Microalgae for aquaculture

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Thesis

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CHAPTER 1

General introduction

1 Introduction

The research of this thesis was imbedded in the Zeeland Sole project. Before describing the actual research performed for the thesis in detail, the project as a whole will be introduced.

The Zeeland Sole Project (Zeeuwse Tong Project)

In 2004, the project 'Zilte Zoom' was initiated in order to develop new economic activities at the boundary of sea and land. One of the ideas was to develop an innovative aquaculture system for the production of sole (*Solea solea*). Sole is the most valuable flatfish, which accounts for 35% of the value of all auctioned fish in the Netherlands. The Dutch quota for sole fisheries decreased from about 15,000 to 9,000 ton in the last 10 years and it was estimated that there would be room for the production of 15,000 ton of sole per year in fish farms for the (export) market.

The Dutch fish farming sector, culturing fish species like catfish, eel and turbot, is mainly using recirculating aquaculture systems (RAS). In these systems fish are reared from fingerlings to the size for consumption. The wastewater produced by the fish is recycled after treatment. Despite the efficient use of water, these systems are still highly capital and labor intensive.

As an innovative and costs reducing alternative for culturing sole, an integrated multi-trophic aquaculture system was suggested (Fig. 1). In this approach, the indoor production of fingerlings was proposed, followed by a grow-out phase in basins outside. The grow-out phase of sole would be combined with ragworm (*Nereis virens*) culture in the same basins. The nutrient-rich wastewater produced by the sole, could be used to grow microalgae. The algae can be used as feed for the ragworms or as feed for shellfish (Broodman, 2006).

Based on this idea, the foundation 'Zeeuwse Tong' was initiated on the 26th of June 2007 (Stichting Zeeuwse Tong, 2009). A consortium of different companies and research institutes was formed, financially supported by the province of Zeeland in the Netherlands.

The overall aim of the Zeeuwse Tong project was to develop a new competitive land-based aquaculture sector, which is producing sole, ragworms, algae, shellfish and saline crops in close harmony with nature. Within the project, two concepts for an

integrated multi-trophic aquaculture were defined: The development of an integrated saline aquaculture farm and the development of an integrated nursery. This last concept forms the direct base for the research described in this thesis.

First concept: The integrated saline aquaculture farm

The integrated saline aquaculture farm is a land-based integrated multi-trophic aquaculture activity. Recycling and reusing water and nutrients is a main part of this type of aquaculture. Together with the sole, ragworms are produced in basins as feed for the sole. The wastewater from the basins is full of nutrients and these are used to stimulate the production of algae and saline crops. The produced algae can then be used as feed for shellfish and ragworms (Fig. 1). The main reasons for coupling the production of ragworms, fish, algae, shellfish and saline crops are to reduce the losses of nutrients to the environment preventing eutrophication and to reuse these nutrients as a valuable source for the assimilation into the food production chain via algae and plants, which are the basis for food production.

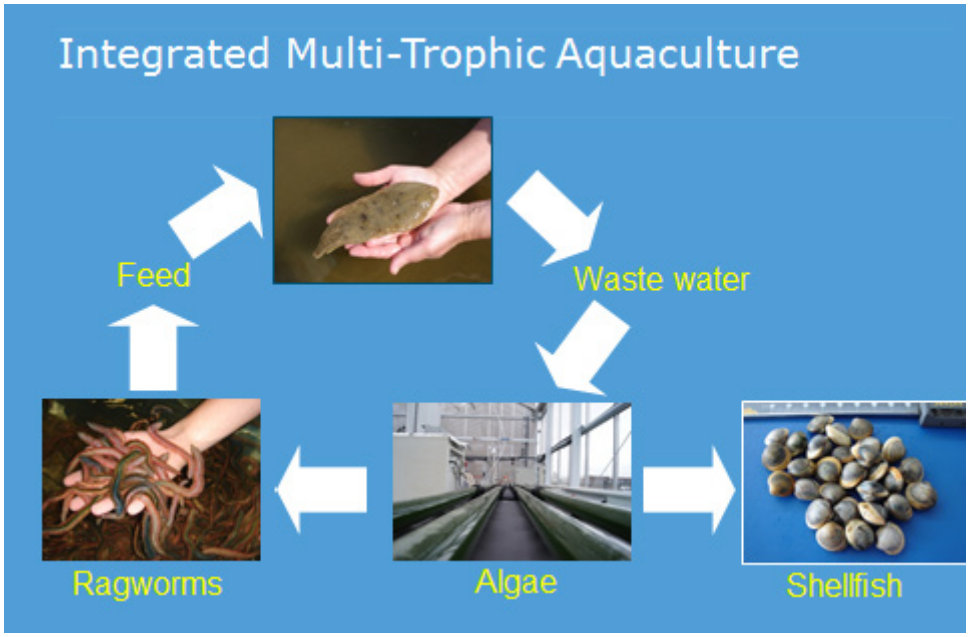

Fig. 1 Integrated multi-trophic aquaculture as concept in the Zeeland Sole project.

A demonstration aquaculture farm 'Zeeuwse Tong' was built in Colijnsplaat, the Netherlands. During 4 years, from 2010 till 2014, research was done on the technical and economic feasibility of this innovative concept of integrated multi-trophic

aquaculture. The demonstration aquaculture farm consisted of 12 experimental basins of 1,000 m^2 each for the cultivation of ragworms, sole, shellfish and algae. They were either produced in multi-culture mode or in separate cultures. Seawater from the Easterscheldt was supplied via pipes, constructed underneath the dike. Before sending the water to the basins it was filtered and its temperature was adjusted. Cooling and heating of the water was achieved by an aquifer thermal energy storage.
In February 2014 this part of the Zeeuwse Tong project was finalized, and the main conclusion was that integrated multi-trophic aquaculture is technically feasible. Currently, negotiations are going on to initiate new businesses at the demonstration aquaculture farm and its facilities in Colijnsplaat, the Netherlands.

Second concept: Integrated nursery

In the integrated nursery the rearing of fingerlings of sole is combined with the cultivation of microalgae as feed for shellfish larvae and spat inside a greenhouse (Bot et al., 2009). The advantages of the integrated nursery in a greenhouse are the lower costs for a greenhouse when it is constructed as a multipurpose use of space, the possibility of combining sole culture with the cultivation of microalgae and shellfish larvae or spat, an integrated thermoregulation and the possibility for reusing the nutrients from the wastewater of the fish basins for microalgal production in closed photobioreactors (PBRs). The idea is to cultivate microalgae in closed PBRs, which can also function as solar collectors. The captured sunlight is not only used for the growth of the microalgae. A lot of the energy can be used to keep the temperature of the basins in which the fingerlings are reared at the optimal temperature. Furthermore, waste heat of power engines needed for the electricity supply can be used as well. Surplus of heat in summer months can be stored in aquifers, which can be used for temperature control in winter. The groundwater of the aquifer will be cooled in winter, which can be applied for cooling the PBRs during warm periods.

For the design of the nursery, different options for the lay-out of the PBRs are possible, of which a layer of horizontal tubular PBRs above the fish basins and racks of horizontal vertically stacked tubular PBRs between the fish basins are depicted in Fig. 2.

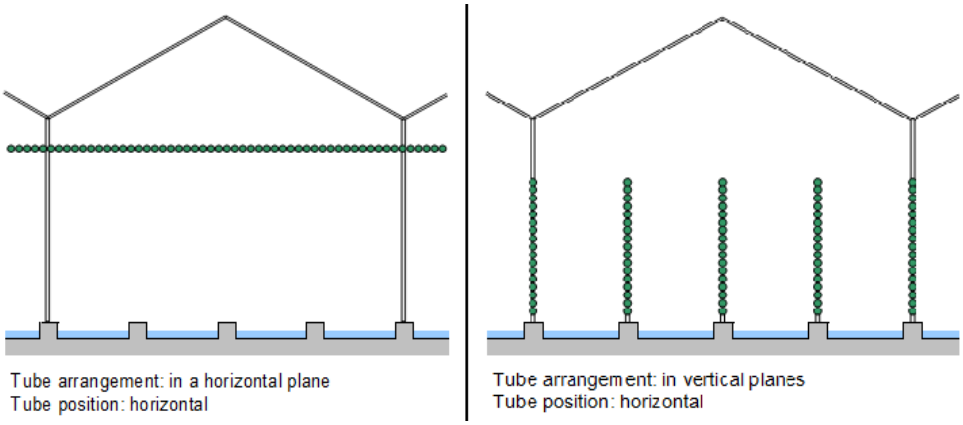

Fig. 2 Different lay-outs of photobioreactors in a greenhouse in combination with basins for rearing fingerlings of sole.

Aim

The aim of this thesis project is to find out if closed tubular PBRs are an interesting alternative for cultivation of microalgae for aquaculture and to investigate if the microalgae production can be integrated in the multi-trophic aquaculture in a sustainable and economically viable way.

Thesis outline

To investigate if the cultivation of microalgae as part of the integrated multi-trophic nursery was possible, a closed photobioreactor system was required. This system should enable the culturing of the shear stress sensitive algal strains that are typically used in hatcheries.

In **Chapter 2** the effects of shear stress on the viability of *Chaetoceros muelleri* was studied. Different levels of shear stress were applied to determine the maximum level of shear stress that can be tolerated by the microalgae. The results could therefore be used for an optimal design of the PBR system. When shear stress levels evoked in PBRs are lower than the maximum level of shear stress that can be tolerated, the viability of the microalgae will not be negatively affected.

Based on this study, a pilot-scale horizontal tubular PBR equipped with a “low shear” pump was designed and built for conducting several experiments (Fig. 3).

Fig. 3 Pilot-scale horizontal tubular photobioreactor in the SEA Lab of HZ University of Applied Sciences in Vlissingen, the Netherlands.

Chapter 3 describes the study on the effect of biomass concentration on the productivity of *Tetraselmis suecica* in the pilot-scale tubular PBR using natural sunlight. The net volumetric productivity and biomass yield on light at 5 different biomass concentrations were determined. When microalgae are cultivated using natural sunlight, losses in productivity in the dark zones of the tubes and losses of photons during high light periods were expected. The optimal biomass concentration was determined at which a high net productivity and a high yield on light were reached while using sunlight as a light source. Yield of biomass on light was used as an important parameter to find the optimal biomass concentration.

Cultivating microalgae in the integrated multi-trophic nursery has two advantages. Wastewater from a fish farm can be purified by microalgae and the wastewater provides free nutrients for the production of microalgae, which can then serve as feed for filter-feeding larvae. In addition, the purified water can be re-used. In **Chapter 4** growth of *Tetraselmis suecica* on wastewater of a fish farm was studied, to verify if indeed waste-water from a fish farm can be applied. The N and P removal efficiencies and the productivity of *Tetraselmis suecica* in a tubular PBR using wastewater from a fish farm were determined. Next to optimizing N and P removal efficiencies, the

volumetric productivity was optimized by studying the effect of addition of the limiting nutrient, at different biomass concentrations in the PBR.

The effect of cooling in the night on the productivity and biochemical composition of *Tetraselmis suecica* is described in **Chapter 5**. This experiment was done to find out if the net productivity can be increased by decreasing the biomass loss due to respiration during the night. Furthermore, the effect of cooling the microalgae during the night on the changes in biochemical composition measured as carbohydrates, proteins and fatty acids were studied. Because the chemical composition of microalgae changes over a day with an increase of carbohydrates during daylight and an increase of proteins in the night, the diurnal changes of the biochemical composition were also determined. Since high light intensities during the day lead to higher concentrations of carbohydrates, light was considered as an important parameter for this study. Therefore, the relation between growth rate during the day and carbohydrate loss rate during the night afterwards was taken into account as well.

The study described in **Chapter 6** was done to determine the shear stress sensitivity of *Tetraselmis suecica*, *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri*. Because these four different microalgal species are frequently used as feed in aquaculture, there is a huge interest to produce these species at larger scale in fully-automated PBRs. However, high shear stress zones in the reactor including the pump can be present, which prevents shear stress sensitive microalgal species to grow. The same method as described in the study of **Chapter 2** to determine the shear stress sensitivity of *Chaetoceros muelleri* was used to quantify the tolerance to shear stress of the other microalgal species. The tubular PBR with a centrifugal pump was used to find out if the four different microalgal species were able to grow, while the growth or lack of growth of microalgae was related to shear stress levels occurring inside the reactor and the determined shear tolerance levels for the four species.

The general discussion (**Chapter 7**) gives an overview of the prospects of microalgal production in tubular photobioreactors in hatcheries. This chapter describes the possibilities of realizing a more cost-effective and sustainable production facility of the microalgae. The main culture conditions are discussed, which are control of contamination, flow velocity in PBRs in relation to shear stress, temperature control, efficient use of CO_2 and nutrients, and light. The costs were calculated for a microalgal production facility of 0.1 ha and 1 ha. For the costs calculations, realistic

productivity data obtained during all the experiments described in **Chapter 3**, **4**, **5** and **6** were used. Furthermore, wastewater as a free nutrient source, temperature control with cogeneration (waste heat from power engine for heating) and cooling with groundwater from an aquifer, and the use of sunlight are important factors for the costs calculations, which can make microalgal production for aquaculture more economic and sustainable.

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CHAPTER 2

Effects of shear stress on the microalgae *Chaetoceros muelleri*

This chapter is published as:

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Abstract

The effect of shear stress on the viability of *Chaetoceros muelleri* was studied using a combination of a rheometer and dedicated shearing devices. Different levels of shear stress were applied by varying the shear rates and the medium viscosities. It was possible to quantify the effect of shear stress over a wide range, whilst preserving laminar flow conditions through the use of a thickening agent. The threshold value at which the viability of algae was negatively influenced was between 1 and 1.3 Pa. Beyond the threshold value the viability decreased suddenly to values between 52% and 66%. The effect of shear stress was almost time independent compared to normal microalgae cultivation times. The main shear stress effect was obtained within 1 min, with a secondary effect of up to 8 min.

Introduction

Microalgae are cultivated to serve as feed for aquaculture (shellfish, shrimps and fish), animal feed (pets and farming), human nutrition, cosmetics and the production of high-value ingredients, such as polyunsaturated fatty acids and pigments (Spolaore et al., 2006). The current market size of microalgal biomass is about 5000 t dry matter/year of which at least 1000 t dry matter/year is produced for aquaculture (Muller-Feuga, 2004; Pulz and Gross, 2004).

We are interested in the production of microalgae as a feed for bivalves. In general, cultivation of microalgae for these applications takes place in transparent plastic bags. Because the capacity of these systems is relatively small, labor costs per amount of feed for bivalve production is high. This explains why alternative large scale production systems for microalgae are being investigated. Such systems can consist of open ponds, bubble columns, flat-plate photobioreactors (PBRs) and tubular PBRs (Borowitzka, 1997; Ugwu et al., 2008; Xu et al., 2009). PBRs seem to be most promising in cultivating these microalgae on a large scale, because of low contamination risk, low space requirement, almost no CO_2 loss, high adaptability to many species and high biomass concentration (Pulz, 2001; Richmond, 2000). However, it is suspected that the large hydrodynamic forces present in the PBR could cause shear stress levels that might be too severe for the sensitive microalgae, which would lead to reduced growth or even cell death (Lebeau and Robert, 2003). Nevertheless, a high mixing intensity is necessary to keep microalgae in suspension, to achieve a sufficient light distribution and to enhance mass transfer. For the optimal design of PBRs, it is therefore important to know the maximum level of shear stress that can be tolerated by the microalgae.

Damage because of shear stress has been demonstrated both in bubble columns due to gas sparging (Alías et al., 2004; Barbosa et al., 2003; Barbosa et al., 2004; Sánchez Mirón et al., 2003; Suzuki et al., 1995) and in tubular PBRs due to pumping action (Alías et al., 2004; Jaouen et al., 1999; Vandanjon et al., 1999). Although cases of shear damage were described, shear stress was only quantified indirectly by using the liquid flow rate or the number and frequency of pump passages. A first step towards quantification of the shear rate or shear stress was done by Contreras et al. (1998) and García Camacho et al. (2007). They used the energy dissipation to calculate an average shear rate or shear stress. For *Phaeodactylum tricornutum*, a shear rate value of 7000 s^{-1} led to the highest growth rate caused by a balance between mass transfer limitations and shear damage (Contreras et al., 1998).

Sensitivity to shear stress is dependent on the microalgae species used. This has been shown in a study with the dinoflagellate *Protoceratium reticulatum*, where a damage threshold was observed already at an average shear stress of 0.16 mPa, which was equivalent to a shear rate of 0.12 s^{-1} (García Camacho et al., 2007).

In order to apply well-defined shear stress, Couette devices have been used in several studies. Couette devices consist of coaxial cylinders, where either the inner cylinder rotates and the outer remains stationary or vice versa. The effect of shear stress on photosynthesis of *Spirulina platensis* has been studied using a Couette device (Mitsuhashi et al., 1995). It was found that the oxygen production rate and the chain length of *Spirulina platensis* started to decrease when shear stress exceeded 0.3 Pa. Experiments on cyanobacteria (Moisander et al., 2002) indicated that a shear rate of as little as 2.2 s^{-1} reduced nitrogenase activity and CO_2 fixation. However, the applied shear stress levels were not given, because the shear viscosity was not measured. Couette shearing devices have also been used to study the effect of shear stress on bioluminescence of different dinoflagellate species (Cussatlegras and Le Gal, 2005; Cussatlegras and Le Gal, 2007; Latz et al., 1994; Maldonado and Latz, 2007). Bioluminescence threshold values were on the order of 0.1 - 1 Pa, where the shear stress was calculated as a function of the shear rate and the viscosity of the medium. Shear stress thus appears to trigger bioluminescence in these cases, but the relationship to cell damage or viability is unclear.

The aim of this study is to determine the shear sensitivity of *Chaetoceros muelleri*. In addition, the time scale in which the effects of shear stress take place will be examined. The purpose was to understand whether the adverse effects were caused by a prolonged exposure to excessive shear stress or if the algal cells were damaged instantaneously. The combination of shear cylinders as Couette devices and rheological measurements were used in order to study the effect of a uniform and well-defined shear stress on the viability of *Chaetoceros muelleri*.

Materials and methods

Culture preparation

The diatom *Chaetoceros muelleri* (CCMP 1316) was obtained from NIOO (Netherlands Institute of Ecology, Yerseke, The Netherlands). *Chaetoceros muelleri* was cultivated in natural seawater from the Easterscheldt (location Yerseke) enriched with Walne

medium modified from Laing (1991). The salinity of the seawater used was 32 g L^{-1}.

Cultures of *Chaetoceros muelleri* were maintained in 10 mL test tubes, 250 mL Erlenmeyer flasks containing 100 mL medium and 3 L Erlenmeyer flasks containing 2 L medium at 20 °C. Light was supplied continuously by white fluorescent light. Every week 10% of the culture was transferred to new medium.

All shear stress experiments were carried out with batch cultures from the 3 L Erlenmeyer flasks after one week of growth, at which point the algae were in the early stationary phase. Locust bean gum (LBG) was applied as a thickener to increase the medium viscosity. In the rest of this article, the term medium refers to the enriched seawater including the thickener. The thickener was used in the following concentrations: 0%, 0.3% and 0.5%. Cell concentrations in the experiments varied between 5 and 10 million per mL. The cell concentrations in the LBG solutions were 50% of the original culture, because 50 mL of the original culture was mixed with 50 mL of 0.6% or 1.0% LBG solution.

Shearing the algae

The effect of shear stress on the viability of *Chaetoceros muelleri* was studied, using four shear cylinders developed at the Laboratory of Food Process Engineering. The shear cylinders have the following dimensions: an inner cylinder with a length of 145.5 mm and a radius of 20 mm and an outer cylinder with a radius of 21 mm. Between the inner and the outer cylinder is a gap of 1 mm. The total volume between the cylinders is 20 mL. The inner cylinder rotates and the outer cylinder is stationary. The shear rate applied in the shear cylinders is proportional to the rotational speed; the conversion factor of rotational speed (rpm) to shear rate (s^{-1}) is 2.157. The maximum shear rate of the shear cylinders is 1,079 s^{-1} obtained at a rotational speed of 500 rpm. The shear rate is given as:

$$\dot{\gamma} = \frac{2R_o R_i \omega}{R_o^2 - R_i^2} \qquad (1)$$

where $\dot{\gamma}$ is the shear rate (s^{-1}), ω is the angular velocity of the outer cylinder (s^{-1}), R_o is the outer cylinder inner radius (m) and R_i is the inner cylinder outside radius (m) with:

$$\omega = \frac{2\pi \cdot n}{60} \qquad (2)$$

where *n* is the rotational speed (rpm) of the shear cylinder.

In the first experiment *Chaetoceros muelleri* without LBG was exposed to different levels of rotational speed, which were 0, 4, 20, 100 and 500 rpm, respectively. The levels of rotational speed applied to the algae in 0.3% LBG were 0, 4, 10, 15, 20, 100 and 500 rpm, respectively. The algae in 0.5% LBG were exposed to the following levels of rotational speed: 0, 2, 4, 10, 20 and 100 rpm. The exposure time to the different levels of shear stress was 1 h and the temperature 4 °C. All exposures in the shear cylinders were done in triplicate.

A second experiment was carried out to investigate the time dependence of shear stress effect. Algae in 0.3% LBG were exposed to a rotational speed of 100 rpm for different times of exposure ranging from 1 to 120 minutes. The temperature was 4 °C as in the first experiment. All tests were done in triplicate.

For all treatments the Taylor number was calculated to find out whether flow instabilities occurred. Taylor vortices will be formed when the Taylor number is higher than the limiting value of 41.3 (Bird, 2002; Mezger, 2006). The Taylor number is defined as:

$$Ta = \left[\omega \cdot \rho \cdot R_i^2 \cdot \left(\delta_{cc} - 1\right)^{3/2}\right] / \eta \qquad (3)$$

where ω is the angular velocity, ρ is 1024 kg m^{-3} for seawater with algae at 4 °C, η is the apparent viscosity (Pa s) and δ_{cc} is the ratio of the outer to the inner cylinder radius, which is 1.05.

Rheological characterization

The shear stress was measured with a sample of 3.6 mL of the algae (without LBG, in 0.3% and 0.5% LBG solution) after exposure in the shear cylinders. A rheometer (type Physica MCR 301, Anton Paar) was used to measure the exact shear stress applied in the shear cylinders. The measurements were done at 4 °C.

From the shear stress, the apparent viscosity can be calculated using the following equation:

$$\eta = \frac{\tau}{\dot{\gamma}} \qquad (4)$$

where τ is the shear stress (Pa), η is the apparent viscosity (Pa s) and $\dot{\gamma}$ is the shear rate (s^{-1}).

For Newtonian fluids, η is independent of $\dot{\gamma}$ and for non-Newtonian fluids, η depends on $\dot{\gamma}$ (Mezger, 2006). The power-law model was used to describe this dependence (Carreau et al., 1997; O'Connor et al., 2002).

$$\eta = m\left|\dot{\gamma}\right|^{n-1} \qquad (5)$$

The parameters *m* and *n* were measured from a log-log plot with apparent viscosity against shear rate.

The same power-law model is used to measure the dependence between shear rate and shear stress (Mezger, 2006).

$$\tau = c\dot{\gamma}^{n} \qquad (6)$$

The parameters *c* and *n* were also measured from a log-log plot of shear stress versus shear rate.

Assessment of the viability

The viability of the algae was measured by using the fluorescein diacetate (FDA) staining method. Esterases in viable cells cleave FDA with the formation of fluorescein as a result. Fluorescein is a compound that fluoresces green in viable cells and does not give fluorescence in non-viable cells (Altman et al., 1993; Rotman and Papermaster, 1966). A FDA stock solution was prepared by dissolving 46 mg FDA (Sigma) in 10 mL acetone (11 mM) and stored in the dark at -4 °C. One milliliter of the sample containing the algae was incubated with 10 µL FDA stock solution for 20 min (Sipkema et al., 2004). Then the total amount of algal cells and the viable cells were counted in a cell chamber (hemocytometer DHC-B02-5 Büker Türk) using a fluorescence microscope (Olympus IX71). Viability of the algae was taken as the percentage of fluorescing algae.

In LBG, the algal cells did not settle at the bottom of the cell chamber, but were suspended over the 0.1 mm depth of the cell chamber. To count the cells, it was therefore necessary to scan over the depth of the cell chamber by adjusting the focus of the microscope.

Results and discussion

Flow regime characterization

Figure 1 shows the relation between the shear rate and shear stress applied to the algae suspension without LBG, in 0.3% LBG and in 0.5% LBG. As expected, higher shear stress levels could be obtained through an increase in either the shear rate or the viscosity, with the help of a thickener. LBG led to a shear-thinning behavior. The slopes of the flow curves with shear stress as a function of shear rate on a logarithmic scale are less steep for the LBG concentrations. This can also be seen in the power-law model functions where $n < 1$ for the algae in LBG, while $n \approx 1$ for the algae without LBG (Eq. 6).

The shear stress levels that were applied in the further experiments were calculated with the power-law model functions in Fig. 1 and are presented in Table 1.

Viscosity as a function of shear rate is shown in Fig. 2 for the algae without LBG, the algae in 0.3% LBG and 0.5% LBG, respectively. The viscosity of the algae without LBG, which is the original culture of *Chaetoceros muelleri* in the medium, is almost independent of the shear rate. Therefore, the algae without LBG behave nearly Newtonian. This Newtonian behavior suggests that there is little interaction between the algal cells in the suspension and that they behave as separate cells, rather than clustered cells.

Non-Newtonian behavior is apparent for the algae in LBG, where shear-thinning is evident. Therefore, the apparent viscosity needs to be determined at the different shear rates for the calculation of the accurate shear stress levels applied. A study of shearing insect cells has also indicated that assuming Newtonian behavior of cell cultures could cause substantial error in shear stress estimates (O'Connor et al., 2002).

The Taylor number for every apparent viscosity value were calculated with Eq. 3 (Table 1). In all experiments laminar flow was maintained, except at the highest shear rate of 1079 s^{-1} in the algae dispersion without LBG. Taylor vortices may have been formed during that experiment and this could have led to secondary flow to a certain extent. The highest shear rate that can be applied for algae without LBG, while keeping the algae in laminar flow, is about 150 s^{-1}. The use of LBG increased the viscosity, leading to a lower Taylor number. For that reason, LBG or other thickeners can be used to reach higher shear stress under stable laminar flow to the extent that they do not affect the viability directly. Maldonado and Latz (2007) also used thickeners to increase the viscosity for applying two different shear stress levels at the same shear rate to study the short-term effect of shear stress on a dinoflagellate.

The dimensions of the shear cylinders are also important in creating a stable laminar flow, because the diameter of the inner cylinder and the gap between the inner and outer cylinder are relatively small. A Couette device with a bigger inner cylinder diameter and a relatively bigger gap leads to a smaller critical angular velocity with the formation of Taylor vortices or turbulent flow as a result (Hondzo et al., 1997; Warnaars and Hondzo, 2006). It is difficult to determine the applied shear stress if flow instabilities are present.

