 97.8%, for filtered and unfiltered samples, respectively (Table 1), suggesting that the presence of particles in the unfiltered wastewater would not hinder light transmission to an algae culturing system. This is a very high light transmissivity, when compared to some other waste streams, such as municipal sludge centrate, which has a low light transmittance (ranging from 0.1% to 21%) with no pretreatment [42]. Using aquaculture wastewater as a growth medium is therefore less challenging when considering this characteristic.

pH values were similar under both axenic and non-axenic conditions. This was probably attributed to the RAS system being well buffered. A pH between 6.5 and 7.5 is considered optimal for most green algae species [12]. The mean COD concentration was slightly higher under non-axenic conditions, most likely due to the presence of

Table 3
Time averaged biomass productivity, chlorophyll and starch content and final lipid and gross calorific content. Mean and standard deviation values are based on measurements from triplicates (NS = data not shown).

		Indigenous	*Chlorella*	*Scenedesmus*
Time-averaged biomass productivity (mg/L/h)	Axenic	5.39 ± 1.35	4.89 ± 0.02	4.85 ± 0.34
	Non-axenic	8.12 ± 1.34	5.18 ± 0.18	10.44 ± 0.14
Max. chlorophyll (mg/g of biomass)	Axenic	6.20 ± 0.03	7.12 ± 0.03	7.57 ± 0.40
	Non-axenic	4.10 ± 0.07	4.59 ± 0.05	10.85 ± 0.19
Maximum starch content (%)	Axenic	9.30 ± 7.50	16.8 ± 2.80	7.50 ± 5.10
	Non-axenic	9.10 ± 3.60	10.7 ± 3.60	6.85 ± 4.70
Final lipid content (%)	Axenic	5.70 ± 2.40	12.5 ± 5.6	NS
	Non-axenic	23.4 ± 3.40	50.4 ± 7.6	NS
Calorific content (MJ/kg)	Axenic	20.2 ± 0.60	22.0 ± 1.0	24.3 ± 0.70
	Non-axenic	22.1 ± 0.60	23.6 ± 0.4	26.5 ± 4.60

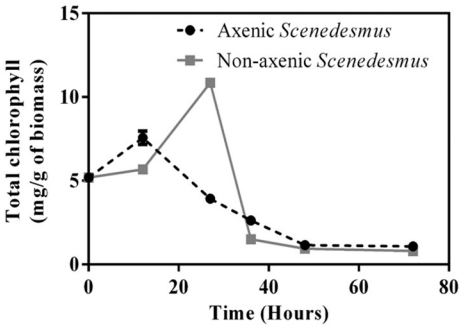

Fig. 4. Total chlorophyll content over a 72 hour period for *Scenedesmus* under axenic and non-axenic conditions.

particulate COD. COD in aquaculture wastewater is attributed to the undigested feed and fish fecal inputs [43]. The presence of COD in the wastewater can provide a source of organic carbon and result in increased growth in mixotrophic algae such as *Chlorella* [44,45]. As expected, HPCs were below detection limits in the filter sterilized feed.

Concentrations of elements (K, Ca, Na, S, Mg, Fe, Zn, Cu, Mn, Al) determined by ICP-OES are also shown in Table 1. Most of concentrations were within the range observed by Martins et al. [46] for RAS wastewaters. Cu concentrations were within the optimal growth range for *Scenedesmus*; however, Zn concentrations were much higher than the optimal range reported in Knauer et al. [47]. Sulfur concentrations were at optimal levels for the growth of *Chlorella vulgaris* based on the Liang et al. [48] and also should not present concerns based on American Society for Testing and Materials biodiesel standards [49].

3.2. Biomass production and intercellular products

The range of heterotrophic counts during different experimental phases is shown in Table 2. As expected, HPCs were below detection limits throughout the experiment for the axenic treatments (data not shown). Under non-axenic conditions, the HPCs increased to more than 10^3 CFU/100 mL within 14 to 38 h in treatments containing algae. After 38 h, HPCs declined in all algae treatments, and were below the detection limit (30 CFU/100 mL) in the indigenous algal culture. Although the control photobioreactor that was not inoculated with algae maintained HPCs above 30 CFU/100 mL throughout the experiment, there were higher counts within the first 49 h, after which the counts declined.