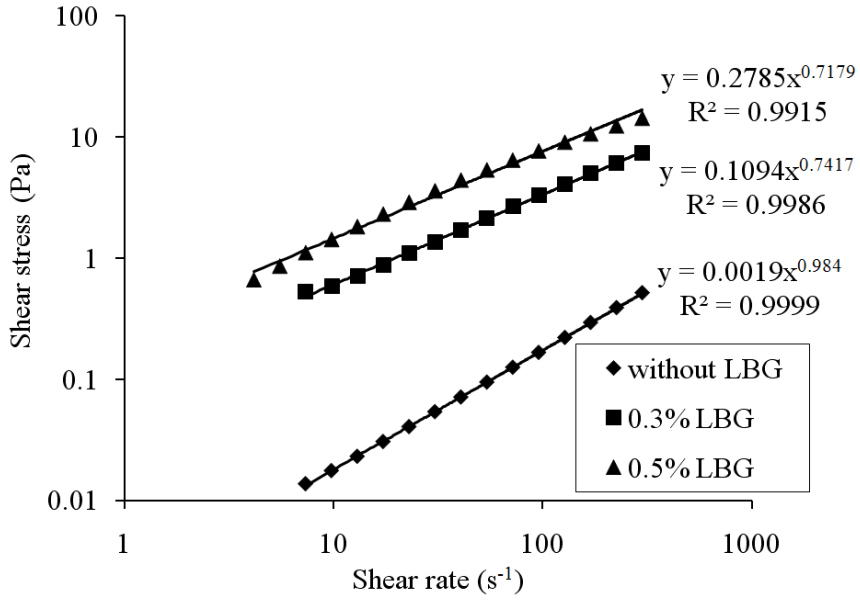

Fig. 1 Shear stress as a function of shear rate: (♦) algae without LBG, (■) algae in 0.3% LBG and (▲) algae in 0.5% LBG.

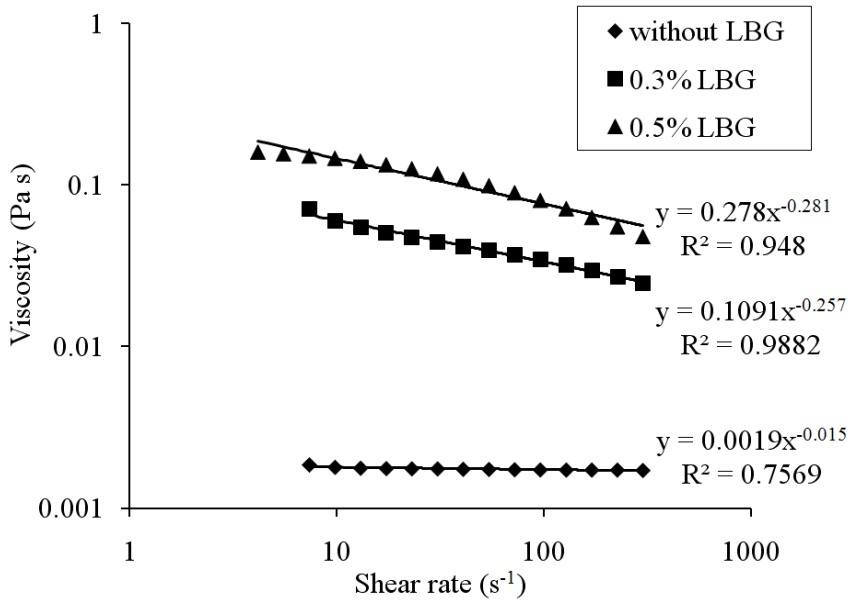

Fig. 2 Viscosity as a function of shear rate: (♦) algae without LBG, (■) algae in 0.3% LBG and (▲) algae in 0.5% LBG.

Table 1 Shear stresses applied and Taylor numbers in relation with shear rates.

Rotational speed (rpm)	Shear rate (s^{-1})	Shear stress without LBG (Pa)	Taylor number 0% LBG	Shear stress 0.3% LBG (Pa)	Taylor number 0.3% LBG	Shear stress 0.5% LBG (Pa)	Taylor number 0.5% LBG
0	0	0	0	0	0	0	0
2	4.31	-	-	-	-	0.795	0.0052
4	8.63	0.016	1.04	0.541	0.031	1.31	0.013
10	21.57	-	-	1.07	0.097	2.53	0.041
15	32.36	-	-	1.44	0.162	-	-
20	43.14	0.077	5.39	1.78	0.232	4.15	0.100
100	215.7	0.376	27.6	5.89	1.76	13.2	0.783
500	1079	1.83	141	19.4	13.3	-	-

The effect of shearing treatment on viability

In all processing conditions tested, the shape of the cells was unaltered. This holds for the viable cells as well as the non-viable cells. The total cell concentration of the algae that were exposed to the high shear stresses did not decrease either.

The viable and non-viable cells could only be discriminated by the FDA staining method, where the viable cells showed fluorescence (Fig. 3). Obviously, the affected cells must be damaged internally without any visible external impact observed in quiescent conditions after the shearing treatment.

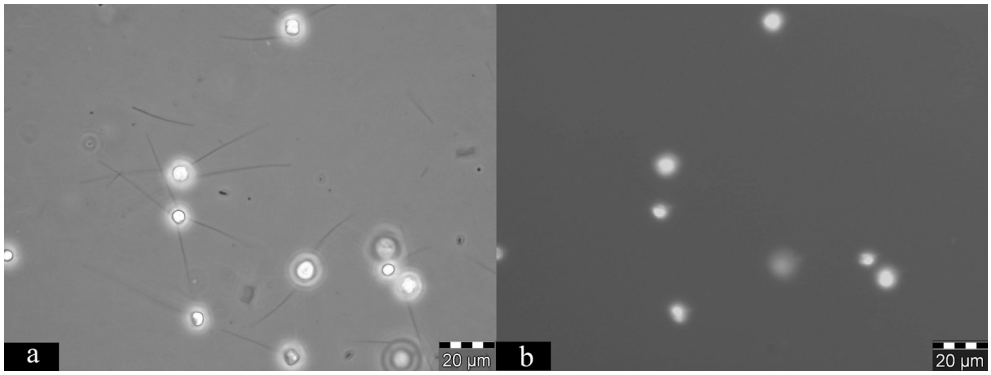

Fig. 3 Microscopic pictures of *Chaetoceros muelleri*; with normal light (a) 11 cells can be counted and with fluorescent light (b) 8 viable cells show fluorescence.

The effect of shear rate on the viability is shown in Fig. 4. Without LBG, no effect of shear rate on the viability of the algae was observed. No effect on the viability of the algae in 0.3% LBG was seen till a shear rate of 22 s^{-1}. Higher shear rates applied to the algae in 0.3% LBG affected the algae negatively, reducing the viability to 56%. At a shear rate of 8.6 s^{-1} applied to the algae in 0.5% LBG, the viability dropped to a value of 59%. Increasing the shear rate reduced the percentage of viable cells slightly to 52%. There seems to be a slight difference between the viability levels of the samples with 0.3% and 0.5% LBG. We believe that these differences might be attributed to the fact that the experiments were done on different days. In addition, LBG did not affect the viability of the algae directly. No negative effect on the viability was seen when the algae in 0.3% and 0.5% LBG were not sheared. Furthermore, 100% viability was seen when low shear rates were applied to the algae in both LBG concentrations.

From Fig. 4, it can be seen that the shear rate is not the determining factor in explaining the negative effect on cell viability. Obviously, a higher viscosity leads to a lower shear rate at which a negative effect can be observed. When analyzing the effect of shear stress on the viability, the effects of shear rate and viscosity can be combined. The effect of all different levels of shear stress on the viability of *Chaetoceros muelleri* can be seen in Fig. 5.

Shear stress values up to 1 Pa had no effect on the viability. At shear stress levels higher than 1.3 Pa, a sharp drop in viability can be seen with resulting viabilities between 52% and 66%. The threshold value of shear stress for *Chaetoceros muelleri* is therefore to be found between 1 and 1.3 Pa. A further increase of the shear stress to 19.4 Pa did not reduce the viability significantly. The effect of shear stress on the viability of *Chaetoceros* muelleri can thus be described as a step response with an almost similar effect beyond the threshold value, which is represented by the dotted line in Fig. 5. This would suggest that only a certain percentage of the cells are sensitive to shear stress and that higher shear stresses than the threshold value, up to the maximum applied value of 19.4 Pa, only affect the sensitive cells. Although it is unclear which cells are more susceptible to shear stress, other studies have speculated that shear stress disrupts cell division (García Camacho et al., 2007; Stoecker et al., 2006). Dividing cells are probably more shear sensitive. Step responses to shear stress have previously been reported for enzymes (Van Der Veen et al., 2004) and starch molecules (Van Den Einde et al., 2004a; Van den Einde et al., 2004b), suggesting a more general role of shear stress in deactivation and breakage processes.

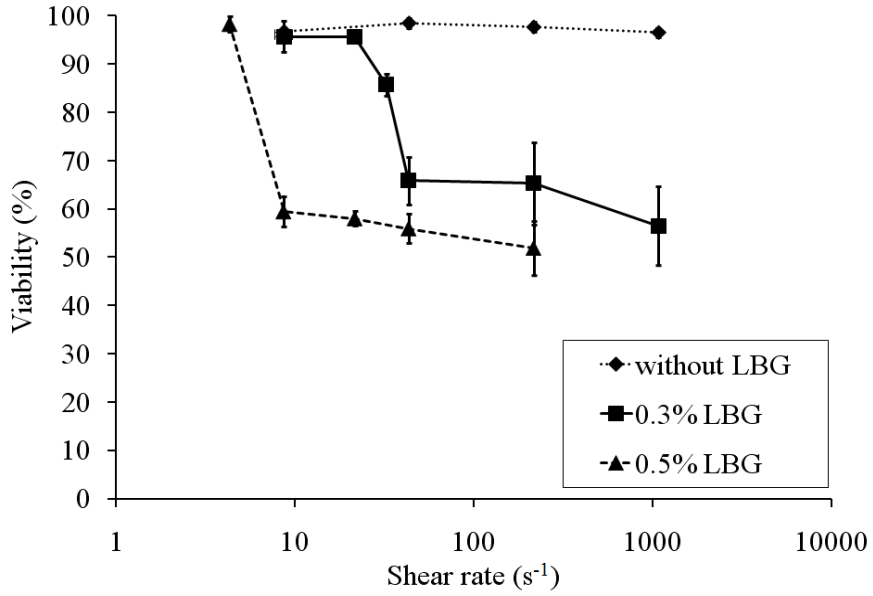

Fig. 4 Effect of shear rate on the viability of *Chaetoceros muelleri*; (♦) without LBG, (■) 0.3% LBG and (▲) 0.5% LBG. Error bars represent 95% confidence intervals.

The only deviation from the general interpretation of our results is that a shear stress of 1.8 Pa could be applied to the algae without LBG at the highest shear rate without any detrimental effect on the viability. Flow instabilities like Taylor vortices could have occurred in that experiment, so that the results could have been influenced in a way that they are not reliable or comparable.

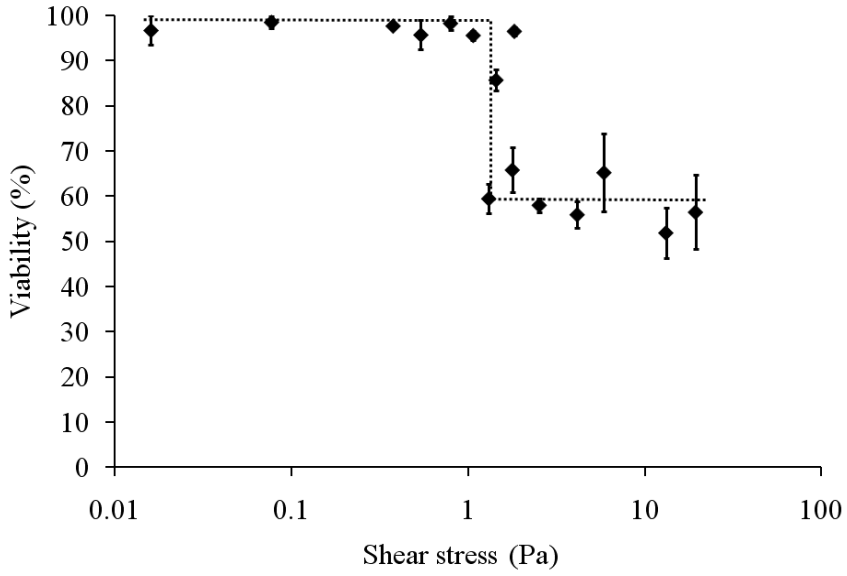

Fig. 5 Effect of shear stress on the viability of *Chaetoceros muelleri* with all treatments (without LBG, 0.3% LBG and 0.5% LBG) taken together. Error bars represent 95% confidence intervals.

These results imply that a PBR for cultivating *Chaetoceros muelleri* should be designed in such a way that the maximum exhibited shear stress is lower than the threshold value. The question that now remains is whether this shear stress can occur in a PBR. Considering the study of Contreras et al. (1998), shear rates up to 14,000 s^{-1} can occur, which implies a local shear stress of about 20 Pa. This value clearly exceeds the value reported above. In other words, shear stress is likely to negatively influence the algae in a PBR. However, reducing the fluid velocity too much can lead to other limitations, such as mass transfer, which also negatively influences the growth rate.

Time dependence of shear stress effect

Chaetoceros muelleri in 0.3% LBG was exposed to a constant shear stress of 5.89 Pa at a shear rate of 215.7 s^{-1} in the shear cylinders to elucidate the time dependence of the shear stress effects. The viability already decreased after one minute to 82% and kept on decreasing at a reduced rate for the following 7 min. Longer exposure times to shear stress did not reduce the viability any further. The viability was found to vary between 65% and 75% for all exposure times longer than 8 min (Fig. 6). This would suggest that the effect of shear stress is almost time-independent. Compared to normal cultivation times, the effect can be considered as almost instantaneous. The fact that the viability does not decrease completely indicates that only the more sensitive cells are susceptible to shear stress, while the more resistant cells are not affected by shear stress even over a longer period. This result is in contrast with a study done with hybridoma cells (Abu-Reesh and Kargi, 1989). The viability of these mammalian cells, which were also sheared for time periods of less than 2 h, decreased over time. However, the shear stress levels applied were much higher and varied between 5 and 100 Pa. The time dependence of shear stress was also investigated with plant cells (Dunlop et al., 1994). Although the main conclusion was that the effect of shear stress on plant cells was time dependent, it was clear that the first minutes of exposure to a certain shear stress were the most detrimental. The study of Dunlop (1994) also showed that similar shear stress levels and exposure times as in this study gave almost the same viability results. Complete cell death was only reached when significantly higher shear stresses (50 - 100 Pa) were applied to the cells. These high shear stress values are expected to be unrealistic in PBRs given the low viscosity of the medium used.

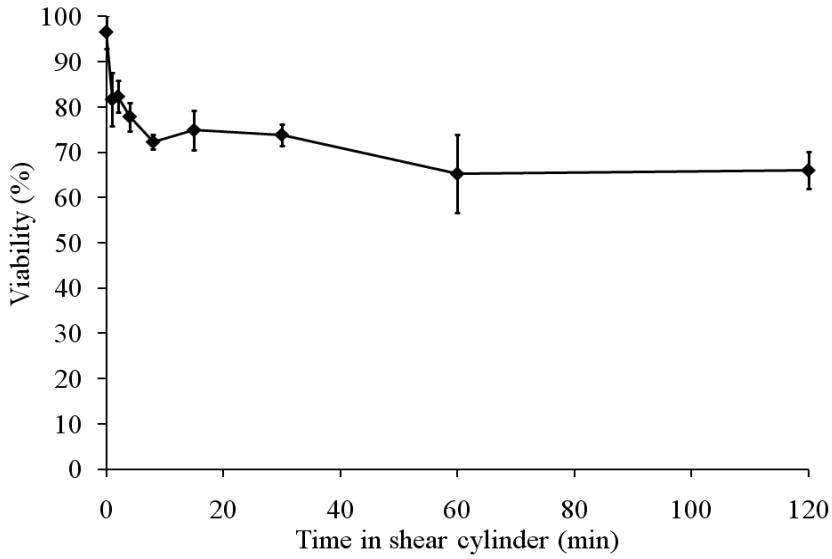

Fig. 6 The effect of time dependency of shear stress on the viability of *Chaetoceros muelleri*. Error bars represent 95% confidence intervals.

Conclusions

Shear stress had an adverse effect on the viability of *Chaetoceros muelleri* with a threshold value between 1 and 1.3 Pa. Shear stress higher than the threshold value caused a sudden decrease in viability to levels between 52% and 66%. It appears that only a certain fraction of the cells are susceptible to shear stress up to 19.4 Pa.

No external damage was observed, meaning that internal damage must have taken place in the affected cells.

The effect of shear stress was almost instantaneous, compared to regular cultivation times. The detrimental effect already took place after 1 min of exposure to shear stress. The viability did not decrease any further with shear stress exposure times longer than 8 min. This would suggest that the sensitive cells were affected by shear stress instantaneously and that high shear stress over a long period did not have a negative effect on the more resistant cells.

Acknowledgment

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CHAPTER 3

Effect of biomass concentration on the productivity of *Tetraselmis suecica* in a pilot-scale tubular photobioreactor using natural sunlight

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Abstract

The effect of biomass concentration on the net volumetric productivity, yield on light and nightly biomass loss rate of *Tetraselmis suecica* was studied using a pilot-scale tubular photobioreactor (PBR) under outdoor light conditions. The net average productivity and yield on light of *Tetraselmis suecica* was optimal at a biomass concentration of 0.7 g L^{-1}. Cultures grown at higher biomass concentrations showed a prolonged respiration period at low light periods, while light was wasted in more dilute cultures at high light intensities. At optimal biomass concentration of 0.7 g L^{-1} the highest average net productivity and yield on light were 0.35 ± 0.03 g L^{-1} d^{-1} and 1.19 ± 0.15 g mol^{-1}, respectively. The highest nightly biomass loss rate was measured for *Tetraselmis suecica* grown at an optimal biomass concentration, which can be explained by higher maintenance costs of the microalgae with a higher growth rate.

This study shows that the productivity and yield on light can be enhanced by optimizing the biomass concentration.

Introduction

The microalga *Tetraselmis suecica* is used in aquaculture as a high-value feed, rich in polyunsaturated fatty acids (PUFAs) (Caers et al., 1999; Camus and Zeng, 2012). Currently, it is mainly produced in plastic bubble-columns using artificial light (Muller-Feuga et al., 1998). To keep up with the increasing demand for microalgae as feed for shellfish, crustaceans and live prey of some fish larvae, alternative cultivation methods are needed that facilitate a high production capacity and a constant quality of the algae produced (Adarme-Vega et al., 2012; Borowitzka, 1997; Muller-Feuga, 2004; Muller-Feuga, 2000; Pulz and Gross, 2004). These requirements can be met by using continuously operated closed photobioreactors for cultivating the microalgae. In these fully-automated photobioreactors (PBRs) mixing, CO_2 and nutrient supply are controlled and the pH and temperature can be kept constant at optimal conditions for productivity and quality of the algae, but the use of artificial light makes the process unsustainable. Therefore, natural sunlight should be used to considerably reduce the energy costs.

At outdoor conditions, however, the available sunlight varies over the day and with the seasons and this will affect the volumetric productivity as well as the quality of the algae (Molina Grima et al., 1994a; Huerlimann et al., 2010; Rebolloso Fuentes et al., 1999). The volumetric productivity is the product of the biomass concentration and the net specific growth rate. This is the combined result of photosynthesis, that occurs only in the light and respiration, that occurs at all light conditions, and it is directly related to the amount of light available for the microalgae (Molina Grima et al., 1999). Light availability for the microalgae inside the culture depends on the photon flux density (PFD) on the PBR surface, the length of the light path in the PBR, and the light attenuation caused by the self-shading effect of the cells (Acién Fernández et al., 1998; Slegers et al., 2011).This makes the biomass concentration a crucial parameter for optimizing the productivity (Slegers et al., 2013; Ugwu et al., 2005).

Fig. 1 shows how the biomass concentration and the light falling on the photobioreactor surface interfere. The ideal situation is when light is fully absorbed and no light is wasted. A too dense culture with a larger dark zone inside the culture will show reduced net specific growth rate. A low biomass concentration, on the other hand, will show high net specific growth rate, but due to the low biomass concentration the overall productivity will be low (Cornet and Dussap, 2009; Posten, 2009; Takache et al., 2010). It is important to know the optimal biomass concentration in relation

with the available light input, to ensure high volumetric productivity in a closed photobioreactor (Camacho et al., 1990; Molina Grima et al., 1997; Moheimani, 2012). As the outdoor light conditions vary, the net specific growth rate will vary and this will cause high variation in productivity at given biomass concentration. Therefore, the effect of biomass concentration on yield on light is also investigated in this study.

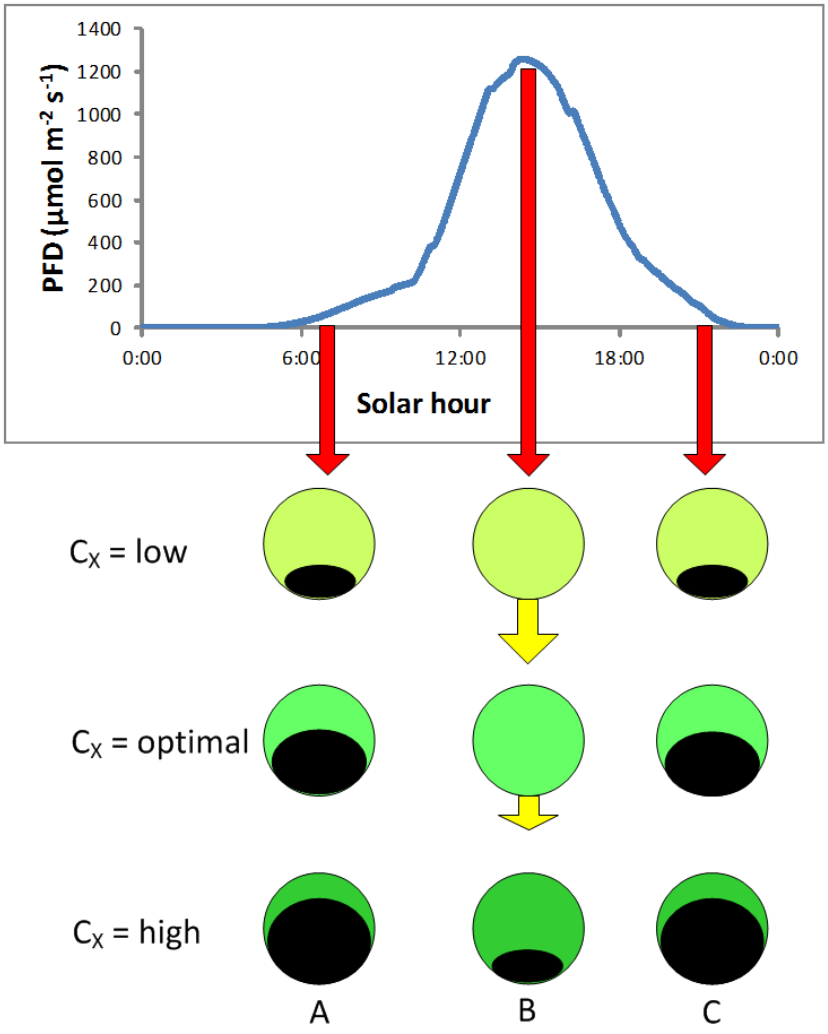

Fig. 1 Schematic view of the influence of PFD and biomass concentration on the light availability inside the tube of a horizontal PBR. Graph shows PFD data obtained on a fairly sunny day in June 2012 at Vlissingen, the Netherlands. Higher biomass concentrations lead to larger dark zones in the tube during low light periods (situation A and C). Lower biomass concentrations lead to inefficient absorbance of light during high light periods (situation B), which in reality means that light is leaving the system (yellow arrow).

Furthermore, during the dark net volumetric productivity and yield on light will be negative due to biomass losses by respiration. Respiration prevails over photosynthesis at night and at low light periods, like during dawn and dusk, but also in the dark zones of the photobioreactor (Takache et al., 2012). Realizing that the net productivity and biomass yield is always a compromise between some losses in productivity in the dark zones where respiration predominates and losses of photons during high light periods, the challenge is to find the optimal biomass concentration at which a high net productivity and a high yield on light are reached while using natural sunlight as a light source.

The quality of the algae in terms of PUFA content may be influenced by the available light as well. At high light conditions, microalgae in general, tend to form saturated fatty acids in the form of triacylglycerol (TAG), especially at nutrient replete conditions in the medium (Pal et al., 2011; Renaud et al., 1991). The fatty acid content and composition may thus vary with the availability of light and thus also with the biomass concentration applied.

The objective of this study is to determine the effect of biomass concentration on the net productivity, yield on light and nightly respiration of *Tetraselmis suecica* in a pilot-scale tubular photobioreactor using natural sunlight conditions and check if the quality in terms of PUFA content is affected.

Materials and methods

Organism and culture medium

Tetraselmis suecica originates from the collection of Seasalter Shellfish (Whitstable) Limited (Kent, United Kingdom) and was obtained from Stichting Zeeschelp (Kamperland, the Netherlands). Walne medium modified from Laing (Laing, 1991) was used for the cultivation of *Tetraselmis suecica* using filtered and de-ironized local saline groundwater with a salinity of 30 g L^{-1}. Walne medium was prepared by mixing 1 L of a stock solution of macro- and micronutrients with 100 mL of a stock solution of vitamins. The stock solution of nutrients contains 0.8 g $FeCl_3$, 0.4 g $MnCl_2 \cdot 4H_2O$, 33.6 g H_3BO_3, 45.0 g EDTA, 20.0 g $NaH_2PO_4 \cdot 2H_2O$, 100.0 g $NaNO_3$, 21 mg $ZnCl_2$, 20 mg $CoCl_2 \cdot 6H_2O$, 9.0 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 20 mg $CuSO_4 \cdot 5H_2O$ in 1 L of distilled water. The stock solution of vitamins contains 1.0 g Vitamin B_1 and 0.05 g Vitamin B_{12} in 1 L of distilled water. The pH of the Walne medium was adjusted to 4.0 with concentrated HCl. Walne medium was supplied to the microalgal

culture during turbidostat operation at a double dose (2.2 ml of Walne medium per 1 L of culture) for obtaining an expected biomass concentration of 0.5 g L^{-1}, a triple dose for 0.7 g L^{-1}, a quadruple dose for 1.0 g L^{-1}, a sixfold dose for 1.5 g L^{-1} and an eightfold dose for 2.0 g L^{-1}.

Tubular photobioreactor

The tubular PBR (Paques, the Netherlands) consists of 20 m long transparent plexiglas tubes with an external diameter of 5 cm joined in a loop, which is connected to a degasser. The loop has three bends with four 5 m long tubes with 7.5 cm space in between. Black paper was placed underneath the tubes to avoid reflection of light from the bottom and enables us to calculate the daily light input accurately. The volume of the tubes is 30 L and the dark volume (volume of the degasser plus the volume of the connecting tubes to the degasser and pump) is 10 L in total.

The tubular PBR is placed inside a greenhouse in Vlissingen, The Netherlands (51°26'45'' N, 3°35'11''). The PBR is almost east-west orientated (110° – 290°) with the length of the tubes directed to the south-southwest (200^0). A LiCor LI190 PAR sensor, placed in front of the tubular PBR inside the greenhouse, measured the photosynthetically active radiation (PAR) every minute as a photon flux density (PFD) in µmol photons m^{-2} s^{-1}.

Operating mode

Tetraselmis suecica was cultivated at five different biomass concentrations (Table 1), which will be further referred to as 0.5, 0.7, 0.9, 1.5 and 2.0 g L^{-1}, respectively. Turbidostat conditions were applied to each biomass concentration for a total period of 14 days.

The turbidity sensor (Inpro 8300 RAMS TCS, Mettler Toledo) and the supervisory control and data management system (SCADA) with programmable logic controller (PLC) were used to control the biomass concentration between a lower and higher set point value for the turbidity. When the higher set point for the turbidity was exceeded for more than 30 seconds, the valve for the incoming water opened automatically and harvesting of the biomass started. At the same time, Walne medium was dosed at a flow rate proportionally to the harvesting rate until the turbidity in the tubes reached the lower set point value.

An example of measured incident light and biomass concentration during a typical turbidostat run at a biomass concentration of 2.0 g L^{-1} is depicted in Fig. 2. The first day in Fig. 2 (25th of June 2012) was a sunny day with cumulus clouds, which is reflected in the scattering of the PFD input data. The second day (26th of June 2012) was a cloudless day and a higher average PFD was monitored, although the maximal PFD was smaller than in the previous day due to little haziness. The harvest moments can be recognized as drops in the biomass concentration from the upper set-point value of 2.04 g L^{-1} to the lower set-point value of 1.96 g L^{-1} and were followed by a growth period till the higher set point for turbidity was reached. The time between harvest moments was shorter during high light periods. During the night the biomass concentration decreased below the lower set-point value, due to prevailing respiration.

Table 1 Biomass concentrations cultivated with average PFD and PF_{in}.

Cultivation period (2012)	C_X (g L^{-1})	Average PFD (µmol m^{-2} s^{-1})	Average PF_{in} (mol d^{-1})
23rd Sep – 7th Oct	0.54 ± 0.014	102 ± 13.9	8.04 ± 1.07
6th Sep – 21st Sep	0.68 ± 0.021	156 ± 23.5	12.4 ± 1.93
17th May – 30th May	0.89 ± 0.020	282 ± 57.1	22.1 ± 4.33
2nd Jun – 15th Jun	1.54 ± 0.044	213 ± 45.7	16.6 ± 3.62
19th Jun – 2nd Jul	1.98 ± 0.022	245 ± 37.8	19.1 ± 3.02

The microalgae were recirculated at a velocity of 0.37 m s^{-1} in the tube, using a centrifugal pump (SealPro KR-32-95, ARBO). Oxygen was measured using a dissolved oxygen electrode (InPro 6800/12/220, Mettler Toledo) and the surplus of produced oxygen was removed in the degasser by sparging air with a flow rate of 450 L h^{-1}. The pH was monitored using a pH electrode (InPro 3250/225/PT100, Mettler Toledo). The pH was set at 8.4 and based on the measured pH, a mass flow controller (MFC) controlled the dose of CO_2. The CO_2 inlet was located just before the recirculation pump.

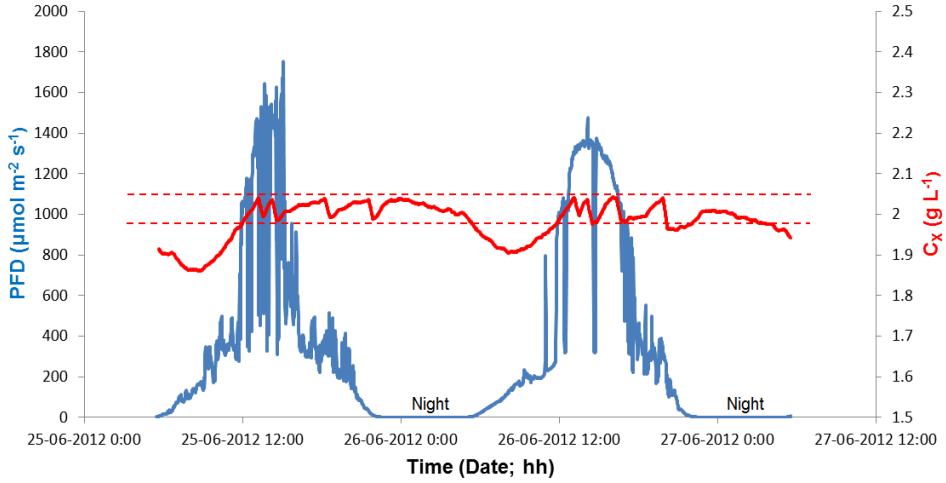

Fig. 2 Typical result of the application of turbidostat regime on the biomass concentration under natural light conditions for two consecutive days. Dashed lines represent the lower and higher set point values for the turbidity.

Water vapor in the off gas from the degasser was condensed and the CO_2 concentration (ppm) in the off gas and in the ambient air was measured with a gas analyzer (Servomex 4100).

The temperature was kept constant at 20 ± 0.5 ^{o}C and controlled by heat exchange through an annular water jacket around the degasser and a recirculation system including a chiller and aquatic heater.

A schematic picture of the tubular PBR is given in Fig. 3.

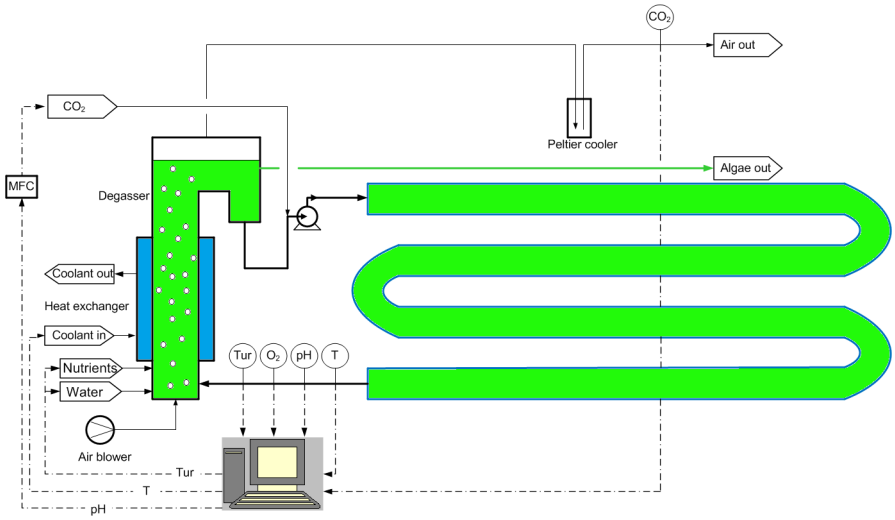

Fig. 3 Schematic picture of tubular photobioreactor.

Analytical methods

Harvest volume and biomass concentration

The volume of the harvested algae was measured with a measuring cylinder every day. A sample from the harvest was taken around noon and used for the biomass concentration and cell concentration analysis. The biomass concentration was determined according to Zhu and Lee (1997). The samples (< 10 mg dry weight) were filtered over a pre-dried and pre-weighed Whatman GF/C glass microfiber filter and washed with 0.5 M ammonium formate. After drying the samples for 24 h at 95 °C, they were cooled down in an exsiccator (2 h) and weighed again.

Fatty acids analysis

Samples at each biomass concentration were taken for the determination of the fatty acid content and composition. The samples were centrifuged for 10 min at 2000 rpm and the pellet was re-suspended with distilled water and centrifuged again for three times to remove the salt. Fatty acid extraction and quantification were performed as described by Lamers et al. (2010) and Santos et al. (2012).

Determination of specific growth rate, net volumetric productivity and yield on light

The net volumetric productivity (P_V, in g L^{-1} d^{-1}) is the product of the net specific growth rate and the biomass concentration (C_X, in g L^{-1}).

$$P_V = \mu \cdot C_X$$

The average net specific growth rate per day (μ, in d^{-1}) corresponds to the dilution rate (D, in d^{-1}) measured at turbidostat conditions (Lee and Shen, 2004). It is calculated from the harvest volume ($V_{harvest}$, in L) per day (t_d = 1 day) and the total volume of the PBR (V_{PBR}, in L).

$$\mu = D = \frac{V_{harvest}}{V_{PBR} \cdot t_d}$$

The yield on light in g per mol of PAR photons supplied ($Y_{X,ph}$) is defined as the biomass harvested per day divided by the daily photon flux supplied to the PBR (PF_{in}, in mol photons d^{-1}).

$$Y_{X,ph} = \frac{V_{harvest} \cdot C_X}{PF_{in} \cdot t_d}$$

Calculation of the daily photon flux supplied to PBR

The photon flux density (PFD in μmol photons m^{-2} s^{-1}) received on the reactor surface was calculated using the horizontally measured PFD, reactor dimensions and solar angles according to the method of Slegers et al. (2013). The total sunlight input of the horizontal tubes, referred to as “daily photon flux”, depends on the light angles. The first step in the method is to derive the horizontal direct and diffuse PFD levels from the measured PFD. The monthly average distributions of direct and diffuse light (Table 2) were used to derive the specific direct and diffuse PFD (Velds et al., 1992). The second step is to transfer the horizontal PFD levels to the PFD levels on the tube surface. Fig. 4 illustrates how part of the tube can be shaded by neighboring tubes. It also illustrates that the highest PFD input is received by only a small part of the tube; the highest direct input is received only at the point perpendicular to the sun rays. The input decreases when moving away from that point. Furthermore, the

back-sides of the tubes are always shaded, as direct PFD cannot reach that side of the system. Therefore, the shaded reactor parts only receive diffuse light. The models presented in Slegers et al. (2013) were used to determine the shading at any time of the day and to determine the PFD levels on the reactor tubes. The cross-sectional tube periphery was divided into 120 calculation points. In the third step the daily photon flux is derived using:

$$PF_{in} = L_{PBR} \cdot \sum_{time} \sum_{N} PFD_{point} \cdot \frac{p_{tube}}{N} \cdot \Delta t$$

where L_{PBR} is the length of the illuminated PBR tube (in m), PFD_{point} (in µmol photons m^{-2} s^{-1}) is the PFD received at each point of the surface of the tube, p_{tube} (in m) is the periphery of the tube, N the number of calculation points and Δt (in s) the time between two measurement points.

Average biomass loss rate during night

The biomass concentrations over the day were calculated via the measured linear relationship between the turbidity and the biomass concentration. The decrease of the biomass concentration during the night is monitored in the same way. The average biomass loss rate due to nightly respiration was determined at each biomass concentration (C_X). The average rate of biomass loss during the night was calculated with the following formula:

$$Average\ biomass\ loss\ \ rate = \frac{ln(C_{X(0)}) - \ln(C_{X(t)})}{\Delta t_{night}}$$

The time period of the night from sunset to sunrise is given as t_{night} (in h) to be able to compare the average biomass loss rates of the different biomass concentrations tested.

Table 2 Average percentages of direct and diffuse light in the Netherlands during the photobioreactor runs[a].

	Direct	Diffuse
May	48%	52%
June	50%	50%
July	47%	53%
September	42%	58%
October	38%	62%

[a] Data derived from C. Velds et al. (1992).

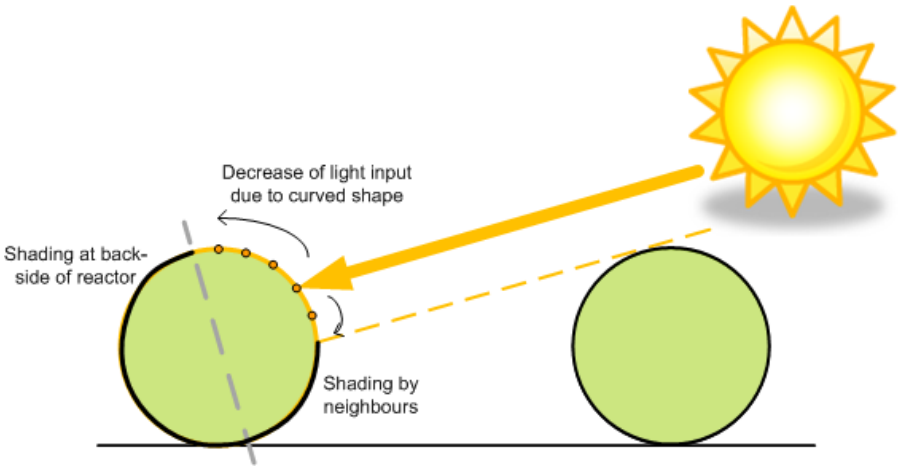

Fig. 4 Schematic overview of the effect of time and reactor design on the light pattern on tubular photobioreactors. The points represent the points used to calculate the total light input.

Statistical analysis

Differences between average values between the five tested biomass concentrations were determined with One-way Anova. The average values with the standard error with a 95% confidence interval were given and used in this paper.

Results and discussion

Specific growth rate, net volumetric productivity and yield on light

Tetraselmis suecica with a biomass concentration of 0.7 g L^{-1} showed significantly higher net volumetric productivities ($p < 0.05$) in comparison with the other biomass concentrations receiving daily photon fluxes between 6 and 12.5 mol photons d^{-1} (Fig. 5). The average net volumetric productivity was 0.35 ± 0.03 g L^{-1} d^{-1} with a highest value of 0.46 g L^{-1} d^{-1}. These results are comparable with the data from Chini Zitelli et al. (2006). Lower average net volumetric productivities, respectively 0.085 (Raes et al., 2013) and 0.11 g L^{-1} d^{-1} (Moheimani, 2012) were reported for *Tetraselmis* sp. and *Tetraselmis suecica* cultivated in different types of PBRs. When similar tubular PBRs were used, the net productivity varied with the algal strain used, and with the culture conditions applied (Acién Fernández et al., 1998; García-González et al., 2005; Ugwu et al., 2005), while also the location (latitude) and the season have an profound effect on the productivity, which makes the comparison with reported average volumetric productivities difficult.

The average net specific growth rate that is measured, depends on the daily photon flux and the biomass concentration (Fig. 6). At biomass concentrations of 0.5 and 0.7 g L^{-1}, the growth rate of *Tetraselmis suecica* shows a similar increase with the daily photon flux at the reactor surface. The highest growth rate measured was 0.68 d^{-1} measured at a biomass concentration of 0.7 g L^{-1} and a daily photon flux of 15.9 mol d^{-1}. At higher biomass concentrations the average net growth rates of *Tetraselmis suecica* were in all cases much lower. The net volumetric productivity, which is the product of the net specific growth rate and biomass concentration, depends on the varying light conditions in the culture. Therefore, yield on light gives more information on how efficient the light is used.

The yield on light (grams of biomass produced per mol of photons in the PAR range supplied) decreased for each biomass concentration at higher photon fluxes (Fig. 7). The observed inversely proportional relationship between yield on light and the daily photon flux is a result of the waste of excess light energy in the form of fluorescence and heat (Müller et al., 2001; Mussgnug et al., 2007). Low-light levels lead to higher photosynthetic efficiencies (Kliphuis et al., 2012; Vejrazka et al., 2011). This graph also shows that the optimal biomass concentration in our experiment is indeed 0.7 g L^{-1}. At this biomass concentration the highest yields on light were obtained, regardless of the daily photon flux. Overall, the average yield on light of *Tetraselmis*

suecica at this biomass concentration was 1.19 ± 0.15 g mol^{-1}. This is high, but comparable with other optimization studies (Cuaresma et al., 2011; Kliphuis et al., 2012; Takache et al., 2010).

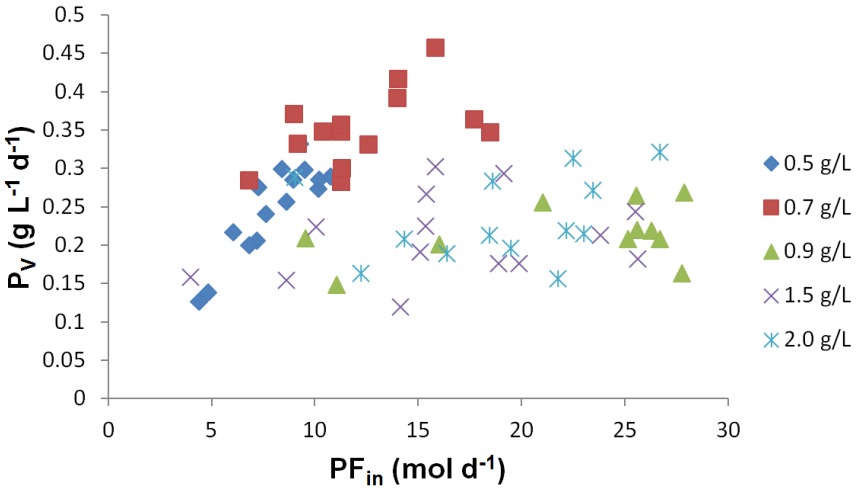

Fig. 5 Net volumetric productivity of *Tetraselmis suecica* with five different biomass concentrations versus the daily photon flux.

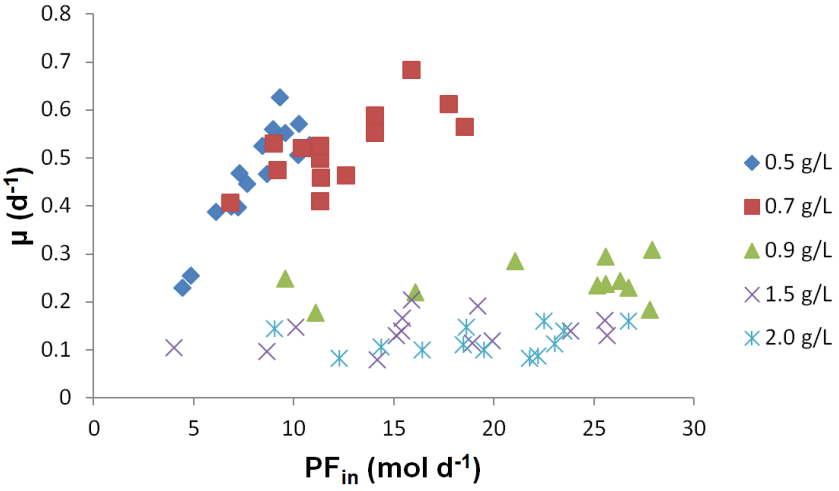

Fig. 6 Specific growth rate of *Tetraselmis suecica* with five different biomass concentrations versus the daily photon flux.

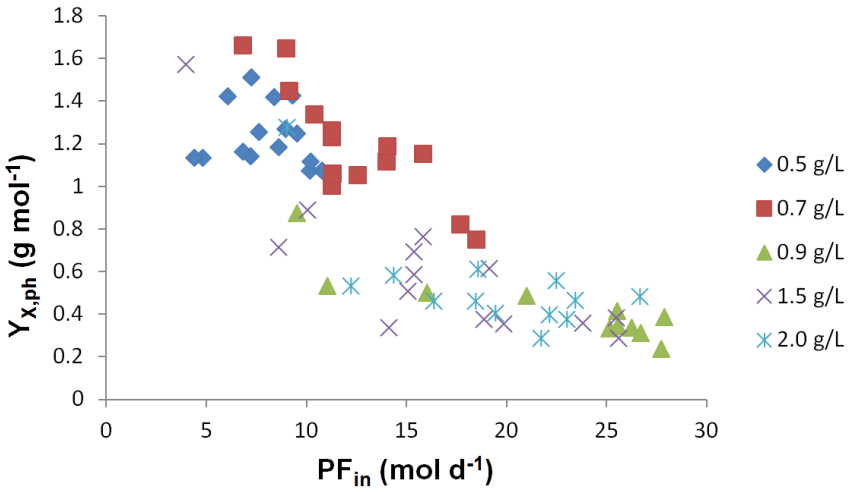

Fig. 7 Yield on light of *Tetraselmis suecica* with five different biomass concentrations versus the daily photon flux.

The values of our calculated yield on light show ups and downs at one biomass concentration and similar daily photon flux. This variation in yields found at similar PF_{in} originates from the calculated photon flux. In the model that is used to calculate the daily photon flux, fixed percentages of diffuse and direct light were used for every day (Table 2) (Slegers et al., 2013). At real-time outdoor conditions this percentage varies with the weather conditions. Cloudless days have a relatively higher percentage of direct light than cloudy days and this results in more variation in the actual total light input than predicted via the model. Despite the scattering in yields, it is obvious that the algae should be grown at 0.7 g L^{-1} observed for PF_{in} levels below about 20 mol d^{-1}. Higher biomass concentrations result in lower yield on light. This is caused by the low supply of light to the microalgae. At these high biomass concentration the self-shading effects of the microalgae prevail, leading to large dark zones in the tubes, especially during dawn and dusk. Higher productivities and photosynthetic efficiencies can be reached when dark zones are avoided (Kliphuis et al., 2010). Lower biomass concentration of 0.5 g L^{-1} also resulted in a lower yield on light. The lower yield is most probably caused by the excess of light, especially during midday, which leads to over-saturation of the photosystem (Vejrazka et al., 2013; Vejrazka et al., 2011). The data confirm once more that for optimizing the productivity of microalgae, that receive seasonal and diurnal changes in light levels, it is better to use yield on light as an optimization parameter.

Average biomass loss rate during night

During the night the biomass concentration decreased (Fig. 2). The respiration is the self-evident process responsible for this. During respiration compounds like carbohydrates are used to provide the cells with sufficient energy for the maintenance of the cell and for formation of compounds like proteins (Fabregas et al., 1995; Torzillo et al., 1991). Energy is used at the cost of biomass.

The highest decrease of biomass overnight was measured for the highest biomass concentration (data not shown). If a similar biomass loss rate for every biomass concentration was expected, higher biomass concentrations would lose more biomass due to prevailing respiration at night. However, the highest average biomass loss rate measured was $6.8 \cdot 10^{-3}$ h^{-1} at a biomass concentration of 0.7 g L^{-1} and was significantly higher ($p < 0.05$) at biomass concentrations of 1.5 and 2.0 g L^{-1} with biomass losses of $4.1 \cdot 10^{-3}$ and $3.6 \cdot 10^{-3}$ h^{-1}, respectively (Fig. 8).

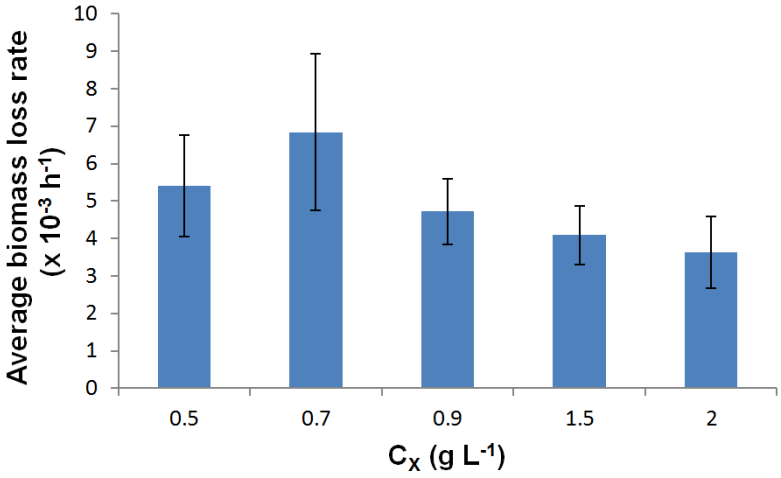

Fig. 8 Average biomass loss rate of five different biomass concentrations. (Error bars represent 95% confidence intervals).

The average biomass loss rates show quite high standard errors at every biomass concentration, especially for the two lowest tested biomass concentrations (Fig. 8), which have the biggest variance in growth rate. Microalgae with a higher growth rate caused by a higher light availability store relatively more energy in the formation of carbohydrates, which can then easier be used in the following night for the maintenance of the cells. Therefore, a linear correlation described between the

specific growth rate and the nightly biomass loss rate of the same day is expected. The results presented in Fig. 9 appear to be in line with this hypothesis (Pearson's correlation coefficient = 0.54). Further research is needed to show if the relationship is linear or that it can better be described by Michaelis-Menten kinetics (Givnish et al., 2004; Kruse et al., 2011). The dependence of the specific growth rate or light input and the biomass loss in the night afterwards was also shown in other studies (Carlozzi, 2003; Doucha and Lívanský, 2006; Grobbelaar, 1991; Torzillo et al., 1991).

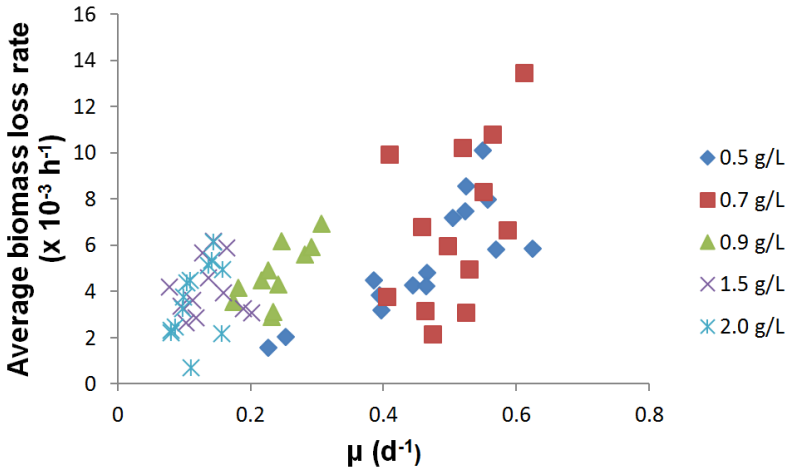

Fig. 9 Average biomass loss rate versus growth rate of five biomass concentrations.

Domination of respiration over photosynthesis also occurs during the day in the dark volume of the PBR with net biomass loss as a result. Furthermore, the larger dark zones in the PBR during dawn, dusk and other low light periods, especially for the higher biomass concentrations, probably had an adverse effect on the net productivity and yield on light. Therefore, the gross productivity can only be calculated if the biomass losses in the dark zones are known as well.

Fatty acids

The fatty acid composition was analyzed to verify if the quality of *Tetraselmis suecica* was affected at the used biomass concentration. PUFAs are of special interest, because many studies showed a positive effect of PUFAs on the development of larvae in hatcheries (Caers et al., 2002; Utting and Millican, 1998). Low light combined with logarithmic growth are likely to promote the formation of PUFAs, which are primarily found in structural lipids such as phospholipids and glycolipids

in membranes (Hu et al., 2008; Olofsson et al., 2012). Although medium conditions can have an effect on the fatty acid profile, nutrient replete conditions and a constant CO_2 concentration by pH control during daylight were applied.

The average fatty acid composition of all biomass concentrations was 10.8 ± 0.9% of the total dry weight. The average percentage as the fraction of PUFAs per total fatty acids of all biomass concentrations was 60.4 ± 2.6%. *Tetraselmis suecica* showed an eicosapentaenoic acid content (EPA or C20:5) varying between 5 and 10% of the total fatty acids (Fig. 10). Similar values were also found by Molina Grima et al. (1994b). The results indicate that only small differences in fatty acid composition of *Tetraselmis suecica* were found for different biomass concentrations, while light conditions inside the culture and growth rates were different. Further research needs to be done to prove if the differences in fatty acid composition are indeed significant.

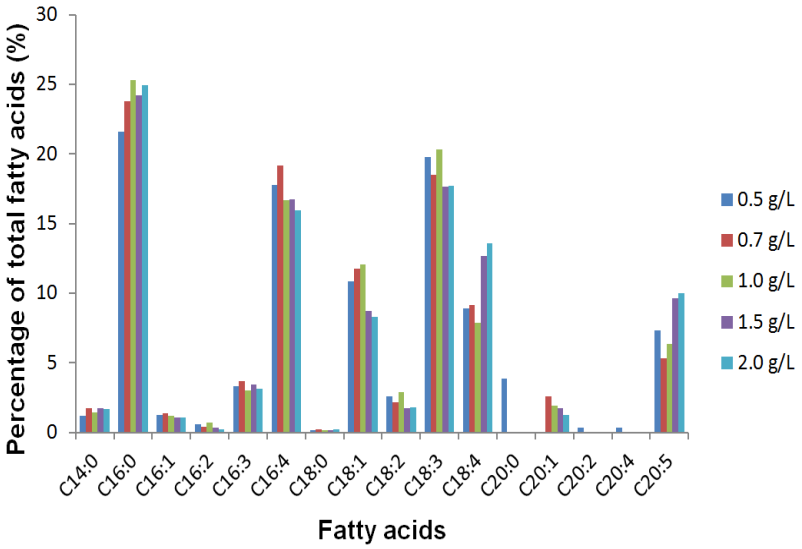

Fig. 10 Fatty acid profile of *Tetraselmis suecica* with five different biomass concentrations. (Values are given as % of total fatty acids).