Growth curves for all cultures under both axenic and non-axenic conditions are shown in Fig. 2. A maximum mean biomass concentration of 414 mg/L was achieved for *Scenedesmus* after 36 h, with no significant differences between the two treatments. This exceeds the amount predicted by the TN concentrations (Section 3.1), possibly due to initial inoculum addition or the algae having a different elemental composition than suggested by the general formula. Similar growth curves were obtained for the indigenous consortium and *Chlorella* (data not shown). Microscopic photographs of *Scenedesmus* (Fig. 3) show dispersed cell growth under axenic conditions and the presence of well-defined aggregates under non-axenic conditions. The presence of indigenous aquaculture microorganisms may have increased *Scenedesmus* autoflocculation by facilitating extracellular polymeric substance (EPS) production. Although no EPS measurements were made in this study, Guo et al. and Manheim [50,51] noted the influence of EPS on algae flocculation. Cell aggregates were not observed with the other cultures.

Time averaged biomass productivity ranged from 4.85 to 10.44 mg/L/h, with no significant differences in time-averaged biomass productivity between axenic and non-axenic conditions within a single culture, as shown in Table 3. *Scenedesmus* had the lowest time-averaged biomass productivity under axenic conditions, while the *Scenedesmus* under non-axenic conditions produced the highest time-averaged biomass productivity (10.44 mg/L/h). This high biomass value may be attributed to algae flocculation, as previously mentioned. The indigenous algal consortium had a moderate productivity, 5.39 and 8.12 mg/L/h, under axenic and non-axenic conditions respectively. Rodolfi et al. obtained similar productivities for both *Scenedesmus* and *Chlorella* cultures of 7.9 mg/L/h and 7.1 mg/L/h, respectively [52], most likely due to similar temperature (25 °C) and continuous illumination (100 μmol/m^2/s). An irradiance regime of 130 μmol/m^2/s (moderate light intensity) should not have presented additional stress to the algae. In addition, literature suggests that having longer photoperiods of light and dark of 16/8 and 24/0 would result in increased productivity [65,66]. Having a full-scale system with artificial lighting may not be feasible.

In this study no negative effects were observed when operating algal systems under non-axenic conditions using aquaculture wastewater, possibly due to the short experimental duration and the small scale at which experiments were conducted. Theegala et al. [53] noted that outdoor cultures usually last for only short periods of time and continuous systems rarely exceeded a few weeks. Mitchell and Richmond [54] showed that the rotifers depleted *Monoraphidium minutum* populations, but only became a problem after four days. Smith and Crews [55] noted that algal species richness increased with water surface area, especially where algal systems were grown under natural, open conditions. Algal ponds were susceptible to contamination and the number of invading species was positively correlated with the physical size of the cultivation system.

The peak in chlorophyll coincided with the log growth period and did not coincide with the highest biomass concentration (Fig. 4). The cultures grew quickly and were N limited after 24 h and quickly matured and formed starch as an intermediate intracellular product. Due to equipment limitations, a 24 hour photoperiod was used, however it is recognized that this would not be practical in a full scale system. No

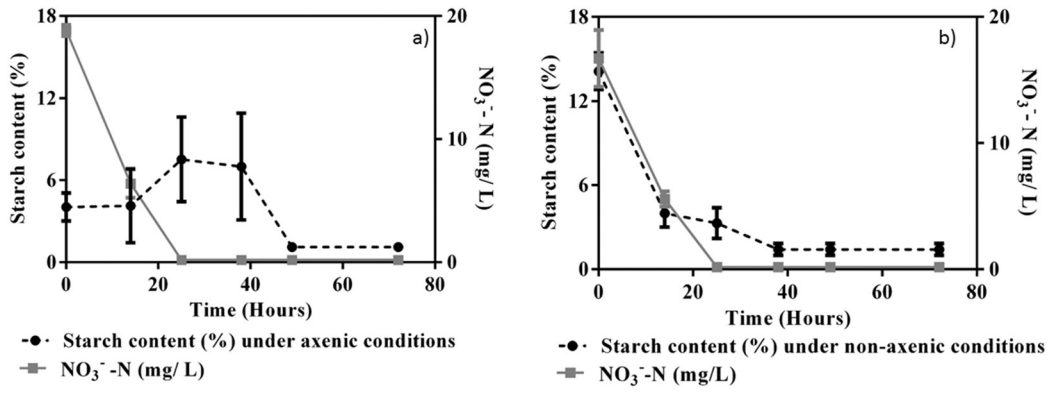

Fig. 5. Starch content and NO_3^--N concentrations over time for *Scenedesmus* under axenic (a) and non-axenic (b) conditions.