Conclusions

The optimal biomass concentration of *Tetraselmis suecica* cultivated in a pilot-scale tubular PBR using natural sunlight was determined. It was apparent that yield on light was a reliable parameter to find the optimal biomass concentration, which

was 0.7 g L^{-1}. In comparison to the other biomass concentrations, a higher average net volumetric productivity and yield on light were found, which were 0.35 ± 0.03 g L^{-1} d^{-1} and 1.2 ± 0.15 g mol^{-1}, respectively. Although the average biomass loss rate of *Tetraselmis suecica* during the night was the highest for this optimal biomass concentration, smaller dark zones during low light periods compared to higher biomass concentrations and less losses of non-absorbed photons during high light periods compared to a lower biomass concentration had a positive effect on the productivity.

The quality in terms of fatty acid content and composition did not seem to be affected by the biomass concentration applied.

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CHAPTER 4

Growth of *Tetraselmis suecica* in a tubular photobioreactor on wastewater from a fish farm

This chapter is published as:

Michels MHA, Vaskoska M, Vermuë MH, Wijffels RH (2014) Growth of *Tetraselmis suecica* in a tubular photobioreactor on wastewater from a fish farm. Water Res 65: 290-296.

Abstract

Wastewater from a fish farm was remediated in a continuously operated tubular photobioreactor in which *Tetraselmis suecica* was cultured. The N and P removal efficiencies and the productivity of *T. suecica* growing on the wastewater were determined. Possibilities to optimize the productivity by adding extra orthophosphate were investigated. At a biomass concentration of 0.5 g L^{-1} on only wastewater, the N and P removal efficiencies were 49.4% and 99.0%, respectively. When extra phosphate was dosed to the wastewater, a 95.7% N removal efficiency and a 99.7% P removal efficiency could be reached at a biomass concentration of 1.0 g L^{-1}. This also resulted in significantly higher average net volumetric productivity ranging from 0.35 g L^{-1} d^{-1} at a biomass concentration of 0.5 g L^{-1} to 0.46 and 0.52 g L^{-1} d^{-1} at biomass concentrations of 0.75 and 1.0 g L^{-1}, respectively.

This study shows the feasibility of an integrated multi-trophic aquaculture approach where wastewater from the fish farms is used to produce feed for juvenile shellfish at high productivity and constant quality.

Introduction

The production of microalgae for aquaculture can become more economically and ecologically sustainable if an integrated multi-trophic aquaculture approach is used (Bunting and Shpigel, 2009; Neori et al., 2007). In this approach, nutrient-rich effluents of fish or shrimp farms are used to culture microalgae as feed in aquaculture (Chopin et al., 2012). By cultivating microalgae in the integrated multi-trophic aquaculture approach the purification of the wastewater from the fish farm is realized, which enables re-use of the water for aquaculture. In addition, the wastewater provides free nutrients for the production of the microalgal feed. *Tetraselmis suecica*, the microalgal species that is used in this study, has one of the broadest applications in aquaculture. It serves as feed for larvae and postlarvae of shellfish, penaeid shrimp larvae, abalone larvae, brine shrimp and rotifers (Becker, 2013).

Photoautotrophic microalgae need dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) besides light and CO_2 for the formation of macromolecules like proteins, DNA, RNA, ATP and phospholipids. Several studies have demonstrated that growing microalgae or macroalgae on wastewater of fish or shrimp farms is possible (Bartoli et al., 2005; Hussenot et al., 1998; Lefebvre et al., 2004; Shpigel et al., 1993). These studies showed that an efficient removal of inorganic nutrients together with the production of algae should be done in a more controlled manner. Continuously operated closed photobioreactors (PBRs) can serve well for this purpose. Culture conditions like CO_2 supply, pH and temperature can be controlled and the biomass concentration can be tuned to use natural available sunlight in an efficient way (Michels et al., 2014).

The uptake of DIN and DIP by the microalgae depends on the nutrient concentrations in the wastewater, the elemental composition of the microalgal species being used and the biomass concentration of the culture (Dalrymple et al., 2013; Lefebvre et al., 2004). High removal efficiencies of both DIN and DIP are only possible if the molar N:P ratio in the wastewater is in balance with the molar N:P ratio of the biomass (Borges et al., 2005). The nutrient concentrations and N:P ratio in the wastewater of fish farms depend mainly on the type of fish feed used. The fish feed may contain animal or vegetable ingredients of which the latter are less digestible. Besides the type of fish feed also the fish species farmed, the size of the fish and rearing temperature may affect the composition of the wastewater (Amirkolaie, 2011; Lemarié et al., 1998). Despite the variation in composition, N:P ratios in marine fish farms effluents are mainly close to the Redfield ratio of 16:1 (Lefebvre et al., 2004), that represents

the average molar N:P ratio for marine phytoplankton (Redfield, 1958).

Next to the wastewater purification, the production of microalgae is the other component of the dual concept of the microalgae incorporated integrated multi-trophic aquaculture approach. This makes a study to find possibilities to optimize the microalgal productivity using nutrient rich wastewater from a fish farm, very relevant. One of the options to improve the productivity is an extra addition of the limiting nutrient to the waste water. This is a way to increase the biomass concentration at which the microalgae can be cultivated, which increases the productivity in the photobioreactor (Gómez et al., 2013).

The aim of the study is to determine the N and P removal efficiencies and the productivity of *T. suecica* in a tubular photobioreactor using wastewater from a fish farm. The produced *T. suecica* in turn can be applied in aquaculture as feed for filter-feeding larvae. The volumetric productivity is optimized and the efficiency of the use of the available light is maximized by studying the effect of addition of the limiting nutrient, at different biomass concentrations in the PBR.

Using wastewater as a free nutrient source to culture microalgae in a fully-automated tubular photobioreactor in outdoor conditions is a step forward in the development of a more environmentally balanced and sustainable aquaculture practice (Chávez-Crooker and Obreque-Contreras, 2010). Our results will show for the first time that it is possible to remediate wastewater from a fish farm with a microalgal species applicable as feed for aquaculture in a highly controlled photobioreactor. This paper contributes to a technically feasible system, in which nutrient removal efficiencies from wastewater and microalgal productivity can be optimized.

Material and methods

Organism and culture medium

T. suecica originates from the collection of Seasalter Shellfish (Whitstable) Limited (Kent, United Kingdom). Wastewater was collected from the fish farm of Grovisco (Stavenisse, the Netherlands), where turbot is raised. Before it was used for the cultivation of *T. suecica* in a tubular PBR (Fig.1), the wastewater was pretreated with an ultrafiltration unit with a filtration pore size of 0.2 µm (Van Antwerpen Milieutechniek B.V.) to remove all suspended solids, zooplankton and bacteria and stored in a storage tank. Water in fish farms is well aerated and therefore it contains

mainly inorganic nutrients and relatively low chemical oxygen demand (COD). The nutrient concentrations of the pretreated wastewater of the turbot farm are shown in Table 1. These were measured regularly and did not change. The wastewater had a soluble COD concentration of 115 mg/L and a pH of 7.00.

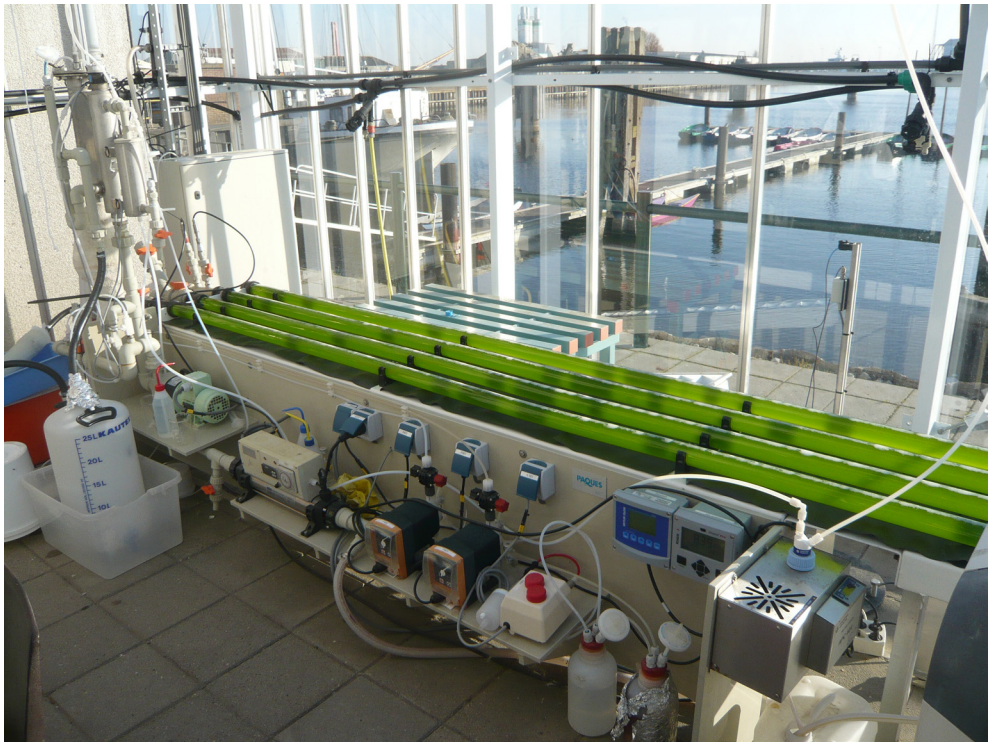

Fig. 1 Tubular photobioreactor.

Table 1 Nutrient composition of waste water from the turbot farm.

NO_3^--N (mg L^{-1})	NO_2^--N (mg L^{-1})	NH_4^+-N (mg L^{-1})	DIN (mg L^{-1})	DIP (mg L^{-1})	N:P (mol mol^{-1})
40.7	0.146	0.48	41.3	4.96	18.4

For the cultivation of *T. suecica* at biomass concentrations higher than 0.5 g/L, extra orthophosphate was dosed. The orthophosphate medium was made by dissolving 10 g $NaH_2PO_4 \cdot 2H_2O$ and 33.6 g H_3BO_3 in 1.0 L of fresh water acidified with chloric acid to a pH of 3.00 and dosed at a flow rate proportionally to the rate of incoming wastewater for obtaining the desired orthophosphate concentration.

Tubular photobioreactor and operating mode

The pH controlled tubular PBR has a working volume of 40 L, which comprises a solar receiver of 30 L and a dark degasser volume of 10 L. The microalgae were recirculated with a centrifugal pump at a rate of 2.0 m^3 h^{-1}. Its location and main operating mode are described by Michels et al. (2014). Turbidostat mode was applied to cultivate *T. suecica* in the tubular PBR during four subsequent runs of 15 days each. The experimental runs were carried out from the 30th of April 2013 till the 5th of July 2013.

For cultivating the microalgae at a biomass concentration of 0.5 g/L, only filtered wastewater was used (run 1). To compensate for the insufficient amount of DIP in the wastewater, extra orthophosphate was dosed to achieve higher biomass concentrations in subsequent runs. For cultivating *T. suecica* at a biomass concentration of 0.75 g/L, two different dosages of extra P were applied: one dosage of 2.5 mg P and another dosage of 5.0 mg P per liter of wastewater with final orthophosphate concentrations of 7.5 mg/L (run 2) and 10.0 mg/L P (run 3) as a result. The latter run was executed to find out if an excess of P would lead to a higher productivity. For a biomass concentration of 1.0 g/L, 5.0 mg P was dosed per liter of wastewater to get an orthophosphate concentration of 10.0 mg/L P (run 4). The 4 consecutive runs will be further referred as 0.5 g/L, 0.75 g/L (7.5 mg/L P), 0.75 g/L (10 mg/L P) and 1.0 g/L (10 mg/L P), respectively. The values between brackets indicate the final orthophosphate concentration in the medium after adding extra DIP.

Analytical methods

The volume of the harvested algae and biomass concentrations were measured every day according to Michels et al. (2014). A sample from the harvest was taken for the determination of the biomass concentration. The samples (<10 mg dry weight) were filtered over a pre-dried and pre-weighed Whatman GF/C glass microfiber filter and washed with 0.5 M ammonium formate. After drying for 24 h at 95 °C and cooling down in an exsiccator for 2 h, the samples were weighed again.

The analyses of ammonium, nitrite, nitrate and orthophosphate in filtrated samples were performed on a UV/VIS Spectrophotometer (DR 5000, Hach Lange, Germany). Standard Hach Lange cuvette tests were used for each parameter, except for nitrate. A calibration curve for nitrate was determined by measuring the absorbance of nitrate standard solutions in seawater at 220 and 275 nm. Twice the absorbance at 275 nm

was subtracted from the absorbance at 220 nm and this was plotted as a function of the nitrate concentration in the UV/VIS Spectrophotometer DR 5000 (Armstrong, 1963).

Ammonium, nitrate and orthophosphate were analyzed daily. Nitrite was measured once a week to check if the nitrite concentration was indeed negligible compared to the sum of ammonium and nitrate concentration.

Calculation of the daily photon flux supplied to PBR

The average daily incident photon flux density (PFD) was measured and translated into daily photon fluxes (PF_{in}, in mol d^{-1}) according to Michels et al (2014). The calculated daily photon flux turned out to be linearly related to the average daily photon flux density, with $PF_{in} = 0.91 \cdot PFD$ with a $R^2 > 0.99$. This relation was used to calculate the daily photon flux, that is experienced by the microalgae when residing in the solar receiver tubes, from the measured PFD.

Determining nutrient removal efficiencies, nutrient uptake, net volumetric productivity and yield of biomass on light

The removal efficiencies of inorganic nitrogen and phosphorus from the wastewater were calculated from the nutrient concentrations in the wastewater and in the harvested microalgal suspension:

$$N\ removal\ efficiency\ (in\ \%) = \frac{DIN_{influent} - DIN_{effluent}}{DIN_{influent}} \cdot 100$$

$$P\ removal\ efficiency\ (in\ \%) = \frac{DIP_{influent} - DIP_{effluent}}{DIP_{influent}} \cdot 100$$

When extra orthophosphate was added, $DIP_{influent}$ was the new DIP concentration after mixing the original DIP concentration in the wastewater with the dosed DIP.

The nutrient uptake (in mg g^{-1}), both for DIN and DIP, was determined by dividing the difference in DIN or DIP concentrations of the influent and of the harvested microalgal suspension by the biomass concentration.

The net volumetric productivity (P_V, in g L^{-1} d^{-1}) and yield of biomass on light ($Y_{X,ph}$, in g mol^{-1}) were determined as described in Michels et al. (2014). The net volumetric

productivity is the product of the net specific growth rate (μ, d^{-1}) and the biomass concentration (C_X, g L^{-1}). During turbidostat operation, the net specific growth rate equals the dilution rate (D, d^{-1}). The yield of biomass on light was determined by dividing the biomass harvested per day (g d^{-1}) by the daily photon flux supplied to the reactor (PF_{in}, mol d^{-1}).

Statistical analysis

One-way Anova was used to determine the differences between average values between the four runs. Due to the difference in the average PF_{in} value of run 2 in comparison with the other runs, only the nine days at the higher PF_{in} values of run 2 were used in One-way Anova to determine differences in net productivity and yield on light. Average values are presented with the standard error with a 95% confidence interval.

Results and discussion

Inorganic nutrient removal efficiencies and nutrient uptake

Assuming most marine microalgae have a N:P ratio of 16 (Redfield, 1958) and contain about 1% (w/w) of P and 9% (w/w) of N (Anderson, 1995), the theoretical maximum attainable biomass concentration was predicted based on the DIP and DIN concentrations in the medium. The N:P ratio of the wastewater of the turbot farm was 18.4 (Table 1) and almost identical to the Redfield ratio. An almost complete uptake of DIN and DIP was therefore expected when *T. suecica* was cultivated at a biomass concentration of 0.5 g L^{-1} on the wastewater with a DIN and DIP concentration of about 41 and 5 mg L^{-1}, respectively (Table 1). When *T. suecica* was grown on wastewater at a biomass concentration of 0.5 g L^{-1} without extra addition of DIP (run 1), the N and P removal efficiencies were 49.4 ± 2.5% and 99.0 ± 0.2%, respectively (Fig. 2). The N removal efficiency was thus lower than expected based on the N:P ratio of 16 reported for marine microalgal species in general (Redfield, 1958). Although, many marine microalgal species have a N:P ratio of around 16, also lower N:P values have been reported (Parsons et al., 1961), especially in nutrient replete conditions (Geider and La Roche, 2002). In particular, *T. suecica* is able to grow at high specific growth rates if cultivated on a medium with sufficient amounts of nutrients but with a N:P ratio lower than 16 (Molina et al., 1991). At those culture conditions the algal biomass that is produced, has relatively low N content (5%) (Whyte, 1987). This would explain the relatively low N uptake of 40.5 ± 2.0 mg g^{-1} and low N:P molar ratio

of 9.2 ± 0.8 of *T. suecica* grown in the wastewater of the turbot farm without any extra addition of DIP (Fig. 3). The P content of the algal biomass obtained in run 1 was 9.8 ± 0.2 mg g^{-1}, which corresponds well with reported values on phosphate content for microalgae in general (Anderson, 1995).

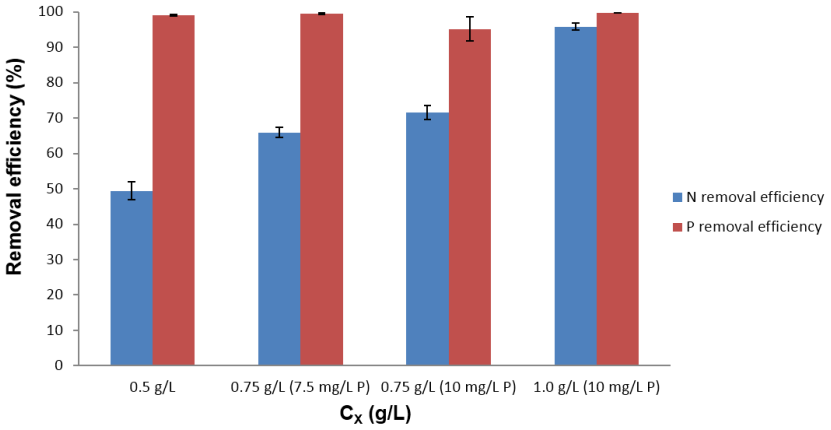

Fig. 2 Effect of biomass concentration and extra DIP addition on N and P removal efficiencies. (The values between brackets indicate that extra DIP was dosed and what the final P concentration of the medium was.)

The balance between the nutrient concentration in the wastewater and the elemental composition of the biomass determines the maximum biomass concentration that can be reached. This balance dictates which nutrient becomes limiting for the growth of the microalgae (Wang and Lan, 2011). A higher N removal efficiency is only possible if extra DIP is added to the wastewater to allow *T. suecica* to grow at a higher biomass concentration. A N removal efficiency of 95.7 ± 1.0% was reached with *T. suecica* cultured at a biomass concentration of 1.0 g L^{-1} on wastewater by increasing the DIP concentration up to 10.0 mg L^{-1} by extra addition of DIP (run 4). The N uptake for run 4 was 39.4 ± 1.0 mg g^{-1} and was not significantly different than for run 1.

Based on the assumption that *T. suecica* contains about 1% (w/w) of P, DIP was added to the wastewater to achieve a biomass concentration of respectively 0.75 g L^{-1} and 1 g L^{-1} in run 2 and run 4. The P removal efficiencies for run 2 and 4 were 99.4 ± 0.2 and 99.7 ± 0.1%, respectively. Again, just like in run 1, an almost 100% P removal efficiency was achieved, with similar P uptake leading to P contents of 9.8 ± 0.2 and 9.9 ± 0.3 mg g^{-1}, respectively.

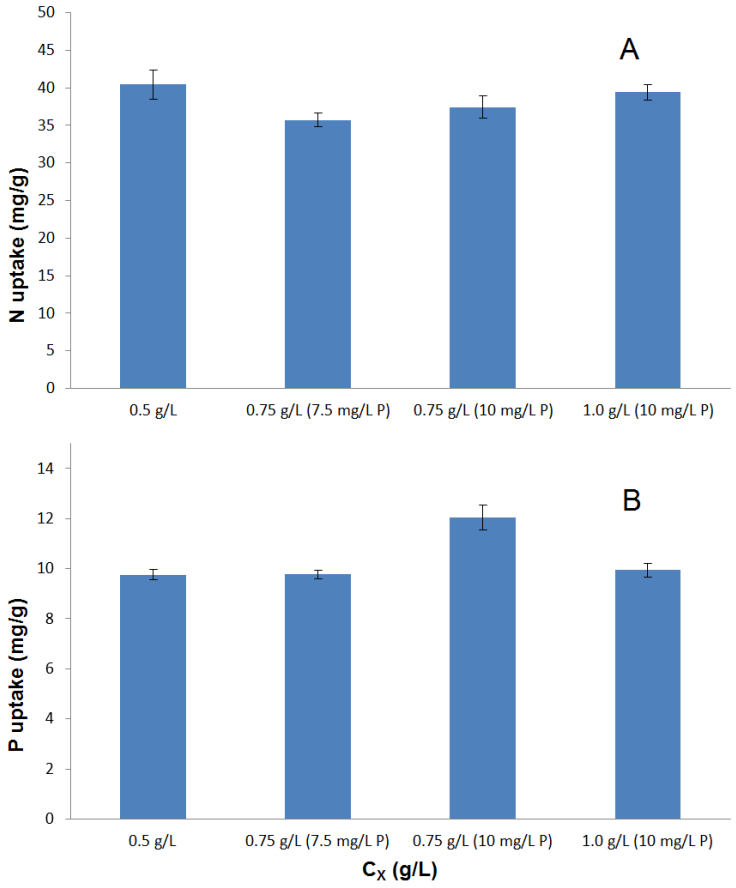

Fig. 3 N uptake (A) and P uptake (B) of *Tetraselmis suecica* at different biomass concentrations. (The values between brackets indicate that extra DIP was dosed and what the final P concentration of the medium was.)

The P removal efficiency in run 3, for which an excess of DIP was added to the incoming waste water, led to a P removal efficiency of 95.1 ± 3.5%. In this case, the P uptake of 12.0 ± 0.5 mg g^{-1} was significantly higher ($p < 0.05$) than was observed in the other runs. The P removal efficiency was therefore higher than the predicted P removal efficiency of 75%, based on the results of former runs. This indicates that *T. suecica* is capable of managing a luxury uptake of DIP. This was also reported in other studies using other microalgal species (Powell et al., 2009; Powell et al., 2011). Despite the luxury uptake of DIP, the P removal efficiency was less constant over the entire run, with values varying from 83.6 to 99.8%.

It has been claimed before that microalgal production can be designed in such a way that the inorganic nutrients from the waste water can be used, at constant N and P removal efficiencies (Chuntapa et al., 2003) and that waste of valuable nutrients is avoided (Acién et al., 2012). Our experimental results proof that this is achieved in practice.

Net volumetric productivity and yield of biomass on light

Fig. 4A shows the net volumetric productivity of *T. suecica* reached per day for the four different runs. The runs were executed at days with varying average daily photon fluxes. Despite this variation in irradiance, they all show a similar trend; the productivity increases with the amount of sunlight received. To be able to compare the net volumetric productivities obtained in the subsequent runs, only the data obtained at daily photon fluxes between 9.5 and 27.6 mol d^{-1} were considered. The average daily photon fluxes for the different runs are similar (Table 2). The average net volumetric productivities ranged between 0.46 and 0.52 g L^{-1} d^{-1}, when *T. suecica* was cultured at biomass concentrations of 0.75 and 1.0 g L^{-1}. The productivities were significantly higher ($p < 0.05$) than the productivity of run 1 (0.35 g L^{-1} d^{-1}), which was operated at a biomass concentration of 0.5 g L^{-1} (Fig. 4A). The lower productivity at low biomass concentrations is probably due to inefficient absorbance of photons at the relatively high local light conditions experienced by the algae in the solar receiver tubes (Takache et al., 2010).

The efficiency of the use of the available light can be expressed as the yield of biomass on light. The yield of biomass on light is decreasing with an increasing daily photon flux (Fig. 4B). This phenomenon is a result of excess light being wasted as fluorescence and heat (Müller et al., 2001; Mussgnug et al., 2007). The yield of biomass on light was the highest for *T. suecica* cultured at biomass concentrations of 0.75 and 1.0 g L^{-1}. The average yield of biomass on light of the microalgae grown determined over the entire run at a biomass concentration of 1.0 g L^{-1} was significantly higher ($p < 0.05$) than at a biomass concentration of 0.5 g L^{-1}, which were 1.09 ± 0.16 and 0.80 ± 0.14 g mol^{-1}, respectively (Table 2).

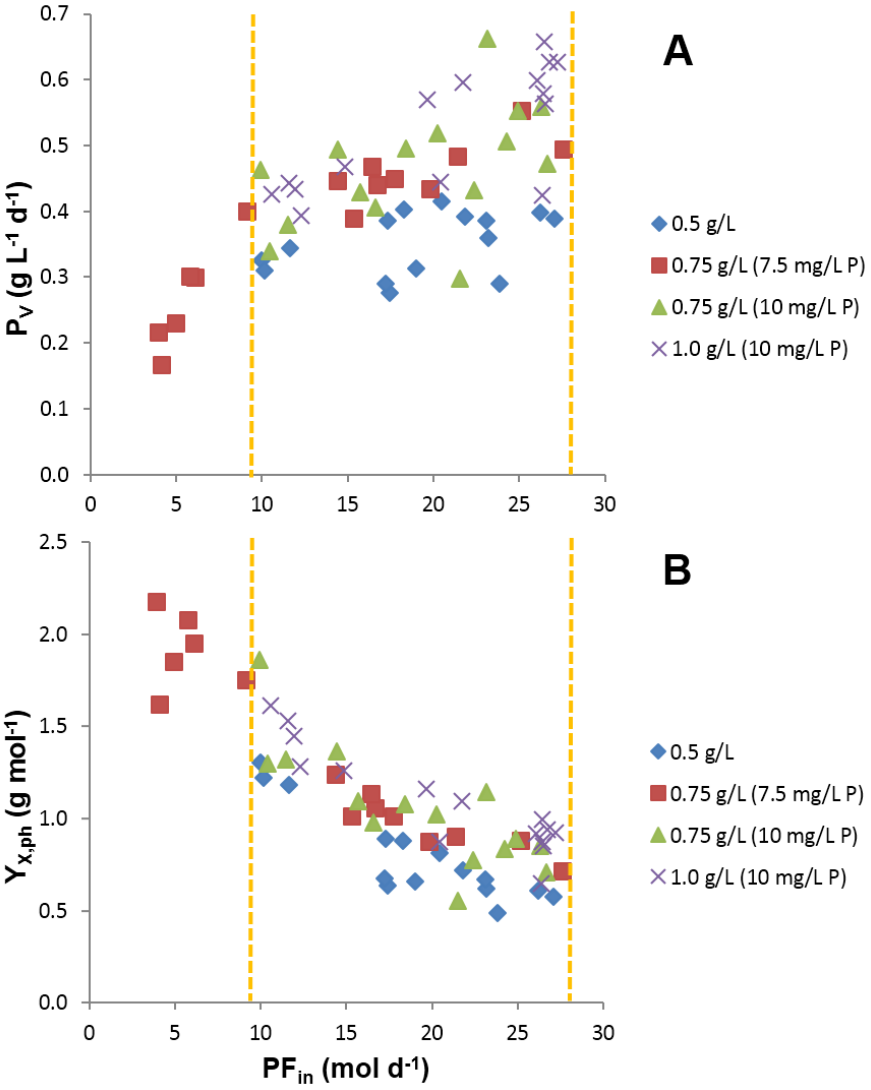

Fig. 4 Net volumetric productivity (A) and yield of biomass on light (B) of *Tetraselmis suecica* versus the daily photon flux. (Dashed lines indicate the range at which the average daily photon fluxes of the different runs were not different.)

Table 2 Average biomass concentrations, daily photon fluxes, net volumetric productivities and yields of biomass on light for the 4 different runs.

Run	Average Cx ($g\ L^{-1}$)	Average PF_{in} ($mol\ d^{-1}$)	Average P_v ($g\ L^{-1}\ d^{-1}$)	Average $Y_{X,ph}$ ($g\ mol^{-1}$)
1) 0.5 g/L	0.50 ± 0.01^{a}	19.1 ± 3.0^{a}	0.35 ± 0.03^{a}	0.80 ± 0.14^{a}
2) 0.75 g/L (7.5 mg/L P)	0.76 ± 0.01^{b}	$19.4 \pm 3.5^{*a}$	$0.46 \pm 0.04^{*b}$	$0.98 \pm 0.12^{*ab}$
3) 0.75 g/L (10 mg/L P)	0.79 ± 0.02^{b}	19.1 ± 3.2^{a}	0.47 ± 0.05^{b}	1.05 ± 0.18^{ab}
4) 1.0 g/L (10 mg/L P)	1.00 ± 0.03^{c}	20.6 ± 3.7^{a}	0.52 ± 0.05^{b}	1.09 ± 0.16^{b}

*Average daily photon flux, net volumetric productivity and yield of biomass on light were calculated from *Tetrasemis suecica* in run 2 receiving daily photon fluxes in the same range as the other runs; between 9.5 and 27.6 mol d^{-1}.

a,b,c Average values not sharing a common superscript were significantly different ($p < 0.05$).

The results do not fully comply with the findings in Michels et al. (2014), who observed highest productivities at an optimal biomass concentration of *T. suecica* of 0.7 g L^{-1} at an average daily photon flux of 12.4 mol d^{-1}. The average daily photon fluxes reported by Michels et al. (2014) were lower than in the subsequent runs described in this paper. This indicates that the biomass concentration optimized for growth in tubular photobioreactors varies with the season; a higher optimal biomass concentration should be used in the summer while in winter a lower optimal biomass concentration should be applied to achieve higher productivities. Furthermore, the composition of the medium used in this experiment, waste water form a turbot farm, is different from the artificial medium used in the experiment of Michels et al. (2014), while the pH was controlled in the same way by CO_2 addition and set at 8.40. The effect of micronutrients or vitamins in the wastewater on the productivity is unknown. Although the micronutrients and vitamins were not analyzed, it seems that the wastewater does not contain any inhibiting substances and is a valuable source of free nutrients.

In addition, an excess of P in the medium does not lead to a significantly higher average net volumetric productivity or average yield of biomass on light ($p < 0.05$) based on comparison of the performance measured in run 2 and run 3 (Table 2).

In this study, some bacteria were present in the culture of *T. suecica* in the tubular PBR. Microscopic analysis of daily samples showed no change in the ratio between microalgae and bacteria over time. Although bacteria are reported to induce biofouling (Holmes, 1986) leading to losses in productivity (Arbib et al., 2013), biofouling was not observed during the 130 days including the trials and the experiment itself. This demonstrates that the cultivation of *T. suecica* was stable without an overgrowth of bacteria.

Conclusion

Growing *T. suecica* at a biomass concentration of 0.5 g L^{-1} on only wastewater from a fish farm resulted in N and P removal efficiencies of 49.4% and 99.0%, respectively. A 95.7% N removal efficiency was obtained at a biomass concentration of 1.0 g L^{-1}, when extra DIP was dosed. The average net volumetric productivity was also higher at higher biomass concentrations.

Therefore, wastewater treatment processes with microalgae can be optimized if the productivity in relation to the available light, nutrient uptake of the microalgal species, biomass concentration and nutrient removal efficiencies are geared to one another.

Acknowledgments

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4

CHAPTER 5

Effect of cooling in the night on the productivity and biochemical composition of *Tetraselmis suecica*

This chapter is published as:
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Abstract

The effect of cooling at night on the 24 hour productivity and biochemical composition of *Tetraselmis suecica* cultivated in a tubular photobioreactor was determined. The hypothesis that cooling at night would decrease the night respiration rate and therefore enhance the net productivity was rejected. The productivity over a 24 hour period and biochemical composition were only influenced by the daily light input. Higher carbohydrate and lower protein content were observed in periods with more light. Carbohydrates produced during daylight were used for the protein synthesis at night, while the fatty acid content stayed constant during the day. The rate of carbohydrate loss at night was linearly related to the specific growth rate and therefore to the light history of the microalgae.

Introduction

The marine microalga *Tetraselmis suecica* is a valuable feedstock in aquaculture, where it is used for direct feed for shellfish or indirect feed for some fish larvae via rotifers, copepods or *Artemia* in hatcheries (Becker, 2013). This phototrophic microalga can best be cultivated at controlled conditions in closed photobioreactors (PBRs). This ensures constant high productivity and high quality of the feedstock. Especially at the larval and post-larval stages of shellfish, crustaceans and fish high-quality algae are needed (Muller-Feuga, 2004).

When placed outdoor the light conditions in the PBR are not constant and change over the day. During daylight CO_2 is captured and photosynthesis is taking place, while the formed carbohydrates are partly used in respiration processes. Because photosynthesis predominates over respiration during daylight, a net increase in biomass during the day will be observed. During the night photosynthesis is no longer possible and respiration prevails. This results in biomass losses during the night. During the night microalgal cells metabolize the carbohydrates for maintenance purposes and for the synthesis of proteins and other cellular compounds. The net productivity is thus the result of both photosynthesis and respiration.

So far, a lot of studies have been dedicated on increasing the net productivity and achieving higher photosynthetic efficiency by optimizing PBR design (Cuaresma et al., 2011), optimizing dilution rate (Camacho-Rodríguez et al., 2014; Sánchez et al., 2008), fine-tuning the biomass concentration in relation to light availability (Michels et al., 2014; Takache et al., 2010), and adjusting CO_2, nutrient supply (González-López et al., 2012) and temperature (Molina et al., 1991). However, little research has been done to verify if the net productivity can be increased by decreasing the biomass loss due to respiration during the night. The hypothesis of this study is that cooling of a PBR during the night will decrease the respiration rate during the night. This would result in less biomass loss during the night and therefore in a higher overall net productivity.

Different microalgal species grown at their optimal temperature resulted in a lower respiration during the night than when they were cultivated at suboptimal temperatures (Grobbelaar and Soeder, 1985; Han et al., 2013; Ogbonna and Tanaka, 1996; Torzillo et al., 1991). The night biomass loss rate depends on the culture temperature during darkness and follows Arrhenius law (Le Borgne and Pruvost, 2013). Apart from the general finding that the respiration rate depends on the temperature, the amount of

light received over the day also seems to affect the respiration losses during the night (Gibson, 1975). High light intensities during the day lead to higher concentrations of carbohydrates in the cell synthesized in the chloroplast. During the night the microalgae convert the surplus of carbohydrates formed during photosynthesis over the day into other building blocks (Smith et al., 1990). The amount of daylight is therefore an important parameter, which influences the biochemical composition of the microalgae during the course of the day (Ogbonna and Tanaka, 1996; Torzillo et al., 1991).

Over the period of one day the chemical composition of microalgae changes. The protein synthesis is low during daylight, when energy is mainly stored in the form of carbohydrates, while in the night an increase in protein content was observed (Rebolloso Fuentes et al., 1999). Whether this circadian rhythm observed for the carbohydrates and protein content of the microalgae is also affecting the fatty acid or lipid content in microalgae is still unclear (Rebolloso Fuentes et al., 1999; Silva Benavides et al., 2013). However, lipids generally serve more for membrane structures and are less used as a storage product, when microalgae are grown under favorable conditions (Hu et al., 2008). Lipids are therefore expected to exhibit minor daily variations in content (Chapin et al., 1990; de Winter et al., 2013).

Cooling the microalgae during the night might therefore not only increase the net productivity via inhibition of the respiration rate, but it might also affect the biochemical composition of the microalgae.

The aim of the study is to determine diurnal changes of the biochemical composition and productivity of *T. suecica* cultivated in a tubular PBR at different temperatures in the night. Since the microalgae in the tubular PBR were grown under outdoor conditions, the influence of daily light input was also considered.

Material and Methods

Organism and culture medium

The chlorophyceae *T. suecica* was obtained from Seasalter Shellfish (Whitsable) Limited (Kent, United Kingdom). The culture medium used was a modification of the Walne medium (Laing, 1991) mixing 1 L of macro- and micronutrient stock solution with 100 mL of vitamin solution. The composition of the nutrients stock solution is 0.8 g $FeCl_3$, 0.4 g $MnCl_2 \cdot 4H_2O$, 33.6 g H_3BO_3, 45.0 g EDTA, 20.0 g $NaH_2PO_4 \cdot 2H_2O$,

100.0 g $NaNO_3$, 21 mg $ZnCl_2$, 20 mg $CoCl_2 \cdot 6H_2O$, 9 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 20 mg $CuSO_4 \cdot 5H_2O$ in 1L of distilled water. The stock solution of vitamins contains 1.0 g thiamine and 0.05 g cianocobalamine in 1 L of distilled water. Concentrated HCl was used in order to adjust the pH to 4.0.

Tubular photobioreactor and operating mode

Experiments were carried out in a horizontal tubular PBR in a greenhouse situated in Vlissingen, The Netherlands (Michels et al., 2014). The effect of cooling at night on the productivity, biomass loss rate and biochemical composition of *T. suecica* was studied. Three different night temperatures, 10, 15 and 20 ºC at night were tested, keeping the temperature during daylight at 20 ºC. Cooling at 10 ºC in the night was chosen as the lowest temperature to avoid too much stress of the microalgae. The temperature sensor and the data management system SCADA with a manual controller were used to keep the temperature constant within a range of ± 0.5 ºC. This was achieved by using heat exchange through an annular water jacket around the degasser and a recirculation system including a chiller/heater device. The temperature set point was manually changed to the night set point at sunset and to the day set point at sunrise. Each night temperature was applied for a period of 14 days in February and March 2014 with daily light irradiances varying between 2 and 17 mol photons per day (Fig. 1). These months were selected for two reasons. First of all, days in March are around the equinox with a similar daylight and night duration. February with night hours longer than 12 was chosen, because a longer duration of cooling in dark was expected to have a greater effect on the productivity, biomass loss rate and biochemical composition. Sunrise and sunset in February were around 7:50 a.m. and 6:15 p.m., respectively. Sunrise and sunset on the sampling days in March were around 6:50 a.m. and 6:50 p.m., respectively.

The PBR operated in turbidostat mode at a biomass concentration of circa 0.6 g L^{-1}. A turbidity sensor (Inpro 8300 RAMS TCS, Mettler Toledo) was used to monitor and control the turbidity. When the upper turbidity set point was exceeded, the seawater input valve was automatically opened and the Walne medium was dosed on demand at a flow rate of 3.3 mL per liter of seawater till the lowest turbidity set point was reached. The culture was harvested over a siphon in a harvest reservoir at the same time.

The recirculation speed in the PBR was 0.37 m s^{-1} and the pH during daylight at the end of the tube was set at 8.40 as described in Michels et al. (2014).

Calculation of the daily photon flux

The local daily photon fluxes experienced by the microalgae in the solar receiver (PF_{in}, in mol d^{-1}) were derived from the measured average daily incident photon flux densities (PFD, in mol m^{-2} d^{-1}) according to Michels et al. (2014). The locally experienced PF_{in} and the measured incident PFD were found to be linearly related via $PF_{in} = 0.91 \cdot PFD$ with $R^2 \geq 0.99$.

Analytical methods

Harvest process and biomass concentration

The harvested culture volume was measured daily with a measuring cylinder. The biomass concentration was quantified by dry weigh determination (Zhu and Lee, 1997). A sample from the harvest was taken every day, around noon. Samples of 8 mL were taken in duplicate, filtered and washed with 10 mL of a 0.5 M ammonium formate solution through a pre-dried and pre-weighed Whatman GF/C glass microfiber filter. Then, the samples were dried for 24 h at 80 ºC, cooled down in a desiccator for 2 h and weighed again.

Biochemical composition analysis

Over a period of 24 h, sampling was carried out at each different temperature to study the effect of both the cooling at night and the irradiance on changes in the biochemical profile of *Tetraselmis suecica* over the day. This was done in February and in March 2014 for each temperature; so the 24 h sampling was done six times in total. A 300 mL sample was taken every 3 h and centrifuged at 2500 rpm for 10 min (Sorvall D-37520 Legend T, Kendro Laboratory Products, Germany). The sample was washed twice with distilled water, stored at -80 °C prior to being freeze-dried and then stored at -20 °C for a maximum of 10 days.

The biochemical composition was determined following established methods: carbohydrates through the phenol-sulphuric acid method (Dubois et al., 1956), proteins via the method which is based on the reaction of protein with alkaline copper tartrate solution and Folin reagent (Lowry et al., 1951) and fatty acid extraction and quantification with gas chromatography (Breuer et al., 2013).

Net volumetric productivity, growth rate, yield on light and CO_2 uptake

The net volumetric productivity (P_V, in g L^{-1} d^{-1}) was calculated as the product of the specific growth rate (μ, in d^{-1}) and the biomass concentration (C_x, in g L^{-1}).

$$P_V = \mu \cdot C_X$$

Under continuous turbidostat mode operation at steady state, the net specific growth rate is equal to the dilution rate (D, in d^{-1}), defined as the volume of harvest ($V_{harvest}$, in L) relative to the volume of the PBR (V_{PBR}, in L) per day (t_d = 1 d) (Lee and Shen, 2004):

$$\mu = D = \frac{V_{harvest}}{V_{PBR} \cdot t_d}$$

The yield on light ($Y_{X,ph}$, in g mol^{-1} of PAR photons) is defined as the amount of biomass harvested per day (g d^{-1}), divided by the daily photon flux supplied to the PBR (PF_{in}, in mol photons d^{-1}).

$$Y_{X,ph} = \frac{V_{harvest} \cdot C_X}{PF_{in} \cdot t_d}$$

The CO_2 uptake (g CO_2 g^{-1} biomass) is defined as the amount of CO_2 supplied to the PBR per day ($CO_{2\,dose}$, in g CO_2 d^{-1}) divided by the harvested amount of biomass per day (g biomass d^{-1}):

$$CO_2 uptake = \frac{CO_{2\,dose}}{C_x \cdot \frac{V_{harvest}}{t_d}}$$

Carbohydrate loss and CO_2 production in the night

The rate of carbohydrate loss during the night was assumed to follow first-order kinetics. From this the carbohydrate loss rate at night (d^{-1}) was deduced to be:

$$Carbohydrate\ loss\ rate = \frac{\ln([CH_2O]_{18:00}) - \ln([CH_2O]_{6:00})}{0.5}$$

where $[CH_2O]_{18:00}$ and $[CH_2O]_{6:00}$ are the carbohydrate concentrations (g L^{-1}) at 6 p.m. and 6 a.m., respectively. A standard dark period of 0.5 day was taken; the night

duration of the three 24 h sampling days in February were between 13.5 and 14 h, while the three 24 h sampling days in March were around the equinox with a night length of 12 h.

The CO_2 concentration (ppm) in the off-gas from the degasser and in the ambient air was measured with a gas analyzer (Servomex 4000) in order to calculate the net CO_2 production at night. The CO_2 production at night (g CO_2 L^{-1} $night^{-1}$) is quantified via the average CO_2 concentration generated by the culture $\{[CO_2]_{off\text{-}gas} - [CO_2]_{air}\}$ (L L^{-1}) times the night length (t_{night}, in h) and the air flow supplied to the degasser (F_{air}, in L h^{-1}), $M(CO_2)$ the molar mass of CO_2 (g mol^{-1}), divided by the volume of the tubular PBR (V_{PBR}, in L) and the molar volume of gas (V_m, in L mol^{-1}),

$$CO_2\ production\ at\ night = \frac{\{[CO_2]_{off-gas} - [CO_2]_{air}\} \cdot F_{air} \cdot M(CO_2) \cdot t_{night}}{V_m \cdot V_{PBR}}$$

Statistical analysis

Average values are given with the standard deviation. One-way ANOVA was used to ascertain if there were differences between average values of net volumetric productivity, yield on light and CO_2 production at the different culture temperatures applied at night.

Results and Discussion

Net volumetric productivity, yield on light and CO_2 uptake

Fig. 1 shows the net volumetric productivity and yield on light of *T. suecica* cultivated at a biomass concentration of 0.59 ± 0.04 g L^{-1} for all experimental days and the three different culture temperatures at night. When the amount of daylight measured as daily photon flux (mol d^{-1}) increased, the net volumetric productivity increased and the yield on light decreased. Yield on light is always higher at low daily photon fluxes, because less photons are being wasted as fluorescence and heat (Müller et al., 2001; Mussgnug et al., 2007).

The average net volumetric productivities and yields on light for each different culture temperature at night were not significantly different at daily photon fluxes between 5 and 13 mol d^{-1}, since *p*-values are larger than 0.05 (Table 1), for which the average daily photon fluxes were comparable. This result is in contrast with the hypothesis

that cooling at night would improve the net productivity. Le Borgne and Pruvost (2013) found that lower temperature in the night enhanced the net productivity, being the overall result of production during daylight and biomass loss at night. However, their experimental data of biomass loss rate at different night temperatures were obtained after applying a dark period of 24 h without a light period afterwards. In this study, the microalgae experienced a natural day-night cycle with different light conditions during the day. The overall effect of cooling at night on the net volumetric productivity and yield on light was found to be negligible. Although lower temperatures at night did not seem to lead to lower night biomass losses, the lower temperature could still have an influence on the metabolic processes during the daylight. Therefore, the changes in biochemical composition and the respiration at night were determined. The effect of the light history of the previous day on the cell composition was incorporated in the study as well.

The CO_2 dosages were divided by the net volumetric productivities of all days to determine the amount of CO_2 that was fixated. Because the pH at the end of the tube of the PBR was set at 8.40 during daylight, no CO_2 was lost based on the equilibria of the carbonate species. At a pH of 8.40 the CO_2 concentration is less than 1% in comparison with the bicarbonate concentration. This was also confirmed by the measurements of the off-gas of the degasser, which showed negligible differences in CO_2 concentration in comparison to the CO_2 concentration in the incoming air. On average, the net amount of fixated CO_2 (in g CO_2 g^{-1} biomass) by *T. suecica* was 1.53 g g^{-1}, which is in accordance with the CO_2 requirement of the algal biomass (Mazzuca Sobczuk et al., 2000). The CO_2 uptake was actually expected to be higher. During the night respiration prevails and CO_2 will be released in the culture medium. The CO_2 released in the medium during the night, causes a decrease in the pH of the medium during the night. This CO_2 was used in the morning till the pH was 8.40 again and CO_2 was automatically supplied to control the pH at the set-point.

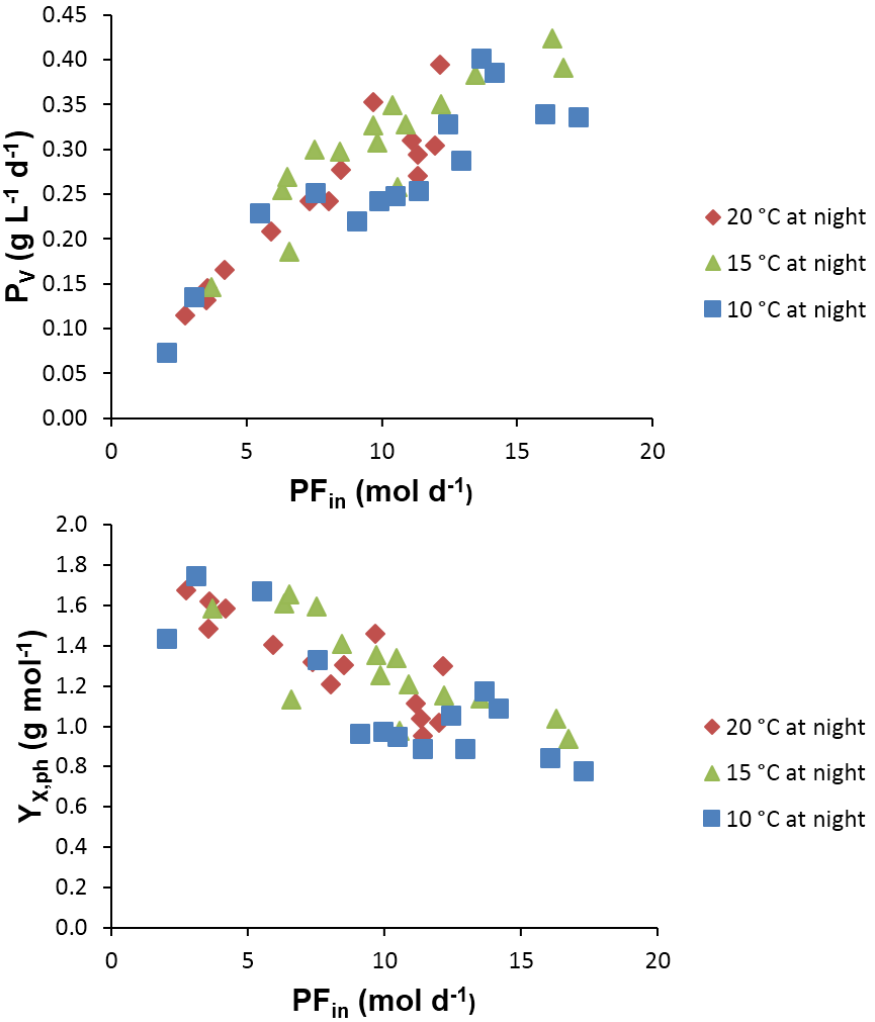

Fig. 1 Net volumetric productivity and yield on light of *Tetraselmis suecica* at culture temperatures of 10, 15 and 20 °C in the night.

Table 1 Average daily photon fluxes, net volumetric productivities, yields of biomass on light and CO_2 production at night for the 3 different night temperatures[a].

T_{night} (°C)	PF_{in} (mol d^{-1})	P_v (g L^{-1} d^{-1})	$Y_{X,ph}$ (g mol^{-1})	CO_2 production at night (g L^{-1} d^{-1})
10	9.92 ± 2.50	0.26 ± 0.04	1.09 ± 0.27	0.030 ± 0.007
15	8.99 ± 2.02	0.30 ± 0.05	1.33 ± 0.22	0.035 ± 0.007
20	9.75 ± 2.18	0.29 ± 0.05	1.21 ± 0.18	0.037 ± 0.010
One-way ANOVA: effect of night temperature on the daily photon fluxes, net volumetric productivities, yields of biomass on light and CO_2 production at night at the 95% confidence level. The data variability is indicated by the *p*-value.				
10 °C vs 15 °C	1.000	0.285	0.074	0.530
10 °C vs 20 °C	1.000	0.553	0.768	0.211
15 °C vs 20 °C	1.000	1.000	0.642	1.000

[a]Average daily photon flux, net volumetric productivity, yield of biomass on light and CO_2 production at night were calculated from *Tetrasemis suecica* receiving daily photon fluxes in the same range; between 5 and 13 mol d^{-1}.

Diurnal changes in biochemical composition

The biochemical composition of *T. suecica* measured as carbohydrates, proteins, total fatty acids and eicosapentaenoic acid (EPA) in percentages of dry weight at culture temperatures of 10, 15 and 20 °C at night in February and March are shown in Fig. 2. The increase of carbohydrates and decrease of proteins during daylight and the reverse trend observed in the dark are evident. During the night carbohydrates were used, while protein was formed. A similar behavior of carbohydrate and protein content in *Spirulina platensis* was observed during a complete day-night cycle (Torzillo et al., 1991).

The average carbohydrate content in March was higher than in February with the consequence that the average protein content was higher in February than in March. Furthermore, the increase of carbohydrate content during the daylight followed by carbohydrate consumption in the night was higher in March than in February. In March, the carbohydrate content at night decreased by 12.1 ± 1.9%. This decrease was significantly higher than in February where an average decrease of carbohydrate content of 3.5 ± 3.0% was measured. The decrease of carbohydrate content at night in February was the lowest at 10 °C, but the increase of carbohydrates during

daylight was also lower due to the low daily photon flux (Table 2). Carbohydrates are the first components being produced by photosynthesis and obviously more in periods with more light and at a higher net volumetric productivity (Table 2). In February, the daily photon flux was highest for the 24 h sampling day with a night temperature of 15 °C (Table 2), which resulted in a higher average carbohydrate content. It was not as high as in March and this was probably caused by the light history of the cells in February. The average daily photon flux in February and March were 6.55 and 11.74 mol d^{-1}, respectively. Therefore, consecutive days of high light lead to a higher average carbohydrate content and lower average protein content. Similar effects have been reported on the biochemical composition of phytoplankton in a natural marine environment (Suárez and Marañón, 2003). Higher carbohydrate and lower protein content in the phytoplankton occurred near the surface in spring and summer, where the light availability was the highest.

In our study the percentage of total fatty acids remained constant during the days measured (Fig. 2), while Rebolloso Fuentes et al. (1999) found a decrease of fatty acid content during daylight, and a decrease of lipid content during the night has also been reported (Silva Benavides et al., 2013). Although the total fatty acid content remained constant, the EPA content decreased during daylight and increased at night for the days at high light conditions (Fig. 2). This can be explained by the importance of EPA as an structural fatty acid in *T. suecica* (Bondioli et al., 2012). EPA is incorporated in cell membranes and accumulates at favorable conditions for growth of the microalgae.

Overall, it can be stated that the biochemical composition and the productivity were mostly influenced by the light history of the microalgal cells. The daily average carbohydrate content is influenced by the light history, being significantly higher in periods with more light (Table 3). Accordingly, a significant drop in the daily average protein accumulation is observed when the daily photon flux increased (Table 3). Cooling in the night did not show a conclusive effect (Fig. 2). This has far-reaching effects on the energy requirements in the process. Especially in early spring and late autumn when the ambient temperature at night drops the temperature control could be switched off. Less energy is needed for heating during the night while the cooling does not affect the net biomass productivity.