Table 4
Summary of nitrate removal efficiency (%) for the different treatments.

		Indigenous	*Chlorella*	*Scenedesmus*	No algae
NO_3^--N removal efficiency (%)	Axenic	99.4 ± 0.8	98.1 ± 0.3	98.7 ± 0.5	NA
	Non-axenic	96.4 ± 0.1	96.3 ± 0.3	99.0 ± 2.0	17.6 ± 0.8

significant differences were observed in chlorophyll contents (mg/g) between axenic and non-axenic conditions within a single culture. *Scenedesmus* produced a slightly higher total chlorophyll content under non-axenic than axenic conditions, as shown in Fig. 4. For the indigenous and *Chlorella* cultures, the maximum total chlorophyll content was slightly higher under axenic conditions. The chlorophyll content (mg/g) for all algal cultures was between 12 and 48 mg/g, as shown in Table 3. In treatments without any inoculated algae, chlorophyll contents ranged from 0.1 to 2.6 mg/g, indicating that some indigenous algae may have been present in the aquaculture wastewater.

Comparisons of starch content values for all three algal cultures and lipid content for *Chlorella* and indigenous cultures under axenic and non-axenic conditions are shown in Table 3. Lipid content results for *Scenedesmus* are not reported because they were inconsistent with previously published literature, possibly due to interferences with the method used due to the presence of chlorophyll or other pigments [64]. *Chlorella* produced the highest overall starch content compared with the other two cultures under both axenic (16.8%) and non-axenic (10.7%) conditions. Final lipid contents for indigenous and *Chlorella* cultures were consistent with published literature and significantly higher under non-axenic conditions.

NO_3^--N and starch concentrations over time are shown in Fig. 5. NO_3^--N concentrations were reduced to less than 10 mg/L within the first 24 h. N limited (<10 mg/L) and N starvation (<1 mg/L) conditions have been shown to result in higher lipid contents as final storage products [14,38–41], with most of the total lipids as TAG (triacylglycerides) produced under N deprived conditions [56]. The results obtained in this study for *Chlorella* and indigenous cultures were generally consistent with other studies. In many cases, starch is formed as an intermediate storage compound [57], and hence the timing of harvesting is important if the process is to be optimized for lipid production. Wang et al. [58] showed that the lipid bodies in a wild type *Chlamydomonas reinhardtii* increased 15 fold after a 48 hour period of N starvation. In this study, starch analyses were performed for each sampling point and used to determine the timing of starch storage depletion and the beginning of lipid accumulation [9,56]. Due to sample size requirements, only final lipid content was measured. For *Scenedesmus* under axenic conditions, the peak starch content (7.5%) was observed at 25 h (Fig. 5a). Under non-axenic conditions; however, the maximum starch content (14.1%) was observed at time zero and steadily decreased over 38 h, after which it remained constant (Fig. 5b). The initial high starch content for *Scenedesmus* under non-axenic conditions can be attributed to the presence of microorganisms and EPS production. When *Scenedesmus* started to grow exponentially between 25 and 38 h, most of the carbon was probably used for growth and not for EPS storage [59].

Gross calorific values varied from 20.2 to 26.5 MJ/kg, as shown in Table 3. The indigenous algal consortium had the lowest calorific value (20.2 MJ/kg), whereas *Scenedesmus* under non-axenic conditions had the highest calorific value (26.5 MJ/kg). Although the calorific values were slightly higher for all cultures under non-axenic conditions, these differences were not significant. A strong correlation between algal lipid content and calorific value has been observed in prior studies [38]. Lipids largely comprise long-chain TAGs, which have an energy value of 2.25 times greater than starch on a weight basis [56]. The presence of other microorganisms may have increased algal physiological stress, under already nutrient limited and starvation conditions, and resulted in a shift in algal storage compounds from starch to lipids between 25 and 48 h. Most researchers focus on lipid and TAG production, as more valuable biofuel derivatives can be produced from this fraction [60].