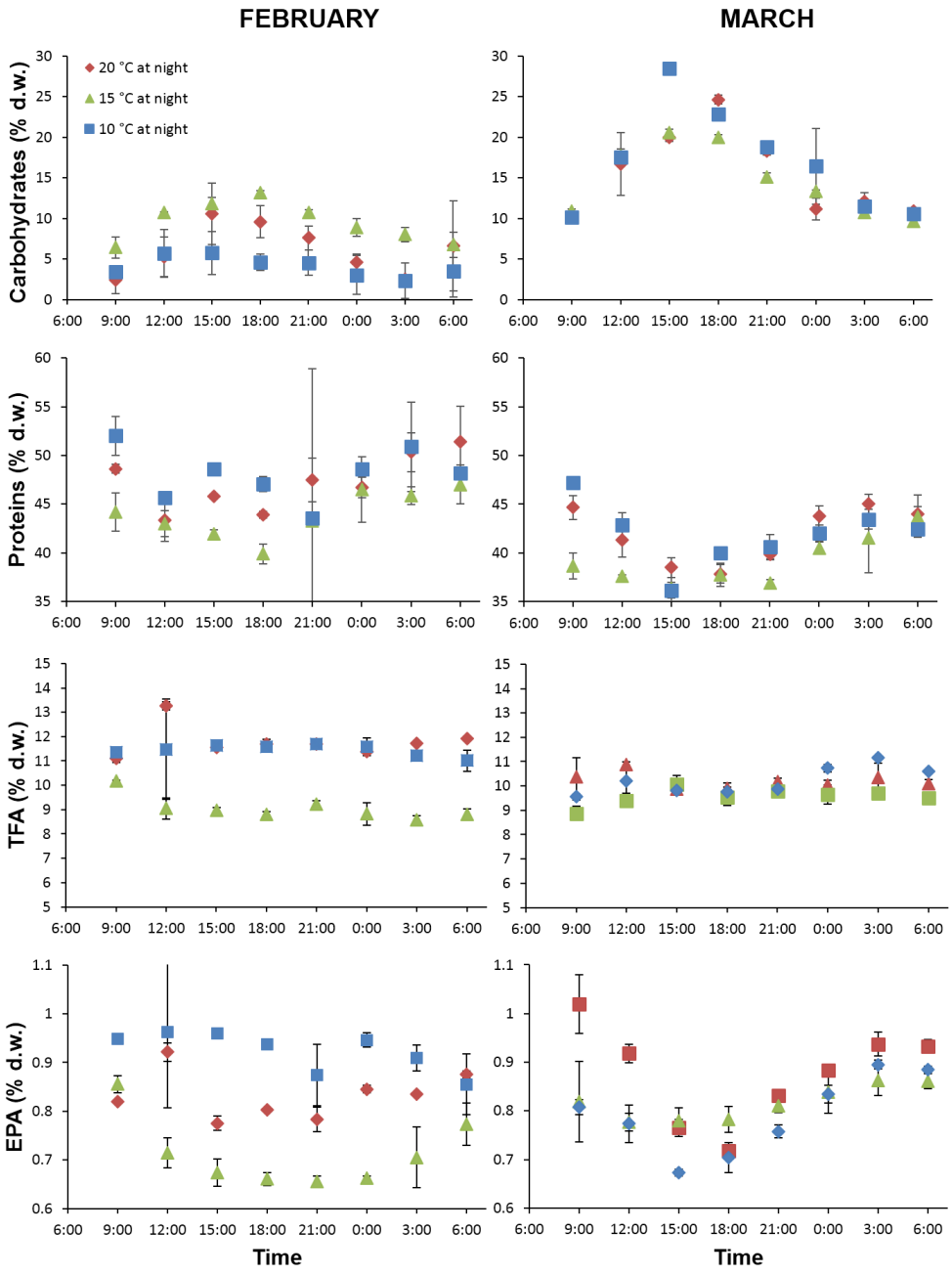

Fig. 2 Carbohydrate, protein, total fatty acid and EPA content (% of dry weight) of *Tetraselmis suecica* at culture temperatures of 10, 15 and 20 °C at night in February and March. Light and productivity data of the six 24 h sampling days are provided in Table 2.

Table 2 Daily photon flux, specific growth rate, net volumetric productivity and carbohydrate loss rate of *Tetraselmis suecica* at three different night temperatures in February and March.

Month	T at night (°C)	PF_{in} (mol d^{-1})	μ (d^{-1})	P_V (g L^{-1} d^{-1})	Carbohydrate loss rate (d^{-1})
February	10	3.08	0.21	0.13	0.55
	15	9.82	0.53	0.31	1.61
	20	5.92	0.37	0.21	1.18
March	10	12.44	0.61	0.33	1.71
	15	10.42	0.55	0.35	1.69
	20	11.34	0.54	0.29	1.70

Table 3 Average carbohydrate and protein content of Tetraselmis suecica at three different night temperatures in February and March.

Month	T at night (°C)	PF_{in} (mol d^{-1})	Carbohydrate content (%)	Protein content (%)
February	10	3.08	4.3 ± 0.9^{a}	48.2 ± 0.9^{a}
	15	9.82	9.7 ± 0.9^{b}	44.0 ± 0.9^{b}
	20	5.92	6.0 ± 0.9^{a}	48.4 ± 0.9^{a}
March	10	12.44	17.3 ± 0.9^{c}	41.9 ± 0.7^{b}
	15	10.42	15.1 ± 0.9^{c}	39.1 ± 0.7^{c}
	20	11.34	14.9 ± 0.9^{c}	41.9 ± 0.7^{b}

$^{a.b.c}$Average values not sharing a common superscript were significantly different.

Although no observed effect of cooling at night was observed in this study, cooling at night might increase the productivity of *T. suecica* when it is cultivated at higher light intensities for a longer period of light per day or at larger differences in day and night temperature. Therefore, further research on the effect of night temperature and light history on daily cycles of this microalgal strain is recommended in order to see, if the productivity and quality of the microalgae is not affected at those conditions.

Carbohydrate loss and CO_2 production at night

The carbohydrate loss rates of *T. suecica* during the six nights at three different temperatures differed a lot in February and were similar in March (Table 2). No relationship between carbohydrate loss rate during the night and the culture temperature at night was found. As previously mentioned, the carbohydrate content of *T. suecica* at the end of the day depends on the light history experienced by the algae during the day itself and experienced over the season. Because carbohydrates are primarily used during the night for maintenance purposes and for synthesis of proteins and other compounds needed for growth, carbohydrate content at the end of the day and carbohydrate loss rate during the night are linked. Therefore, the influence of light history on the carbohydrate loss rate was analyzed in more detail.

The specific growth rate as function of the daily photon flux was described by the Monod equation:

$$\mu = \frac{\mu_{max} \cdot PF_{in}}{K_m + PF_{in}}$$

This Monod equation was used to fit the observed specific growth rate. The best fit was obtained with μ_{max} of 1.42 d^{-1} as the maximum specific growth rate and K_m of 17.7 mol d^{-1} as the daily photon flux at which the specific growth rate is the half of the maximum (Fig.3A). These are similar to data reported in literature with values of μ_{max} of 1.42 d^{-1} and K_m of 18.1 mol d^{-1} for *T. suecica* (Molina Grima et al., 1994). The dependence of carbohydrate loss rates on the light received in the day follows similar kinetics and can be described as:

$$Carbohydrate\ loss\ rate = \frac{k_{max} \cdot PF_{in}}{K_{CH2O} + PF_{in}}$$

with k_{max} of 4.12 d^{-1} as the maximum carbohydrate loss rate and K_{CH2O} of 16.01 mol d^{-1} as the daily photon flux at which the carbohydrate loss rate is the half of the maximum carbohydrate loss rate (Fig. 3B). When the carbohydrate loss rate is plotted versus the specific growth rate that is observed at a given light input over the day, a linear relation is found (Fig. 3C). This confirms that the light history of the cells is directly coupled to the carbohydrate loss rate.

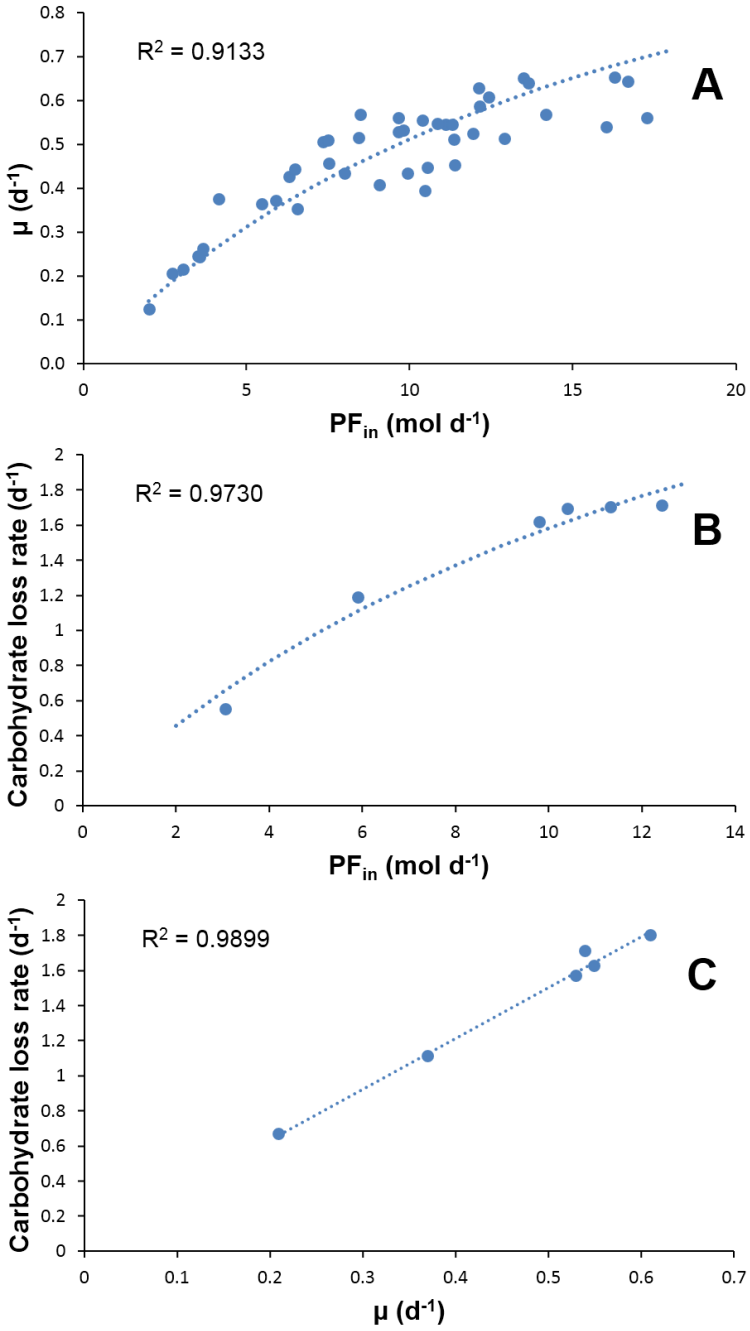

Fig. 3 Influence of daily photon flux on growth rate (A), carbohydrate loss rate (B) and the relationship between growth rate and carbohydrate loss rate (C). The dotted lines represent the fitted Monod equation for the specific growth rate using μ_{max} = 1.42 d^{-1} and K_m = 17.7 mol d^{-1} in Fig. 3A and k_{max} of 4.12 d^{-1} and K_{CH2O} of 16.01 mol d^{-1} to fit the carbohydrate loss rate in Fig. 3B.

The measured CO_2 production due to respiration at night is related to the net specific growth rate (Fig. 4) and thus also to the amount of daylight received (Fig. 3). It seems that the CO_2 production at night was lower at lower temperatures at night, but the differences were not significant (*p*-value > 0.05; Table 1). Respiration is the sum of the costs of maintenance and biosynthesis. CO_2 is being produced for both processes of which maintenance respiration is the basis. The biosynthesis respiration costs are mainly due to the formation of proteins for which carbohydrates are used as well (Geider et al., 1998; Penning de Vries et al., 1974).

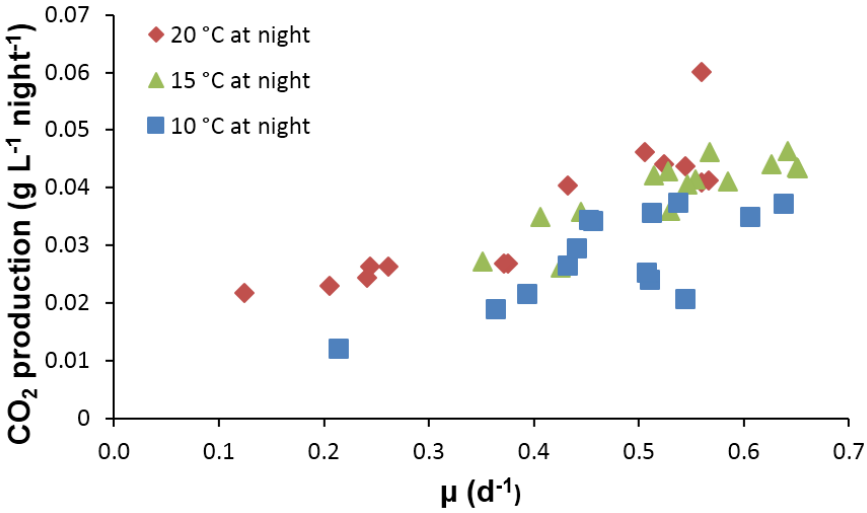

Fig. 4 CO_2 production at night versus net specific growth rate.

Conclusion

In months of relatively low light input per day, the productivity as well as the biomass composition were influenced by the daily light input. During daylight a carbohydrate build-up is observed together with a protein content decrease, while at night the inverse was observed. The carbohydrate loss rate at night depended on the growth rate and therefore on the light conditions during the daylight period. The net productivity of *T. suecica* was not enhanced by cooling the culture at night. Because cooling does not affect the productivity and the biochemical composition negatively either during periods with relatively low light input, energy can be saved by turning off the temperature control at night during these periods.

Acknowledgments

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CHAPTER 6

Cultivation of shear stress sensitive and tolerant microalgal species

This chapter has been submitted for publication as:
Michels MHA, Van Der Goot AJ, Vermuë MH, Wijffels RH. Cultivation of shear stress sensitive and tolerant microalgal species.

Abstract

The tolerance to shear stress of *Tetraselmis suecica*, *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* was determined in shear cylinders. The shear tolerance of the microalgal species strongly depends on the strain. *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* exposed to shear stress between 1.2 and 5.4 Pa resulted in severe cell damage. *Tetraselmis suecica* is not sensitive to stresses up to 80 Pa.

The possibility to grow these algae in a tubular photobioreactor (PBR) using a centrifugal pump for recirculation of the algae suspension, was studied. The shear stresses imposed on the algae in the circulation tubes and at the pressure side of the pump were 0.57 and 1.82 Pa, respectively. The shear stress tolerant *Tetraselmis suecica* was successfully cultivated in the PBR. Growth of *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* in the tubular PBR was not observed, not even at the lowest pumping speed. For the latter shear sensitive strains, the encountered shear stress levels were in the order of magnitude of the determined maximum shear tolerance of the algae. A model was used to predict the effect of local high shear zones on the percentage of microalgae that survive the passage through such zone. This model shows that a culture of shear stress sensitive species is bound to collapse after only limited number of passages. This shows that shear stress is a major process parameter in future design of closed PBRs for microalgal cultivation of shear sensitive strains.

Introduction

Skeletonema, *Chaetoceros*, *Phaeodactylum*, *Isochrysis*, *Pavlova* and *Tetraselmis* are frequently used microalgae in feed applications for shellfish in hatcheries (Muller-Feuga, 2013). These species would be candidates for large scale production given the increasing demand for microalgal feed of constant and high quality for aquaculture. An increased level of microalgae (components) in fish feed is interesting for the development of fish feed with reduced environmental impact (Draganovic et al., 2013).

For controlled large-scale cultivation of microalgae, closed photobioreactors (PBRs) are often advocated (Acién et al., 2012; Borowitzka, 1999). Closed PBRs combine low contamination risk and almost no CO_2 losses leading to a high productivity. Furthermore, the cultivation can be fully automated and culture conditions can be highly controlled (Pulz, 2001). However, hydrodynamic forces evoked by turbulent mixing in closed PBRs may limit the application of these closed systems. These forces must not exceed the level that would lead to detrimental effects (Tredici, 2010), suggesting the importance of limiting the shear stress in such PBR systems to levels that can be tolerated by the microalgae.
This is especially important when considering the fact that digestibility of microalgal species is positively related to the fragility of the cells (Gladue and Maxey, 1994) and therefore to the way many microalgal species used in aquaculture can be cultivated.

Table 1 summarizes the culture systems being employed for the cultivation of the most frequently used microalgal species for aquaculture. All these species can successfully be grown in bubble columns, bags and carboys; systems that use aeration for mixing. Other types of PBRs, like horizontal tubular PBRs with a shorter light path to produce microalgae at a higher density, are used by aquaculture facilities more often lately (Zmora et al., 2013). Mixing in these systems is induced through recirculation of the microalgal culture in the tubular PBR using pumps, of which centrifugal pumps and airlift pumps are the most common ones (Alías et al., 2004; Molina et al., 2001). Centrifugal pumps are efficient in gas-liquid mass transfer (Fadavi and Chisti, 2005) and energy use (Norsker et al., 2011). However, microalgal productivity can be influenced negatively by using centrifugal pumps, due to cell damage occurring inside the pump (Carvalho et al., 2006). This could be an explanation for the little record of microalgal species used in aquaculture, which have been successfully cultivated in tubular PBRs with centrifugal pumps (Table 1). It is still not clear if shear stress sensitivity of certain microalgal species hardly enables

successful cultivation in closed PBRs. Although shear stress sensitive species are being cultivated in tubular PBRs with airlift pumps, shear stress levels occurring in the tubes of PBRs could already be too high resulting in loss of productivity. Therefore, there is a need to study the relationship between shear stress sensitivity of microalgal strains and shear stress levels encountered in closed PBRs.

The aim of the study is to determine the tolerance to shear stress of four different microalgal species. Shear cylinders were used to quantify the threshold values of shear stress for the different microalgal species. These microalgal species were all tested in a tubular PBR with a variable-frequency-drive centrifugal pump to determine the capability of growth. Growth or lack of growth of microalgae will be related to estimated shear stress levels inside the reactor and shear tolerance levels determined for all four species.

Table 1 Most frequently used microalgal species as feed for larvae of mollusks, shrimp and live prey for fish larvae and their culture systems.

Class	Species	Culture system	Mixing	References
Bacillariophyceae	*Skeletonema costatum*	Raceway	Air	Hussenot et al. (1998)
		Polyethylene bags	Air	Pronker et al. (2013)
		Airlift PBR, bubble column	Airlift, air	Monkonsit et al. (2011)
	Chaetoceros muelleri, Chaetoceros gracilis, Chaetoceros calcitrans	Polycarbonate carboys	Air	Camus and Zeng (2012)
		Polyethylene bags	Air	Kaspar et al. (2014); Pronker et al. (2013)
		Bubble column	Air	Lee et al. (2011)
		Airlift PBR, bubble column	Airlift, air	Krichnavaruk et al. (2005)
	Phaeodactylum tricornutum	Polyethylene bags	Air	Pronker et al. (2013)
		Bubble column	Air	Lee et al. (2011)
		Tubular PBR	Airlift	Acién et al. (2000)
		Tubular PBR	Centrifugal pump	Silva Benavides et al. (2013)
Prymnesiophyceae	*Isochrysis galbana*	Polycarbonate carboys	Air	Camus and Zeng (2012)
		Polyethylene bags	Air	Dunstan et al. (1993); Kaspar et al. (2014); Pronker et al. (2013)
		Bubble column	Air	Lee et al. (2011)
		Airlift PBR	Airlift	Loubière et al. (2009)
		Tubular PBR	Airlift	Grima et al. (1994); Van Bergeijk et al. (2010)
		Tubular PBR	Centrifugal pump	Van Bergeijk et al. (2010)
	Pavlova lutheri, Pavlova salina	Polycarbonate carboys	Air	Camus and Zeng (2012)
		Polyethylene bags	Air	Dunstan et al. (1993); Pronker et al. (2013)
Prasinophyceae	*Tetraselmis suecica, Tetraselmis chuii*	Polycarbonate carboys	Air	Camus and Zeng (2012)
		Polyethylene bags	Air	Moheimani (2013); Pronker et al. (2013)
		Bubble column	Air	Lee et al (2011)
		Annular column	Air	Chini Zittelli et al. (2006)
		Green wall panel reactor	Air	Bondiolo et al. (2012)
		Tubular PBR	Centrifugal pump	Michels et al. (2014)

Material and methods

Organisms and medium

Tetraselmis suecica, *Isochrysis galbana* and *Skeletonema costatum* were obtained from Seasalter Shellfish (Whitsable) Limited (Kent, United Kingdom) and *Chaetoceros muelleri* (CCMP 1316) was provided by NIOZ (Royal Netherlands Institute for Sea Research). Walne medium modified from Laing (Laing, 1991) was used for the cultivation of the microalgal species. Walne medium consists of solution A (macro- and micronutrients), C (vitamins) and D (silicate, which is only needed for diatoms). Solution A contains 0.8 g $FeCl_3$, 0.4 g $MnCl_2 \cdot 4H_2O$, 33.6 g H_3BO_3, 45.0 g EDTA, 20.0 g $NaH_2PO_4 \cdot 2H_2O$, 100.0 g $NaNO_3$, 21 mg $ZnCl_2$, 20 mg $CoCl_2 \cdot 6H_2O$, 9.0 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 20 mg $CuSO_4 \cdot 5H_2O$ in 1 L of distilled water, of which the pH was adjusted to 4.0 with concentrated HCl. Solution C consists of 1.0 g Vitamin B_1 and 0.05 g Vitamin B_{12} in 1 L of distilled water. Solution D contains 40.0 g $Na_2SiO_3 \cdot 5H_2O$ in 1 L of distilled water. Medium for maintaining the cultures in Erlenmeyer flasks was made by adding 1 ml solution A, 0.1 ml solution C and 2 mL solution D per L of filtered and de-ironized saline groundwater (30 g L^{-1}). A double dose of solution A, C and D was supplied to the culture in the tubular photobioreactor during turbidostat operation to avoid nutrient depletion. Solution D was only added for the cultivation of the diatoms *Skeletonema costatum* and *Chaetoceros muelleri*.

Generation of shear stress levels tolerance

Batch cultures of *Tetraselmis suecica*, *Isochrysis galbana* and *Skeletonema costatum* from 3 L Erlenmeyer flasks containing 2 L medium after 1 week of growth at 20 °C under white fluorescent light (150 µmol PAR photons m^{-2} s^{-1}) were used for shear stress experiments. The three microalgal strains were exposed to different shear stress levels in shear cylinders as Couette devices (Van Riemsdijk et al., 2010). Shear stress exposed to the microalgae can be determined with:

$$\tau = \dot{\gamma} \cdot \eta$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}) and η is the apparent viscosity (Pa s). Shear stress levels can be varied by increasing the shear rate or by increasing the apparent viscosity of the medium. The shear rate applied in the shear cylinders is related to the rotational speed with a conversion factor of rotational speed (rpm) to shear rate (s^{-1}) of 2.157 (Michels et al., 2010). Locust bean gum (LBG), which does not affect the viability directly (Michels et al., 2010), was used as

a thickener to increase the apparent viscosity.

Skeletonema costatum and *Isochrysis galbana* with 0.3% LBG in the medium were exposed to different shear rates, which were 0, 8.6, 43, 216 and 1,079 s^{-1}, respectively. *Tetraselmis suecica* was also exposed to the same shear rates, but with two LBG concentrations: 0.5% and 0.75% LBG, respectively to obtain higher viscosity. All exposures were done in triplicate at 4 °C with an exposure time of 1 h. Since LBG solutions can be described as non-Newtonian fluids, the apparent viscosity was measured at the different shear rates in order to calculate the applied shear stress levels. The shear stress levels applied in the shear cylinders were determined with a rheometer (type Physica MCR 301, Anton Paar) at 4 °C according to the method described in Michels et al. (2010). The power-law functions that describe the relation between shear rate and shear stress applied to algae in 0.3%, 0.5% and 0.75% LBG, shown in Fig. 1, were used to calculate the shear stress levels applied at different rotational speed levels and LBG concentrations. Table 2 presents the calculated shear stress levels related to rotational speed, shear rate and LBG concentration.

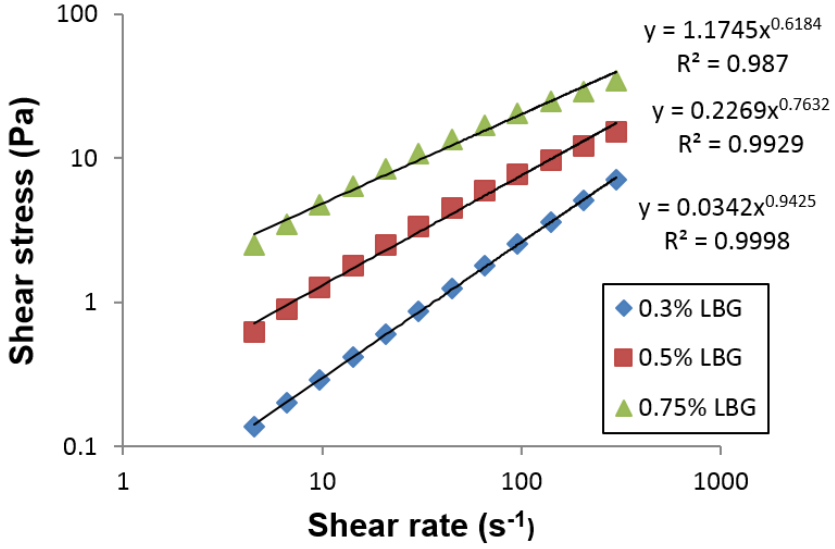

Fig. 1 Shear stress as a function of shear rate with different LBG concentrations used as a thickener.

Table 2 Shear stress applied in relation with rotational speed, shear rate and LBG concentration.

Rotational speed (rpm)	Shear rate (s^{-1})	Shear stress 0.3% LBG (Pa)	Shear stress 0.5% LBG (Pa)	Shear stress 0.75% LBG (Pa)
0	0	0	0	0
4	8.6	0.26	1.2	4.5
20	43	1.2	4.0	12
100	216	5.4	14	33
500	1,079	25	47	88

Measurement of effect of shear stress on viability

The effect of applying shear stress on the viability of *Tetraselmis suecica*, *Skeletonema costatum* and *Isochrysis galbana* was measured by using fluorescein diacetate (FDA). Viable cells contain esterases that convert FDA into fluorescein and diacetate. Viable cells show fluorescence caused by fluorescein (Altman et al., 1993; Rotman and Papermaster, 1966). After exposure in the shear cylinders, 1 mL of algae was incubated with 10 µL FDA solution (11mM) for 20 min. The total cell concentration and viable cell concentration were determined with a cell chamber (hemocytometer DHC-B02-5 Büker Türk) using a fluorescence microscope. The viability of the sheared algae was calculated as the percentage of fluorescing algae and compared to the shear stress tolerance of *Chaetoceros muelleri* (Michels et al., 2010).

Because *Skeletonema costatum* can form chains up to 8 - 10 cells per chain, the effect of shear stress on the distribution in the number of cells per chain was also measured. The amount of cells per chain was counted for at least 50 chains per sample. The Mann-Whitney *U* test was done to determine any statistical differences in cells per chain distribution per applied shear stress.

Growth of microalgae in a tubular photobioreactor

Growth tests with *Tetraselmis suecica*, *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* were carried out in a tubular PBR with a total volume of 40 L. The tubular PBR consists of 20 m long loop connected to a degasser. The loop is made of Plexiglas tubes with an external diameter of 50 mm and an internal diameter 43.6 mm. This culture system is equipped with a variable-frequency-drive centrifugal pump (SealPro KR-32-95, ARBO) with a pressure side diameter of 32

mm, to circulate the microalgal culture. The location and main operating mode of the tubular PBR are described in Michels et al. (2014).