Timing of harvesting algae should correspond with the maximum production of the targeted end-product. If pigments are the desired end product, harvest time should correspond with the peak chlorophyll content. Some processes, such as pyrolysis, are optimized using algae with higher carbohydrate or starch contents, which were observed during the middle of the growth period. Since the primary activity of most algal cells is photosynthesis, there was little accumulation of starch and lipids in the young cells [61], indicating that harvesting should be delayed if lipids are the desired end product.

3.3. Nitrogen and organic matter removal

Since 97% of the initial TN was in the form of NO_3^- (Table 1), only NO_3^- was measured during the algal growth experiments. For all treatments with algae, NO_3^- concentrations were reduced to less than 10 mg/L within the first 14 h (N depletion) and to less than 1 mg/L within 24 h (N starvation). Overall NO_3^- removal efficiencies ranged from 96.4 to 99.4% for all systems inoculated with algae, as shown in Table 4, with no significant differences between algal cultures or treatments. The removal efficiency for the treatment that was not inoculated with algae had a NO_3^- removal efficiency of only 17.6%, indicating that the presence of algae was needed for N removal in aquaculture wastewater under these conditions.

The removal of COD over time for *Scenedesmus* under both axenic and non-axenic conditions is shown in Fig. 6a. The overall COD removal efficiencies are shown in Fig. 6b. Under axenic conditions, approximately 25% COD removal was observed in algal treatments, most likely due to the mixotrophic growth of algae. Prior studies have shown that lipid production is increased for green algae under mixotrophic and heterotrophic conditions [49,62,63]; however, due to the use of real RAS

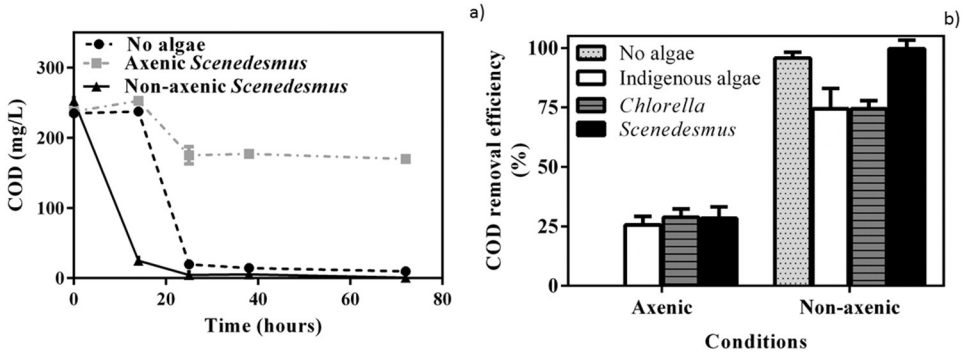

Fig. 6. a) COD removal for *Scenedesmus* under axenic and non-axenic conditions. b) COD removal efficiency for all algal cultures under axenic and non-axenic conditions as well as RAS wastewater with no inoculated algae.

wastewater no comparisons could be made on lipid production with or without COD in this study. COD removal (74.4 to 99.7%) was significantly higher under non-axenic conditions for all cultures (Fig. 6b), indicating that the microorganisms present in the aquaculture wastewater were needed to achieve high COD removal efficiencies required for wastewater treatment.

4. Conclusions

Algae and fish co-cultivation has the potential to improve water quality and fish health, while producing a feedstock for onsite energy production and/or feed supplementation. However, maintaining large-scale algal cultivation systems under axenic conditions is impractical. Results from this study showed that biomass and lipid productivity are improved under non-axenic conditions. Final lipid content was significantly higher for *Chlorella* and indigenous cultures under non-axenic conditions, due to competition for N by indigenous microorganisms. In addition, the presence of both indigenous RAS microorganisms and algae produced a treated wastewater effluent with low N and COD concentrations. Algae were mainly responsible for N removal, while microorganisms were mainly responsible for COD removal. Negative consequences of contamination of algal cultures with RAS microorganisms were not observed. This was probably due to the short growth period (72 h) in the batch system and small scale of the system.

Acknowledgments

Funding for this research was provided by the Norwegian Research Council (NRC; project number: O-08031), the US Department of Education Graduate Assistance in Areas of National Need (GANN; project number: P200A090162) program and the US-Norway Fulbright Foundation student grant program. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funding agencies.

References

[1] Nations FaAOotU, FAO Global Aquaculture Production Statistics, Food and Agriculture Organization of the United Nations, 2011. (ftp://ftp.fao.org/FI/news/GlobalAquacultureProductionStatistics2011.pdf. Accessed November 30th 2013).
[2] A. Graber, R. Junge, Aquaponic systems: nutrient recycling from fish wastewater by vegetable production, Desalination 246 (1–3) (2009) 147–156, http://dx.doi.org/10.1016/j.desal.2008.03.048.
[3] A.E. Ghaly, M. Kamal, N.S. Mahmoud, Phytoremediation of aquaculture wastewater for water recycling and production of fish feed, Environ. Int. 31 (1) (2005) 1–13, http://dx.doi.org/10.1016/j.envint.2004.05.011.
[4] P. De Schryver, R. Crab, T. Defoirdt, N. Boon, W. Verstraete, The basics of bio-flocs technology: the added value for aquaculture, Aquaculture 277 (3–4) (2008) 125–137, http://dx.doi.org/10.1016/j.aquaculture .2008.02.019.
[5] C.M. Drapcho, D.E. Brune, The partitioned aquaculture system: impact of design and environmental parameters on algal productivity and photosynthetic oxygen production, Aquac. Eng. 21 (3) (2000) 151–168, http://dx.doi.org/10.1016/S0144-8609(99)00028-X.
[6] M.E. Azim, D.C. Little, The biofloc technology (BFT) in indoor tanks: water quality, biofloc composition, and growth and welfare of Nile tilapia (Oreochromis niloticus), Aquaculture 283 (1–4) (2008) 29–35, http://dx.doi.org/10.1016/j.aquaculture.2008.06.036.
[7] K. Haglund, M. Pedersén, Outdoor pond cultivation of the subtropical marine red alga *Gracilaria tenuistipitata* in brackish water in Sweden. Growth, nutrient uptake, co-cultivation with rainbow trout and epiphyte control, J. Appl. Phycol. 5 (3) (1993) 271–284, http://dx.doi.org/10.1007/BF02186230.
[8] E.W. Becker, Micro-algae as a source of protein, Biotechnol. Adv. 25 (2) (2007) 207–210, http://dx.doi.org/10.1016/j.biotechadv.2006.11.002.
[9] I. Branyikova, B. Marsalkova, J. Doucha, T. Branyik, K. Bisova, V. Zachleder, M. Vitova, Microalgae—novel highly efficient starch producers, Biotechnol. Bioeng. 108 (4) (2011) 766–776, http://dx.doi.org/10.1002/bit.23016.
[10] J. Seyfabadi, Z. Ramezanpour, Z.A. Khoeyi, Protein, fatty acid, and pigment content of *Chlorella vulgaris* under different light regimes, J. Appl. Phycol. 23 (4) (2011) 721–726, http://dx.doi.org/10.1007/s10811-010-9569-8.
[11] Y. Avnimelech, Bio-filters: the need for an new comprehensive approach, Aquac. Eng. 34 (3) (2006) 172–178, http://dx.doi.org/10.1016/j.aquaeng.2005.04.001.
[12] R.A. Anderson (Ed.), Algal Culturing Techniques, Elsevier Academic Press, London, UK, 2005.
[13] A. Singh, P.S. Nigam, J.D. Murphy, Mechanism and challenges in commercialisation of algal biofuels, Bioresour. Technol. 102 (1) (2011) 26–34, http://dx.doi.org/10.1016/j.biortech.2010.06.057.
[14] S.A. Scott, M.P. Davey, J.S. Dennis, I. Horst, C.J. Howe, D.J. Lea-Smith, A.G. Smith, Biodiesel from algae: challenges and prospects, Curr. Opin. Biotechnol. 21 (3) (2010) 277–286, http://dx.doi.org/10.1016/j.copbio.2010.03.005.
[15] L. Xu, P.J. Weathers, X.-R. Xiong, C.-Z. Liu, Microalgal bioreactors: challenges and opportunities, Eng. Life Sci. 9 (3) (2009) 178–189, http://dx.doi.org/10.1002/elsc.200800111.
[16] O. Pulz, Photobioreactors: production systems for phototrophic microorganisms, Appl. Microbiol. Biotechnol. 57 (3) (2001) 287–293, http://dx.doi.org/10.1007/s002530100702.
[17] M. Gantar, J.P. Berry, S. Thomas, M. Wang, R. Perez, K.S. Rein, Allelopathic activity among Cyanobacteria and microalgae isolated from Florida freshwater habitats, FEMS Microbiol. Ecol. 64 (1) (2008) 55–64, http://dx.doi.org/10.1111/j.1574-6941.2008.00439.x.
[18] R. Costanza, Ecosystem health and ecological engineering, Ecol. Eng. 45 (0) (2012) 24–29, http://dx.doi.org/10.1016/j.ecoleng.2012.03.023.
[19] H. Thi Vu, S. Otsuka, H. Ueda, K. Senoo, Cocultivated bacteria can increase or decrease the culture lifetime of *Chlorella vulgaris*, J. Gen. Appl. Microbiol. 56 (5) (2010) 413–418.
[20] W.H. Bell, Bacterial utilization of algal extracellular products 3. The specificity of algal–bacterial interaction, Limnol. Oceanogr. 28 (1983) 1131–1143.
[21] E.J. Olguín, Dual purpose microalgae–bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a Biorefinery, Biotechnol. Adv. 30 (5) (2012) 1031–1046, http://dx.doi.org/10.1016/j.biotechadv.2012.05.001.