The four microalgal species were separately used for inoculation of the tubular PBR with a minimal starting cell concentration of 200,000 cells mL^{-1}. Temperature was controlled at 20 ± 0.5 °C. The pH is measured at the end of the tube before the degasser and controlled at 8.40 via CO_2 supply. The initial pumping speed at which the microalgae were recirculated was 0.37 m s^{-1}.
The increase in cell concentration was followed by taking daily samples for 7 days. The run in the PBR was terminated when the cell concentration of an algal species did not increase during this period. The PBR was cleaned thoroughly and inoculated with the same species for a second time. The daily samples were inspected with a microscope to check the shape of the microalgal cells, the motility of the flagellates and potential bacterial occurrence.
The runs in which the algal concentration increased during the first 7 days, were further used to study the effect of pumping speed on the net volumetric productivity at turbidostat conditions, with the biomass concentration set at about 0.5 g L^{-1}. The pumping speed of the recirculation pump was 2.0, 2.4, 2.8, 3.2 and 3.6 m^3 h^{-1}, respectively. Those trials were done for a period of 14 days.

Calculation of shear stress levels in the tubular photobioreactor

The corresponding flow velocities and Reynolds numbers in the tubes and at the pressure side of the pump were calculated, with a known culture density of 1,024 kg m^{-3} and an apparent viscosity of $1.8 \cdot 10^{-3}$ Pa s (Michels et al., 2010). Those conditions were used to estimate the average shear stress levels in the tubes. The Blasius equation was used to calculate the shear stress at the wall of the tube where flow will be laminar (Durst et al, 1996). The equation reads:

$$\tau = C_f \frac{1}{2} \rho \bar{u}^2 \text{ with } C_f = 0.0791 Re^{-1/4}$$

where τ is the average shear stress (Pa), C_f is the Fanning friction factor (dimensionless), ρ is the density (kg m^{-3}), $\bar{u}$ is the average flow velocity (m s^{-1}) and Re is the Reynolds number. The Reynolds number is expressed by:

$$Re = \frac{\rho \cdot \bar{u} \cdot D}{\eta}$$

where D is the internal diameter (m).

Determination of the net volumetric productivity

The net volumetric productivity (P_V, g L^{-1} d^{-1}) was determined as the product of the net specific growth rate (μ, d^{-1}) and the biomass concentration (C_X, g L^{-1}). At turbidostat conditions, the net specific growth rate is equal to the dilution rate (D, d^{-1}), defined as the daily harvest volume divided by the volume of the PBR (Michels et al., 2014). The daily photon fluxes were derived from the measured photosynthetically active radiation (PAR) as an average daily incident photon flux density (PFD, mol m^{-2} d^{-1}) (Michels et al., 2014). The PFD was measured with a LiCor LI190 PAR sensor. The derived PF_{in} and measured PFD were found to be linearly related with $PF_{in} = 0.91 \cdot PFD$ and a $R^2 > 0.99$.

The growth experiments were carried out in the fall of 2012 and 2013, during which daily light intensities varied.

Results and discussion

Shear stress tolerance

Fig. 2 shows the effect of shear stress on the viability of *Tetraselmis suecica*, *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri*. An adverse effect was found for *Isochrysis galbana*, which viability decreased suddenly to 74.9% evoked by a shear stress level of 5.4 Pa. The viability of *Isochrysis galbana* was not reduced any further when the shear stress increased to 25 Pa. *Isochrysis galbana* is a flagellate and a member of the non-calcified coccolithophytes with only a plasma membrane covering, which makes the naked cell fragile (Graham et al., 2009b; Zhu and Lee, 1997).

The effect of shear stress on *Skeletonema costatum* was a bit different. The viability of *Skeletonema costatum* decreased further from 80.5% to 73.4 %, when the shear stress increased from 5.4 to 25 Pa. This observation can be ascribed to a change in morphology of *Skeletonema costatum* due to the increased shear stress. Since this diatom forms chains, breakages of cell chains was expected at higher shear stress levels (Sauriau and Baud, 1994). The effect of shear stress on the distribution of cells per chain is shown in Fig. 3. The distribution of cells per chain of unexposed *Skeletonema costatum* and cells exposed to shear stress of 0.26 Pa did not differ, while significant differences in distribution of cells per chain were found between all the exposed shear stress levels. Higher shear stress levels than 0.26 Pa caused a

significant reduction in the average chain length with a progressive increase of chains with 1–3 cells. The average chain length reduced linearly on a semi-logarithmic scale from 3.46 ± 0.18 to 2.49 ± 0.04 cells per chain with corresponding shear stresses of 0.26 and 25 Pa. Although a shear stress of 1.2 Pa already caused a reduction of the average number of cells per chain, the viability did not decrease significantly (Fig. 2). Shear stress probably has first an impact on the intercellular junctions causing chain breakage, which is then followed by other cell structures like the siliceous frustules being damaged causing mortality (Sauriau and Baud, 1994).

Data of *Chaetoceros muelleri* were used as reference, and were obtained from Michels et al. (2010). *Chaetoceros muelleri* was found to be shear stress sensitive with a threshold value of shear stress between 1 and 1.3 Pa. Higher shear stress levels caused a decrease in viability with a certain fraction of the cells being sensitive to shear stress (Michels et al., 2010).

Tetraselmis suecica exposed to shear stress levels up to 88 Pa did not show any adverse effects on the viability (Fig. 2). The tolerance to high shear stress of *Tetraselmis suecica* is probably caused by the rigid cell wall composed of layers of scales attached to the cell membrane (Graham et al., 2009a).

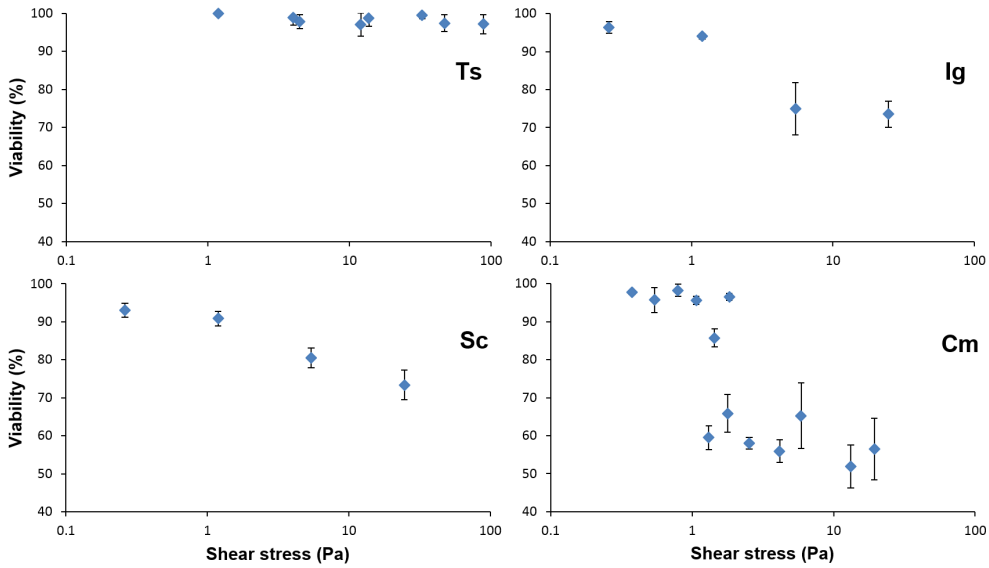

Fig. 2 Effect of shear stress on *Tetraselmis suecica* (Ts), *Isochrysis galbana* (Ig), *Skeletonema costatum* (Sc) and *Chaetoceros muelleri* (Cm). Data from effect of shear stress on *Chaetoceros muelleri* were obtained from Michels et al. (2010).

For the algal species that were susceptible to shear stress, the viability was decreased with 20 to 40%. The fact that not all cells were inactivated could be explained by the fact that cells are not equally susceptible to shear damage during the full growing cycle. It is reported that microalgae are more vulnerable to shear stress during cell division (García Camacho et al., 2007; Stoecker et al., 2006). This will cause a loss of viability of only a certain percentage of the shear stress sensitive species *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* (Fig. 2). A plausible explanation why the flagellate *Tetraselmis suecica* is not shear stress sensitive during cell division, is that the flagella are shed during the division prior to mitosis, and cytokinesis takes place within the rigid parental wall (Graham et al., 2009a).

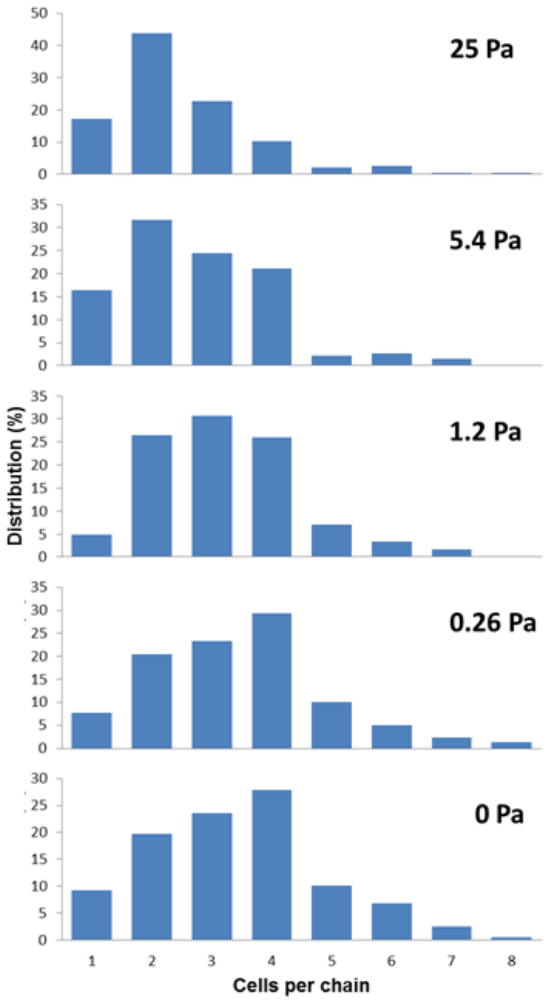

Fig. 3 Effect of shear stress on the distribution of cells per chain of *Skeletonema costatum*.

Growth tests with shear stress sensitive and tolerant microalgal species

Tetraselmis suecica, Isochrysis galbana, *Skeletonema costatum* and *Chaetoceros muelleri* were all tested for their capability to grow in a tubular PBR in which the culture is recirculated using a centrifugal pump. *Tetraselmis suecica* was the only species tested that grew well in the tubular PBR. The net volumetric productivity of *Tetraselmis suecica* cultivated at different pumping speeds is shown in Fig. 4. The biomass concentration was kept constant at 0.52 ± 0.05 g L^{-1} applying turbidostat conditions. The net volumetric productivity linearly increased with the daily photon flux received. During the entire experiment the microalgae were exposed to relatively low light intensity. At these low light intensities, no light saturation or light inhibition occurs. A linear increase of productivity with light input is therefore to be expected (Geel et al., 1997).

No differences in net volumetric productivity of *Tetraselmis suecica* receiving similar light were found between the cultivation periods at different pumping speeds. Higher pumping speed evokes increased shear stress. It could affect the net productivity negatively by its potential detrimental effects on the microalgae. On the other hand, it can also have a positive effect on the net volumetric productivity due to a better mass and gas transfer and shorter day/light cycles, that is realized when mixing is increased (Contreras et al., 1998; Leupold et el., 2013; Vejrazka et al., 2012). Neither negative nor positive effects of pumping speed on the productivity of *Tetraselmis suecica*, however, were found during this study.

The average net volumetric productivities with corresponding average daily photon fluxes for the five runs with a different pumping speed are given in Table 3. During the experimental period at limiting light conditions during the fall, the maximum net volumetric productivity of the microalgae receiving a daily photon flux of 10.7 mol photons d^{-1} was 0.34 g L^{-1} d^{-1}, while daily light inputs lower than 1 mol d^{-1} resulted in a zero or close to zero productivity (Fig. 4). The daily photon flux of 1 mol d^{-1}, which corresponds to an average daily PFD of 12.8 µmol m^{-2} s^{-1}, was obviously the compensation point of photosynthesis, at which the rate of photosynthesis equals the respiration rate. The compensation point of photosynthesis of *Tetraselmis suecica* was similar to the value of *Chlamydomonas reinhardtii* (Takache et al., 2010).

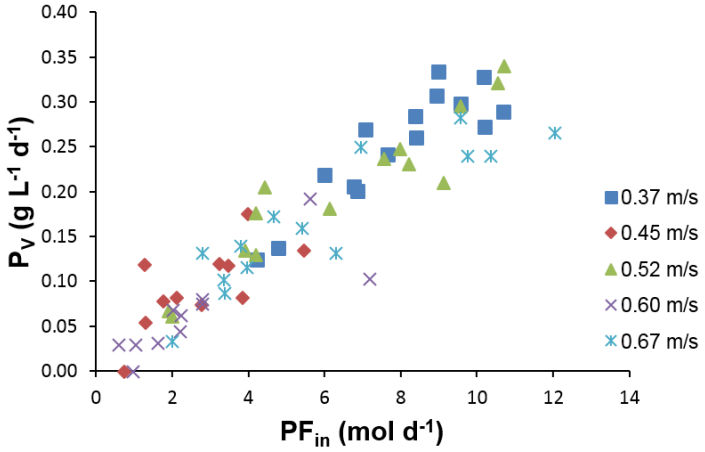

Fig. 4 Net volumetric productivity of *Tetraselmis suecica* versus the daily photon flux at different pumping speeds.

Table 3 Average net volumetric productivity and average daily photon flux at different runs.

Pumping speed (m^3 h^{-1})	Net volumetric productivity (g L^{-1} d^{-1})	Daily photon flux (mol d^{-1})
2.0	0.25 ± 0.06	7.94 ± 0.14
2.4	0.09 ± 0.05	2.74 ± 1.43
2.8	0.20 ± 0.09	6.47 ± 3.04
3.2	0.07 ± 0.05	2.67 ± 2.02
3.6	0.17 ± 0.08	6.04 ± 3.22

Biofouling at high pumping speed

Although the highest pumping speed did not lead to a lower net volumetric productivity, the culture of *Tetraselmis suecica* was affected negatively. Biofouling started to occur in the tubes one day after the pumping speed was set at 3.6 m^3 h^{-1} (Fig. 5), at which the average shear stress in the tubes and at the pressure side of the pump were 1.60 and 5.10 Pa, respectively (Table 4). Due to the presence of most of the biofouling at the bottom of the tubes, the cells were still able to receive light, which did not result in a lower net volumetric productivity. Biofouling in the tubes increased enormously during the two weeks *Tetraselmis suecica* was cultivated at this high pumping speed. Therefore, *Tetraselmis suecica* should be cultured at lower pumping speeds to ensure cells will not be damaged. An additional benefit of applying a lower pumping speed is the lower energy costs.

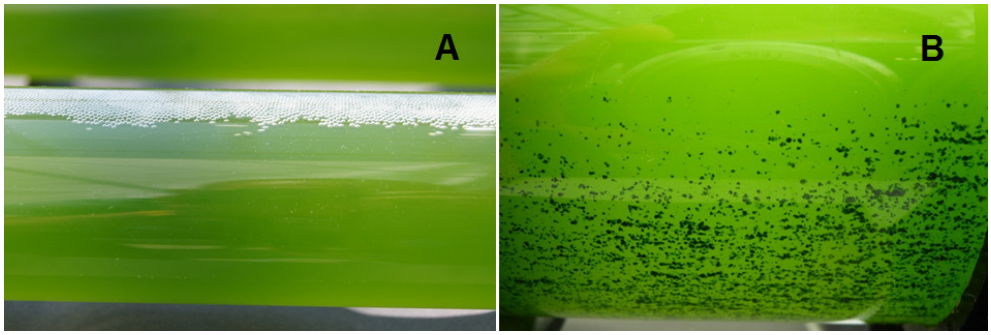

Fig. 5 No attached growth during cultivation at lower pumping speeds (A) and biofouling at highest pumping speed (B).

Growth of recirculated *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* in the tubular PBR was not observed, not even at the lowest pumping speed of 2.0 m^3 h^{-1}. The cell concentration of these three microalgal species did not increase during the 7 days tested. General observations in chronological order were a decrease of cell concentration within one or two days, an increase of number of bacteria, followed by foam formation and finally biofouling. In the case of *Isochrysis galbana*, the remaining viable cells did not lose their motility and no alteration of the shape could be observed. Regarding *Skeletonema costatum*, longer chains disappeared after one day with more short chains and single cells as a result. Broken and disintegrated cells of *Skeletonema costatum* were seen after a few days. The shape of the remaining cells of *Chaetoceros muelleri* did not change immediately, but only after a few days the cells became spherical and lost their spines. Shear stress most likely caused the incapability of *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* to grow in the tubular PBR.

Relative high shear-stress levels encountered in the photobioreactor

Lack of growth of these microalgal strains could be correlated to the shear stress sensitivity of the cells investigated. Unfortunately, exact shear stress values are difficult to predict in turbulent flow, but the stress at the wall can be calculated easily, due to the fact that flow is laminar at the wall. The shear stress at the wall is 0.57 Pa at a pumping speed of 2.0 m^3 h^{-1}. A similar calculation leads to a wall shear stress of 1.82 Pa in the pressure side of the pump (Table 4). It can be expected that even higher shear stress values can occur in other parts of the reactor. For example, maximum shear stress levels of about 2 Pa were reported in bends of tubes with similar diameters (5 cm) and at similar velocity (0.35 m s^{-1}) in the tubes based on computational fluid dynamics (Ramírez-Duque and Ramos-Lucumi, 2011).

Table 4 Flow velocities, Reynolds numbers and average shear stress levels in tubes and at pressure side of the pump at different pumping speeds.

	Tubes			Pressure side of pump		
Pumping speed ($m^3\ h^{-1}$)	Flow velocity ($m\ s^{-1}$)	Reynolds number	Average shear stress (Pa)	Flow velocity ($m\ s^{-1}$)	Reynolds number	Average shear stress (Pa)
2.0	0.37	$9.2 \cdot 10^3$	0.57	0.69	$1.3 \cdot 10^4$	1.82
2.4	0.45	$1.1 \cdot 10^4$	0.79	0.83	$1.5 \cdot 10^4$	2.51
2.8	0.52	$1.3 \cdot 10^4$	1.03	0.97	$1.8 \cdot 10^4$	3.29
3.2	0.60	$1.5 \cdot 10^4$	1.30	1.11	$2.0 \cdot 10^4$	4.15
3.6	0.67	$1.7 \cdot 10^4$	1.60	1.24	$2.3 \cdot 10^4$	5.10

Even higher shear stress levels will occur in the cavity of the centrifugal pump, where the shear forces are not equally distributed. Comparing these shear stress levels with the viability of the cells exposed to such shear stress (Fig. 2), it is obvious that shear stress at the pressure side of the pump and in the bends could already cause shear stress related damage to the cells. In other words, local conditions inside the reactor might give rise to shear damage to the cell. However, a large part of the reactor might still have favorable conditions for cell cultivation.

Simulation of the possible damage caused by high circulation rates

Unfortunately, the high circulation rate of the culture will inevitably lead to the situation that all cells will pass a high shear zone and most of them will pass the zone several times. This can be demonstrated by a simple simulation as shown below. In this simulation it is assumed that a shear sensitive cell will be damaged if the cell enters a high shear stress zone inside a certain part of the reactor (e.g. in the cavities of the circulation pump), where the shear stress level is beyond the threshold of the algae. Computational fluid dynamics (CFD) analyses on shear stress in centrifugal pumps show that the probability of cells entering regions inside the pump with shear stress levels that damage the cells, depends on the residence time of the cells inside the pump, the rotational speed of the impeller and the shape of the impeller (Song et al., 2003; Takiura et al., 1998; Zhou et al., 2003). The magnitude of the damage is therefore related to the proportion of the cells φ that pass this high shear stress zone per passage and the amount of passages n over time:

$$\frac{N_r}{N_0} = (1 - \varphi)^n$$

where N_r is the cell concentration of the sensitive algae remaining viable and N_0 is the cell concentration of sensitive algae before exposure. Figure 6 shows the percentage of intact cells remaining over time assuming that 1, 5 or 10% of the cells are being damaged per passage.

The amount of passages through the pump depends on the length of the tubular PBR and the flow velocity. At a pumping speed of 2 $m^3\ h^{-1}$, the culture is recirculated 50 times per hour in the PBR. This means that the number of passes per day is about 1200. Even when only 1% of the cells passes a high shear zone, it is clear that all sensitive cells would be inactivated within one day. However, not all cells might be shear stress sensitive. Most likely, especially the cells which are in the dividing stage are vulnerable to shear stress. Assuming that at a given time about 30% of

the microalgal cells are in the dividing stage (Coats and Heinbokel, 1982) and that the generation time is about 1 day, the time that a cell is vulnerable to shear stress is about 7.2 hours per day. In those 7.2 hours, the number of passages is 360, which leads to an inactivation of 97% of the sensitive cells. Only 3% will remain for further growth, which is clearly too low to maintain or even increase the number of microalgal cells. This means that in the tubular PBR, almost all cells are expected to be inactivated within one or a few days, which is in line with the experimental observations.

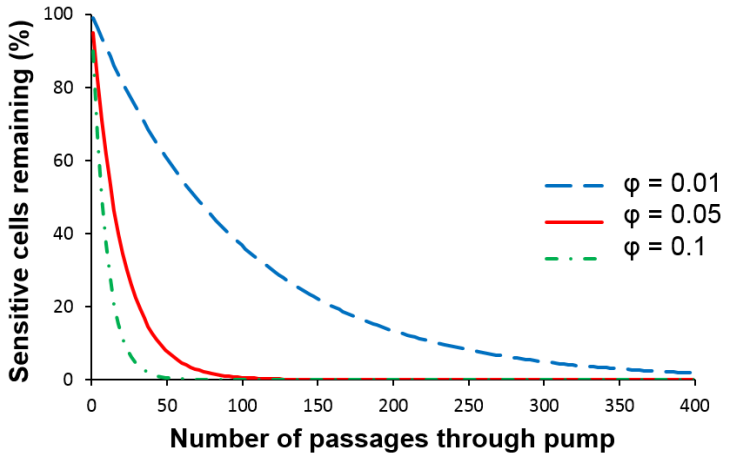

Fig 6 Percentage of remaining cells as an effect of passages through a pump and proportion of cells being damaged per passage.

Design of closed photobioreactor systems for shear sensitive microalgae

Theoretically, two possible routes are possible to avoid cell damage due to shear stress. The first one is reducing or avoiding high shear stress zones in the reactor. This option, however, might be quite difficult to achieve. Lowering the flow rate too much will lead to other problems, because high turbulence is needed for keeping the microalgae suspended and the use of light and nutrients is enhanced by turbulent mixing (Richmond, 2013). Another option is to reduce the number of passages. Many commercial tubular PBRs consists of longer tubes with a length of 100 m (Pulz et al., 2013) and with flow velocities applied in PBRs between 0.3 and 0.5 m s^{-1} (Norsker et al., 2011). This reduces the amount of passages of the shear stress sensitive cells, which are in the dividing stage, to about 90 per day. In the case of 1% of the cells being affected per pump passage in a commercial PBR, 60% of the

sensitive cells would still be damaged. It is obvious that more cells will be damaged, if a higher percentage of the cells is affected per passage through the pump (Fig. 6). Considering that the time needed for cell proliferation is about one day, the time is too short to overcome the damage done by high shear stress, even if the high shear stress region is small.

The tolerance to shear stress of various strains seems to be selective to the choice of recirculation pumps. Microalgae with rigid cell walls are shear stress tolerant, while species lacking a cell wall, coccolithophores with calcium carbonate containing coccospheres and diatoms with fragile siliceous frustules are sensitive to shear stress (Leupold et al., 2013; Moheimani et al., 2011; Vandanjon et al., 1999). *Phaeodactylum tricornutum* is an exception among the diatoms. Its cell wall structure does not contain silica but higher concentrations of polysaccharide rendering a more rigid cell wall (Tesson et al., 2009). It is therefore successfully cultivated in tubular PBRs with centrifugal pumps, although some reduction of productivity has been reported at higher flow rates causing high shear zones in the reactor (Alías et al., 2004; Silva Benavides et al., 2013).

The fact that *Isochrysis galbana*, *Skeletonema costatum* and a species of the genus *Chaetoceros* were reported to be successfully cultivated in tubular PBRs recirculated with airlift pumps (Molina Grima et al., 1994; Krichnavaruk et al., 2005; Loubière et al., 2009; Monkonsit et al., 2011; Van Bergeijk et al., 2010) suggests that hydrodynamic forces exerted in the tubes of PBRs were probably not high enough to negatively affect the growth. Airlift pumps, which are causing lower shear stress than centrifugal pumps (Carvalho et al., 2006), seem to be the best option for the recirculation of shear stress sensitive microalgae.

Conclusion

Four different microalgal species used as feed for shellfish in hatcheries were tested for their shear stress sensitivity. *Tetraselmis suecica* was found to be the only shear stress tolerant species tested, which viability was not negatively affected by a maximum applied shear stress level of 88 Pa. *Tetraselmis suecica* was also successfully grown in a tubular PBR driven by a centrifugal pump. *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* were not able to grow in the tubular PBR recirculated by a centrifugal pump at its lowest speed. Shear stress levels between 1.2 and 5.4 Pa caused a reduction in viability of the shear stress

sensitive species *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri*.

In order to increase the feasibility of the production of microalgae for aquaculture in fully-automated PBRs, high shear stress zones in the reactor (including the pump) should be avoided when designing a culture system for shear stress sensitive microalgal species.

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CHAPTER 7

General discussion: Prospects of microalgal production in tubular photobioreactors for hatcheries

Introduction

Over the last decade the contribution of aquaculture to the world fish production has increased from 34.6 to 62.7 million tons (FAO, 2013). The associated demand for microalgae for aquaculture has increased as well. The annual production of microalgae between 1999 and 2010 for feeding mollusks, crustaceans and fish in hatcheries increased from around 530 t DW (Muller-Feuga, 2004) to 1,800 t DW (Muller-Feuga, 2013).

The microalgae that are mainly used to feed the larval stages of these organisms are selected from the genera *Chaetoceros*, *Skeletonema*, *Thalassiosira, Tetraselmis, Isochrysis* and *Pavlova* (Spolaore et al., 2006). Cultivation of these microalgae in hatcheries is commonly done at relatively small scale using cylindrical polyethylene bags with artificial light. The productivity in these systems is relatively low and high costs for labor, electricity and nutrients are involved. In addition, reliable and stable constant production is hardly possible as the cultures often crash due to contamination of bacteria, other algal species or zooplankton (Borowitzka, 1997).

To realize higher production rates of microalgae combined with higher biomass concentration of constant quality, closed tubular and flat-plate photobioreactors (PBRs) have been proposed (Zmora et al., 2013). The design of these closed systems, however, should be tailored to meet the specific demands for production of microalgae for aquaculture. A better quality control should be realized, which minimizes the chance of contamination and a variation in composition of the microalgal biomass produced (Hemaiswarya et al., 2011). In addition, the current energy demand and operational as well as investment costs for production of microalgae in these systems are relatively high (Molina Grima et al., 2003; Norsker et al., 2011). Improvements are needed in the cost-effectiveness of microalgal production in hatcheries by lowering the costs of electricity, labor and nutrients and in scaling-up of closed fully-automated PBRs systems.