[22] P. Syrett, Nitrogen metabolism of microalgae, Can. Bull. Fish. Aquat. Sci. 1981 (1981).
[23] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[24] L.B. Christenson, R.C. Sims, Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products, Biotechnol. Bioeng. 109 (7) (2012) 1674–1684, http://dx.doi.org/10.1002/bit.24451.
[25] O.K. Dalrymple, T. Halfhide, I. Udom, B. Gilles, W.J., Q. Zhang, S.J. Ergas, A preliminary estimation of the algal feedstock production potential of Tampa Bay utilizing CO_2 emissions and wastewater effluent, J. Aquat. Biosyst. 9 (2) (2013).
[26] A.D. Eaton, L.S. Clesceri, E.W. Rice, A.E. Greenberg (Eds.), Standard Methods: For the Examination of Water and Wastewater, American Public Health Association, American Water Works Association, Water Environment Federation, 2005.
[27] M.C. Franco, M.F. Buffing, M. Janssen, C.V. Lobato, R.H. Wijffels, Performance of Chlorella sorokiniana under simulated extreme winter conditions, J. Appl. Phycol. 24 (4) (2012) 693–699, http://dx.doi.org/10.1007/s10811-011-9687-y.
[28] H. Liechtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Methods Enzymol. 148 (1987) 350–382.
[29] O.-I. Lekang, Aquaculture Engineering, Blackwell Publishing Limited, 9600 Garsington Road, Oxford OX4 2DQ, United Kingdom, 2007.
[30] J.M. Ebeling, M.B. Timmons, J.J. Bisogni, Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems, Aquaculture 257 (1–4) (2006) 346–358, http://dx.doi.org/10.1016/j.aquaculture.2006.03.019.
[31] O. Perez-Garcia, F.M.E. Escalante, L.E. de-Bashan, Y. Bashan, Heterotrophic cultures of microalgae: metabolism and potential products, Water Res. 45 (1) (2011) 11–36, http://dx.doi.org/10.1016/j.watres.2010.08.037.
[32] T. Källqvist, A. Svenson, Assessment of ammonia toxicity in tests with the microalga, *Nephroselmis pyriformis*, Chlorophyta, Water Res. 37 (3) (2003) 477–484.
[33] J. Park, H. Fjin, B.R. Lim, K.Y. Park, K. Lee, Ammonia removal from anaerobic digestion effluent of livestock waste using green algae *Scenedesmus* sp. Bioresour. Technol. 101 (22) (2010) 8649–8657.
[34] L. Taiz, E. Zeiger, Plant Physiology, Sinauer Associates Inc., Sunderland, MA 01375, 2006.
[35] A. Abeliovich, Y. Azov, Toxicity of ammonia to algae in sewage oxidation ponds, Appl. Environ. Microbiol. 31 (1976) 801–806.
[36] S. Porrello, M. Lenzi, E. Persia, P. Tomassetti, M.G. Finoia, Reduction of aquaculture wastewater eutrophication by phytotreatment ponds system: I. Dissolved and particulate nitrogen and phosphorus, Aquaculture 219 (1–4) (2003) 515–529, http://dx.doi.org/10.1016/S0044-8486(02)00656-7.
[37] I.T.D. Cabanelas, J. Ruiz, Z. Arbib, F.A. Chinalia, C. Garrido-Pérez, F. Rogalla, I.A. Nascimento, J.A. Perales, Comparing the use of different domestic wastewaters for coupling microalgal production and nutrient removal, Bioresour. Technol. 131 (0) (2013) 429–436, http://dx.doi.org/10.1016/j.biortech.2012.12.152.
[38] A.M. Illman, A.H. Scragg, S.W. Shales, Increase in Chlorella strains calorific values when grown in low nitrogen medium, Enzyme Microb. Technol. 27 (8) (2000) 631–635, http://dx.doi.org/10.1016/S0141-0229(00)00266-0.
[39] T. Tornabene, G. Holzer, S. Lien, N. Burris, Lipid-composition of the nitrogen starved green-alga *Neochloris oleoabundans*, Enzyme Microb. Technol. 5 (6) (1983) 435–440.
[40] J.L. Harwood, A.L. Jones, Lipid metabolism in algae, in: J.A. Callow (Ed.), Advances in Botanical Research, vol. 16, Academic Press, 1989, pp. 1–53, http://dx.doi.org/10.1016/S0065-2296(08)60238-4.
[41] G.O. James, C.H. Hocart, W. Hillier, H. Chen, F. Kordbacheh, G.D. Price, M.A. Djordjevic, Fatty acid profiling of *Chlamydomonas reinhardtii* under nitrogen deprivation, Bioresour. Technol. 102 (3) (2011) 3343–3351, http://dx.doi.org/10.1016/j.biortech.2010.11.051.
[42] B. Rusten, A.K. Sahu, Microalgae growth for nutrient recovery from sludge liquor and production of renewable bioenergy, Water Sci. Technol. 64 (6) (2011) 1195–1201, http://dx.doi.org/10.2166/wst.2011.722.
[43] A. Meriac, E.H. Eding, J. Schrama, A. Kamstra, J.A.J. Verreth, Dietary carbohydrate composition can change waste production and biofilter load in recirculating

aquaculture systems, Aquaculture 420–421 (0) (2014) 254–261, http://dx.doi.org/10.1016/j.aquaculture.2013.11.018.
[44] C. Yang, Q. Hua, K. Shimizu, Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions, Biochem. Eng. J. 6 (2) (2000) 87–102, http://dx.doi.org/10.1016/S1369-703X(00)00080-2.
[45] J. Ogbonna, H. Yoshizawa, H. Tanaka, Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms, J. Appl. Phycol. 12 (3–5) (2000) 277–284, http://dx.doi.org/10.1023/A:1008188311681.
[46] C.I.M. Martins, M.G. Pistrin, S.S.W. Ende, E.H. Eding, J.A.J. Verreth, The accumulation of substances in Recirculating Aquaculture Systems (RAS) affects embryonic and larval development in common carp *Cyprinus carpio*, Aquaculture 291 (1–2) (2009) 65–73, http://dx.doi.org/10.1016/j.aquaculture.2009.03.001.
[47] K. Knauer, R. Behra, L. Sigg, Effects of free Cu^{2+} and Zn^{2+} ions on growth and metal accumulation in freshwater algae, Environ. Toxicol. Chem. 16 (2) (1997) 220–229, http://dx.doi.org/10.1002/etc.5620160218.
[48] Y. Liang, N. Sarkany, Y. Cui, Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions, Biotechnol. Lett. 31 (7) (2009) 1043–1049, http://dx.doi.org/10.1007/s10529-009-9975-7.
[49] M.B. Johnson, Z. Wen, Production of biodiesel fuel from the microalga *Schizochytrium limacinum* by direct transesterification of algal biomass, Energy Fuel 23 (10) (2009) 5179–5183, http://dx.doi.org/10.1021/ef900704h.
[50] S.-L. Guo, X.-Q. Zhao, C. Wan, Z.-Y. Huang, Y.-L. Yang, M. Asraful Alam, S.-H. Ho, F.-W. Bai, J.-S. Chang, Characterization of flocculating agent from the self-flocculating microalga *Scenedesmus obliquus* AS-6-1 for efficient biomass harvest, Bioresour. Technol. 145 (0) (2013) 285–289, http://dx.doi.org/10.1016/j.biortech.2013.01.120.
[51] D.C. Manheim, Improved Microbial Biomass Harvesting Using Optimized Environmental Conditions and Bacterial Bioflocculants, California Polytechnic State University, San Luis Obispo, 2012.
[52] L. Rodolfi, G. Chini Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M.R. Tredici, Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, Biotechnol. Bioeng. 102 (1) (2009) 100–112, http://dx.doi.org/10.1002/bit.22033.
[53] C. Theegala, R. Malone, K. Rusch, Contaminant washout in a hydraulically integrated serial turbidostat algal reactor (HISTAR), Aquac. Eng. 19 (4) (1999) 223–241, http://dx.doi.org/10.1016/S0144-8609(98)00051-X.
[54] S.A. Mitchell, A. Richmond, The use of rotifers for the maintenance of monoalgal mass cultures of Spirulina, Biotechnol. Bioeng. 30 (2) (1987) 164–168, http://dx.doi.org/10.1002/bit.260300205.
[55] V.H. Smith, T. Crews, Applying Ecological Principles of Crop Cultivation in Large-scale Algal Biomass Production, Research, Algal, 2013.
[56] M. Siaut, S. Cuine, C. Cagnon, B. Fessler, M. Nguyen, P. Carrier, A. Beyly, F. Beisson, C. Triantaphylides, Y. Li-Beisson, G. Peltier, Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves, BMC Biotechnol. 11 (1) (2011) 7.
[57] Y. Li, D. Han, G. Hu, M. Sommerfeld, Q. Hu, Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*, Biotechnol. Bioeng. 107 (2) (2010) 258–268.
[58] Z. Wang, N. Ullrich, S. Joo, S. Waffenschmidt, U. Goodenough, Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*, Eukaryot. Cell 8 (12) (2009) 1856–1868.
[59] N.M. Córdoba-castro, A.M. Montenegro-jaramillo, R.E. Prieto, G.E. González-Mariño, Analysis of the effect of the interactions among three processing variables for the production of exopolysaccharides in the microalgae *Scenedesmus obliquus* (Utex 393), Vitae 19 (2012) 60–69.
[60] P. Schenk, S. Thomas-Hall, E. Stephens, U. Marx, J. Mussgnug, C. Posten, O. Kruse, B. Hankamer, Second generation biofuels: high-efficiency microalgae for biodiesel production, Bioenergy Res. 1 (1) (2008) 20–43, http://dx.doi.org/10.1007/s12155-008-9008-8.
[61] Y. Zhu, K.O. Albrecht, D.C. Elliott, R.T. Hallen, S.B. Jones, Development of hydrothermal liquefaction and upgrading technologies for lipid-extracted algae conversion to liquid fuels, Algal Res. 2 (4) (2013) 455–464, http://dx.doi.org/10.1016/j.algal.2013.07.003.
[62] T. Heredia-Arroyo, W. Wei, B. Hu, Oil accumulation via heterotrophic/mixotrophic chlorella protothecoides, Appl. Biochem. Biotechnol. 162 (7) (2010) 1978–1995, http://dx.doi.org/10.1007/s12010-010-8974-4.
[63] V.O. Adesanya, M.P. Davey, S.S. Scott, A.G. Smith, Kinetic modelling of growth and storage molecule production in microalgae under mixotrophic and autotrophic conditions, Bioresour. Technol. (2012), http://dx.doi.org/10.1016/j.biortech.2014.01.032.
[64] L.M.L. Laurens, T.A. Dempster, H.D.T. Jones, E.J. Wolfrum, S. Van Wychen, J.S.P. McAllister, M. Rencenberger, K.J. Parchert, L.M. Gloe, Algal biomass constituent analysis: method uncertainties and investigation of the underlying measuring chemistries, Anal. Chem. 84 (4) (2012) 1879–1887, http://dx.doi.org/10.1021/ac202668c.
[65] Z.A. Khoeyi, J. Seyfabadi, Z. Ramezanpour, Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae, *Chlorella vulgaris*, Aquac. Int. 20 (1) (2012) 41–49, http://dx.doi.org/10.1007/s10499-011-9440-1.
[66] M. Ponraj, M. Fadhil Md Din, Effect of light/dark cycle on biomass and lipid productivity by *Chlorella pyrenoidosa* using Palm Oil Mill Effluent (POME), J. Sci. Ind. Res. 72 (11) (2013) 703–706 (http://nopr.niscair.res.in/bitstream/123456789/22633/1/JSIR%2072%2811%29%20703-706.pdf).