The aim of this general discussion of the thesis is to discuss the design of PBR systems for production of microalgal feed for aquaculture hatcheries and to review possibilities of realizing a more cost-effective and sustainable production facility. The effect of implementation of cost-reducing methods will be evaluated. The evaluation of the PBR system and the proposed costs-reducing methods will contribute to the realization of integrated multi-trophic aquaculture.

Specific design criteria for microalgae production for aquaculture

Microalgae used as feed for aquaculture, like *Chaetoceros muelleri*, *Skeletonema costatum*, *Tetraselmis suecica* and *Isochrysis galbana* should be cultured in closed systems. Flow velocity, temperature, pH, biomass concentration as well as medium supply can be controlled in fully-automated PBRs and if artificial light is used even the light supply can be tuned (Pulz et al., 2013). These culture conditions must be optimized to obtain high productivity at lower costs. The most important culture conditions and how these culture conditions can be optimized will be discussed here.

Control of contamination by unwanted species

Although the chances of invasions by unwanted species are small in closed systems (Borowitzka, 1999; Pulz, 2001; Wang et al., 2013), contamination may still occur. Especially, the supply of fresh medium can lead to the entry of zooplankton or other algal species with a higher growth rate than the cultivated species (Forehead and O'Kelly, 2013). Zooplankton, such as ciliates and rotifers, graze on the microalgae with often a culture collapse within two days as a result (Richmond, 2013). Since ciliates are capable of surviving extreme conditions, like high salinity, extreme temperatures, desiccation and anoxia (Olendzenski, 1999; Scott et al., 2001), the proliferation of these grazers is difficult to control. Grazing as a significant biological constraint to microalgal production is highly overlooked leading to a lack of effective ways to reduce or prevent contamination by zooplankton (Day, 2013). Therefore, early detection and control measures are needed to overcome these problems (Day et al., 2012a; Day et al., 2012b).

To prevent contamination, medium for microalgal cultivation in hatcheries is commonly sterilized by pasteurization, filtration, UV radiation, chlorination followed by neutralization, ozonation or a combination of methods (Lavens and Sorgeloos, 1996; Lekang, 2013). Filtration of medium was chosen as the method for medium sterilization for the cost calculation.

Avoid high shear forces

Tubular PBRs require a minimum flow velocity to keep the microalgae in a turbulent regime, which is needed for a sufficient mass transfer of nutrients, CO_2 and oxygen and light supply (Norsker et al., 2011). Recirculation of the microalgal culture is commonly done with airlift or centrifugal pumps (Zittelli et al., 2013). Most microalgal

species that are used in aquaculture, however, are shear stress sensitive (Chapter 2 and 6). Cell damage caused by hydrodynamic forces inside the recirculation pumps should be avoided. The microalgal strains that are to be cultured, will determine the choice of pumps. Although airlift pumps are less energy efficient than centrifugal pumps (Norsker et al., 2011), low-shear airlift pumps are essential for the cultivation of shear stress sensitive species. Shear stress tolerant species can be successfully cultivated in tubular PBRs with centrifugal pumps (Chapter 3, 4, 5 and 6), but lower pumping speeds are still favorable for achieving lower shear stress levels (Michels et al., 2014b; Vandanjon et al., 1999).

Temperature control

Temperature is another important critical culture condition that needs to be controlled. The optimal temperature of the strain and its tolerance to varying temperatures determine to what extent temperature control is needed. When microalgae are grown in tubular PBRs outdoors or in a greenhouse, cooling during daylight is required, while temperature control during cold periods by heating is also needed. Cooling and heating is mainly done with heat exchangers in or around the degassing unit or with spraying water causing evaporative cooling (Chisti, 2007).

In our costs calculations we assumed that a heat exchanger was present to maintain a constant temperature of about 20 °C and gas and energy prices of €0.40/m^3 and €0.20/kWh, respectively (Bot et al., 2009).

pH control and efficient use of CO_2

The pH of a microalgal culture can be controlled by a direct supply of CO_2, which is required for microalgal growth. Microalgae consume between 1.5 and 2.0 kg of CO_2 to produce 1.0 kg of biomass (Mazzuca Sobczuk et al., 2000). The efficiency of the use of CO_2 in PBRs however, is not always optimal. CO_2 losses in the degasser lead to a higher demand for CO_2 per kg biomass produced (Acién et al., 2012). A reduction of CO_2 losses was achieved in our PBR by controlling the pH just before the degasser at a pH of around 8.3 (Chapter 3, 4, 5 and 6). Free CO_2 will be depleted at this pH value, where HCO_3^- is the principal ion of the carbonate system (Goldman et al., 1972). Therefore, CO_2 dosed at the beginning of the tube was taken up by the microalgae completely before it reached the degasser.

Nutrient supply

In continuous cultures, new medium is directly supplied to the culture during harvest. Based on the elemental composition of the cultivated species, the biomass concentration at which the microalgae are cultivated and the dilution rate, the addition of a balanced and sufficient quantity of nutrients can easily be calculated for an efficient nutrient use (Chapter 4). Regular monitoring of nutrients is required to check if the availability of nutrients is sufficient for growth (Richmond, 2013).

Continuous cultivation; chemostat versus turbidostat mode of operation

The main types of continuous operation for microalgae production systems are chemostat and turbidostat (Lee and Shen, 2004). Chemostat is the condition, where the dilution rate is constant. The rate of new medium addition is the same as the rate at which the culture is harvested and the biomass concentration in the medium depends on the available light. When turbidostat is applied, the turbidity and thus the associated biomass concentration are kept constant. The dilution rate is no longer constant, but depends on the light availability (Chapter 3, 4, 5 and 6).

Chemostat is easy applicable in practice, because the dosing pump for new medium coupled to the harvest can be set on a certain rate. For turbidostat operation, the biomass concentration is kept constant at a pre-set value controlled by a turbidity sensor and a programmable logic controller (PLC) (Michels et al., 2014b). The advantage of maintaining the biomass concentration at a constant level is that the nutrient supply can better be adjusted to avoid waste of nutrients and CO_2 for the production of microalgae with a constant quality, which will save costs as well.

Costs calculation for the production of microalgae in tubular photobioreactors at 0.1 and 1 ha scale

The costs involved in a microalgal production facility using fully-automated tubular PBRs in a greenhouse were evaluated based on the techno-economic model of Norsker et al. (2011). The model was adjusted to evaluate the costs involved in microalgal production at a scale of 0.1 ha and 1 ha, while using productivity data obtained from the experiments described in Chapter 3, 4, 5 and 6 (Michels et al. 2014a; Michels et al., 2014b; Michels et al., 2014c). Calculations were based on the design of tubular PBRs with a tube diameter of 5 cm, a horizontal distance between

the tubes of 2 cm and 28 L of solar receivers per m^2 ground surface (Fig. 1). During year-around experiments in our tubular PBR in Vlissingen, the Netherlands, an average volumetric productivity of 0.30 g L^{-1} d^{-1} was measured at an average biomass concentration of 0.7 g L^{-1} and at an average photosynthetically active radiation (PAR) measured as photon flux density of 11.8 mol m^{-2} d^{-1}. This is equivalent to an areal productivity of about 30 ton ha^{-1} yr^{-1} and a photosynthetic efficiency (PE) of 3% on total solar light.

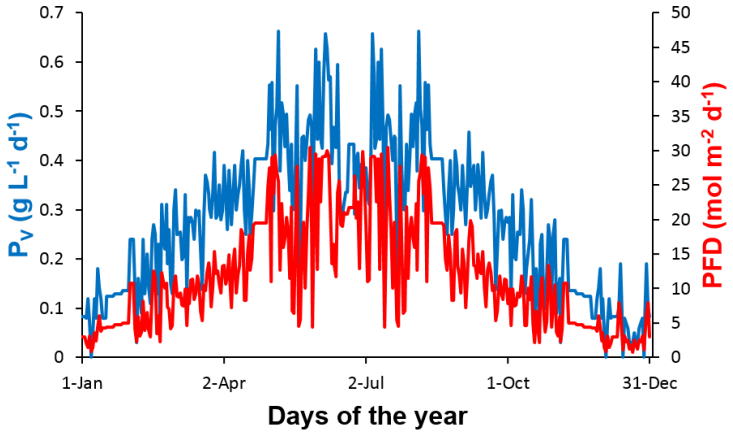

Fig. 1 Net volumetric productivity of *Tetraselmis suecica* and photon flux density in tubular PBR over a year in the Netherlands. (Data are obtained of experiments done in tubular PBR in greenhouse in Vlissingen, the Netherlands. Missing data were interpolated.)

Costs calculations include the costs for the major equipment and other capital costs (installation costs, instrumentation and control, piping and buildings), operational costs for raw materials, power and labor as listed in Norsker et al. (2011). The list of major equipment was extended with a temperature control unit. The centrifuge was discarded in the calculation, because the algal culture does not need to be concentrated when feeding it directly to shellfish or fish larvae via rotifers, copepods or *Artemia*. Costs of food-grade fertilizers used for microalgal cultivation for aquaculture were estimated to be €2 per kg DW of microalgae (personal communication of several anonymous hatchery operators in the Netherlands and UK). Using the model, the effect of several methods to reduce the costs were estimated.

Several costs (filters for medium sterilization, power supply to the recirculation pump and temperature control, etc.) are related to the control of culture conditions in PBRs to maintain stable microalgal production. Critical conditions for the production of microalgae in PBRs are recirculation, temperature, pH, CO_2 and nutrient supply, and light. The control of these critical culture conditions and how the control is applied

have an influence on the costs.

The total costs were determined for different scenarios (Table 1). Assuming medium sterilization with (ultra)filtration, recirculation by airlift pumps, constant temperature control, full uptake of CO_2, the use of fertilizers, and the use of sunlight, the total costs were calculated. For this base case at a scale of 0.1 ha and 1 ha in a greenhouse in the Netherlands, the costs were found to be €53.70 and €30.90, respectively.

The costs for labor, overhead and maintenance as percentage of total costs are high for both cases. The highest reduction in total costs per kg of microalgae produced can therefore be realized by producing microalgae at bigger scale. This reduces the costs for labor, maintenance and overhead from €17.60 at a scale of 0.1 ha to €6.30 per kg at a scale of 1 ha (Fig. 2).

Since aquaculture hatcheries depend on a constant supply of microalgae for feed all year around and the productivity over the year varies if the algae are cultivated on natural sunlight (Fig. 1), additional artificial light could be necessary. This will eventually lead to extra costs. When algae are produced on artificial light only, production costs will increase with about €20 per kg (Blanken et al., 2013). In the case that only artificial light is supplied during the winter months (25% of the year), the total production costs will be €5 per kg higher than on only sunlight (case 2).

A way to reduce the costs for nutrient supply is to make use of wastewater containing inorganic nutrients (Chapter 4). Wastewater is a free source of nutrients. A costs reduction of around €2 per kg is therefore possible, if wastewater is used instead of fertilizers (case 3). Reduction of the costs is not the only reason for using the nutrients available in wastewater for microalgal production. Use of wastewater also contributes to the development of a more sustainable aquaculture production. Fish farms produce nutrient-rich wastewater, which can be remediated by microalgae. The microalgae in turn can be used as feed for the larvae in hatcheries. This integrated multi-trophic aquaculture approach combines the two advantages: purification of the wastewater from the aquaculture and the provision of free nutrients for the production of microalgae (Michels et al., 2014c).

Temperature control is the other major part of the total costs, which is neglected in most costs calculations in literature. Temperature control would lead to investments, maintenance and energy costs of €59 per m^2 and therefore €19.70 per kg DW of microalgae, assuming a gas and energy price of €0.40/m^3 and €0.20/kWh,

respectively (Bot et al., 2009). Additional energy can be saved by turning off the temperature control at night, because colder temperatures at night did not affect the productivity negatively as can be seen in Chapter 5 (Michels et al., 2014a). The costs savings by turning off the temperature control at night, which can be realized in about 9 months per year, are estimated to be €7.40 per kg. The total costs for temperature control would then be €12.30 per kg (case 4). This results in total costs of €44.30 and €21.50 per kg at a scale of 0.1 ha and 1 ha, respectively.

For temperature control, other options next to conventional cooling and heating are also possible. Cooling and heating can be achieved with storage of waste heat in an aquifer in summer which can be used to heat the culture in winter. The groundwater can later be used for cooling during summer months winter (Bot et al., 2005). The costs for temperature control will then be €12 per kg of biomass with costs savings of €7.70 per kg. The cheapest option is the use of groundwater from an aquifer for cooling in combination with cogeneration. Cogeneration is the combined production of power and heat. The power engine produces power for the energy needed for pumps, ventilators, etc., and at the same time the waste heat is used for heating the PBRs during cold periods. The costs for temperature control are then €6.70 per kg of microalgal biomass (Bot et al., 2009). If the temperature control at night will be turned off as well, the total costs for temperature control would then be €4.20 per kg. The scenario (case 5) for which sunlight, wastewater and the cheapest temperature control was used, results in total costs of €36.20 and €13.40 per kg at a scale of 0.1 ha and 1 ha.

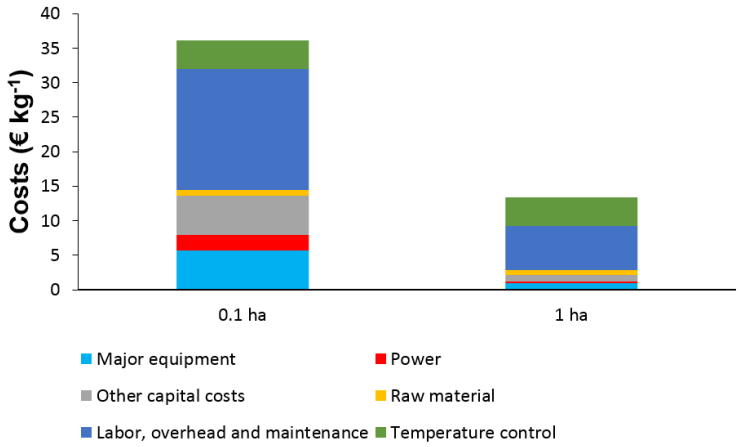

Fig. 2 Costs of microalgae in a production facility in a greenhouse at 0.1 and 1 ha scale, based on productivity and light availability in the Netherlands. For this scenario, microalgae are cultivated on sunlight, wastewater is used as the nutrient source and the cheapest temperature control is applied (Case 5).

Table 1 Costs for microalgal production in tubular PBRs for hatcheries in the Netherlands for different scenarios at scale of 0.1 ha and 1 ha.

Scenario	Conditions	Costs for 0.1 ha (€ per kg DW)	Costs for 1 ha (€ per kg DW)
Base case 1; based on model of Norsker et al. (2011) with 3% PE in tubular PBR	Filtration of medium No centrifuge Fertilizers Temperature control Only sunlight	53.70	30.90
Case 2	Base case with artificial light in winter	58.70	35.90
Case 3	Base case with wastewater instead of fertilizers	51.70	28.90
Case 4	Base case with wastewater instead of fertilizers and no temperature control at night	44.30	21.50
Case 5	Base case with wastewater instead of fertilizers, no temperature control at night and use of cogeneration in combination with cooling with groundwater	36.20	13.40

Future prospects

This study showed that the production of microalgae for hatcheries is not only feasible, but that there is a lot of room to improve the economic viability. Since labor is the major part of the costs, research is needed to reduce this by extra process control and implementation of process analytical technology (PAT). By measuring the critical process parameters (CCPs), the process could be automatically monitored and controlled without increasing labor costs. On-line turbidity measurement is an important process tool to control a constant biomass concentration, which ensures a high productivity without chance on wash-out conditions.

Possible improvements of productivity can be realized by using horizontal vertically stacked tubular PBRs or flat plate PBRs (Slegers et al., 2013; Slegers et al., 2011). However, more research is needed on shear stress levels encountered by the microalgae in these systems and their pumps to enable the production of shear stress sensitive strains.

Further energy savings for temperature control are possible, when a stable microalgal production could be realized at varying temperatures. This would allow the culture temperature to increase during daylight and decrease at night or cold periods. Less energy would be needed in comparison with maintaining a constant culture temperature during the day as well as over the seasons. Therefore, research on the effects of temperature on productivity and the production costs is therefore needed. Focus should be on development of less temperature sensitive production strains.

It is clear that the use of sunlight as much as possible will lead to lower production costs, but especially in winter time additional artificial light is still needed to meet the demands of constant supply of fresh microalgal feed. An alternative method would be to store preserved live microalgae produced in times when an excess is produced, which can then later be used in periods with a lower productivity (Núñez-Zarco and Sánchez-Saavedra, 2011). However, it is still difficult to preserve live microalgae for longer times than a few weeks. Further research on storage of viable microalgal cell cultures and its quality could therefore help to reduce energy costs for the application of artificial light.

Although some obstacles still need to be overcome, this study provides an important contribution to develop a more economically and sustainable production facility of microalgae as part of an integrated multi-trophic aquaculture.

Acknowledgments

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SUMMARY

In 2007, the project 'Zeeuwse Tong' (Zeeland Sole) was founded with support of the province of Zeeland, the Netherlands. The aim of the Zeeuwse Tong project was to establish an innovative land-based integrated multi-trophic aquaculture sector, which is producing sole, ragworms, algae, shellfish and saline crops in close harmony with nature. The project was divided into two sub-projects: The integrated saline aquaculture farm and the integrated nursery.
The research described in this thesis resides within the integrated nursery sub-project. In this project the rearing of fingerlings of sole would be combined with the cultivation of microalgae as feed for shellfish larvae and spat inside a greenhouse. An integrated nursery in a greenhouse has several advantages: a greenhouse with a multipurpose use of space, sole culture combined with the cultivation of microalgae and shellfish larvae or spat, an integrated thermoregulation and the reuse of nutrients from the wastewater of the fish basins for the production of microalgae in closed photobioreactors (PBRs).
For this thesis, a horizontal tubular PBR needed to be designed and constructed to investigate the productivity and yield of microalgae applied as feed for shellfish larvae or spat, within the context of an integrated nursery.

The hydrodynamic forces in the PBR systems cause shear stress levels that potentially damage shear stress sensitive microalgae. The effect of shear stress on the viability of *Chaetoceros muelleri* is described in **Chapter 2.** Shear cylinders were used, in which different levels of shear stress were applied by varying the shear rates and the medium viscosities to determine the shear sensitivity of *Chaetoceros muelleri*. The viability of *Chaetoceros muelleri* was negatively affected by shear stress levels higher than 1 and 1.3 Pa. A sudden decrease in viability to levels between 52% and 66% occurred, when *Chaetoceros muelleri* was exposed to shear stress beyond the threshold value. The effect of shear stress was almost instantaneous, compared to normal cultivation times. Exposure to high shear stress over a longer period of time did not have an adverse effect on the resistant cells. This indicated that internal damage must have taken place in the sensitive cells, as external damage was not observed.

In **Chapter 3**, the effect of biomass concentration on the net volumetric productivity, the yield of biomass on light and the nightly biomass loss rate of *Tetraselmis suecica* was studied in the pilot-scale tubular PBR using only natural sunlight. The challenge was to find the optimal biomass concentration at which a high net productivity and

a high yield on light were reached. At optimal biomass concentration, theoretically all available light would be absorbed by the biomass. A too dense culture would create a larger dark zone and this will lead to losses in productivity due to respiration. A too low biomass concentration on the other hand will lead to losses of photons, especially during high light periods. This hypothesis was confirmed in our experiments. The optimal biomass concentration of *Tetraselmis suecica* cultivated in a pilot-scale tubular PBR using natural sunlight was 0.7 g L^{-1} with the highest average net productivity and yield on light of 0.35 ± 0.03 g L^{-1} d^{-1} and 1.19 ± 0.15 g mol^{-1}, respectively. The average net productivity and yield on light were lower for cultures grown at other biomass concentration due to the waste of light in cultures with a lower biomass concentration, while a prolonged respiration period during low light periods was evident for cultures grown at higher biomass concentrations. Although the nightly biomass loss rate for *Tetraselmis suecica* grown at an optimal biomass concentration was highest due to higher maintenance requirements of the microalgae growing at a higher growth rate, the productivity was positively affected by smaller dark zones during low light periods compared to higher biomass concentrations and less losses of photons during high light periods compared to a lower biomass concentration.

The production of microalgae in an integrated multi-trophic aquaculture can become more economically and ecologically sustainable, if wastewater is used. In particular, the nutrient-rich wastewater of fish farms can be used to cultivate microalgae in hatcheries. The wastewater from fish farms is not only purified by the microalgae, but it also provides free nutrients for the production of the microalgal feed. Therefore, the objective of **Chapter 4** was to determine the N and P removal efficiencies and the productivity of *Tetraselmis suecica* in a fully-automated tubular PBR using wastewater from a fish farm. When *Tetraselmis suecica* was grown at a biomass concentration of 0.5 g L^{-1} on only wastewater, N and P removal efficiencies of respectively 49.4% and 99.0% were obtained. The average net volumetric productivity in this case was 0.35 g L^{-1} d^{-1}. Because phosphate was the limiting nutrient, extra P was dosed to cultivate *Tetraselmis suecica* at higher biomass concentrations. This resulted in a 95.7% N removal efficiency, a 99.7% P removal efficiency and a significantly higher average net volumetric productivity of 0.52 g L^{-1} d^{-1} at a biomass concentration of 1.0 g L^{-1}. The results show that the use of wastewater as a free nutrient source for the production of microalgae in PBRs for aquaculture promotes a more sustainable aquaculture practice.

In **Chapter 5**, the effect of cooling at night on the 24 hour productivity and diurnal changes of the biochemical composition of *Tetraselmis suecica* cultivated in a tubular PBR was determined. This study was done to investigate if the net productivity can be increased by decreasing the biomass loss due to respiration during the night. The hypothesis was that cooling during the night would reduce the respiration rate with less biomass loss during the night as a result. As the net productivity is the result of both photosynthesis and respiration, a higher overall net productivity was expected. Our results showed that cooling at night did not lead to a decrease of the respiration rate at night and an increase in the net productivity. The net volumetric productivity and biochemical composition were only affected by the daily light input. Periods with more light resulted in microalgal cells with a higher carbohydrate and lower protein content. A build-up of carbohydrates during daylight was observed. At night these carbohydrates are used for the protein synthesis. The fatty acid content stayed constant over the entire day, except for the EPA content. During daylight the EPA content decreased, and at night it increased. This reflects the function of EPA as a structural fatty acid present in the polar phospholipid membranes. Furthermore, the carbohydrate loss rate at night was linearly related to the specific growth rate and therefore to the light conditions during daylight.
Although cooling does not increase the productivity or affects the biochemical composition, energy can be saved by turning off the temperature control at night.

A literature research revealed that limited number microalgal species used in aquaculture have been successfully cultivated in tubular PBRs with centrifugal pumps. This suggests that the hydrodynamic forces occurring in PBRs and in the recirculation pumps are too high for shear stress sensitive microalgae. The threshold values of shear stress for *Tetraselmis suecica*, *Isochrysis galbana* and *Skeletonema costatum* were determined with the shear cylinders, which were also used for *Chaetoceros muelleri* in **Chapter 2**. The capability to grow of these four species in a tubular PBR with a variable-frequency-drive centrifugal pump was studied to determine the relationship between shear stress sensitivity of microalgal strains and shear stress levels encountered in tubular PBRs (**Chapter 6**).
Tetraselmis suecica was found to be a shear stress tolerant species, which could sustain maximum applied shear stress level of 88 Pa without loss of viability. *Tetraselmis suecica* was also successfully cultivated in the tubular PBR driven by a centrifugal pump. Only when *Tetraselmis suecica* was recirculated at the highest pumping speed, biofouling occurred.
Isochrysis galbana, *Skeletonema costatum* and *Chaetoceros muelleri* were found to be shear stress sensitive. Cells were damaged when exposed to shear stress

levels between 1.2 and 5.4 Pa. *Isochrysis galbana*, *Skeletonema costatum* and *Chaetoceros muelleri* were also not able to grow in the tubular PBR, not even at the lowest pumping speed. In the circulation tubes and pressure side of the pump, the microalgae were exposed to shear stresses of 0.57 and 1.82 Pa, respectively. These shear stress levels were in the order of magnitude of the maximum shear tolerance of the three sensitive species. Higher shear stress levels probably occur in the recirculation pump, which could explain why shear stress sensitive algae cannot be successfully grown in a tubular PBR with a centrifugal pump. Future design of closed PBRs for microalgal cultivation of shear stress sensitive strains should therefore take shear stress into account.

Chapter 7 discusses the possibilities of realizing a more cost-effective and sustainable production facility of microalgae for hatcheries, which is needed to meet the requirements for a stable production of microalgae with a constant quality. Fully-automated closed PBRs equipped with low-shear pumps are advocated to avoid culture collapses due to contamination and to create low shear stress conditions for stable production. The costs of the production of microalgae in fully-automated tubular PBRs were estimated for a scale of 0.1 ha and 1 ha. This resulted in an estimated cost price of €53.70 per kg DW microalgae at 0.1 ha and €30.90 at 1 ha scale. This showed that labor costs are enormously reduced by increasing the scale of production. The incorporation of the production of microalgae into the concept of integrated multi-trophic aquaculture in which wastewater is used instead of fertilizers is another way to reduce the costs. Other important costs reducing methods are turning off the temperature control at night, or by using waste heat from power engines and cooling with groundwater to control the temperature. If these costs reducing methods are applied, costs can be reduced respectively from €53.70 and €30.90 to €36.20 and €13.40 per kg DW microalgae at 0.1 ha and 1 ha scale.
Based on these results, further research is suggested to further develop and implement a more economically and sustainable production facility of microalgae for aquaculture.

SAMENVATTING

In 2007 werd de Stichting Zeeuwse Tong opgericht met steun van de Provincie Zeeland. Het doel van het Zeeuwse Tong project was om een binnendijkse geïntegreerde multitrofische aquacultuur sector tot stand te brengen, die tong, zagers, algen, schelpdieren en zilte gewassen produceert in harmonie met de natuur. Het project was verdeeld in twee deelprojecten: Het gemengd zilt bedrijf en de geïntegreerde kwekerij.
Het onderzoek, dat beschreven is in deze thesis, valt onder het deelproject geïntegreerde kwekerij. In dit project zou de kweek van pootvis van tong gecombineerd worden met de kweek van algen als voedsel voor schelpdierlarven en schelpdierbroed in een kas. Een geïntegreerde kwekerij in een kas heeft meerdere voordelen: een kas met een multifunctioneel gebruik van ruimte, tongkweek gecombineerd met algenkweek en schelpdieren, een geïntegreerde warmtehuidhouding en het hergebruik van nutriënten van het afvalwater van de visvijvers voor de productie van microalgen in gesloten fotobioreactoren.
Voor deze thesis is een horizontale tubulaire fotobioreactor ontworpen en gebouwd om de productiviteit en opbrengst aan microalgen te onderzoeken, die toegepast kunnen worden als voedsel voor schelpdierlarven en schelpdierbroed in de context van de geïntegreerde kwekerij.

De hydrodynamische krachten in fotobioreactoren veroorzaken schuifspanning, die mogelijkerwijs microalgen kunnen beschadigen die gevoelig zijn voor schuifspanning (shear stress). Het effect van schuifspanning op de levensvatbaarheid van *Chaetoceros muelleri* is beschreven in **Hoofdstuk 2.** Hiervoor zijn shear cilinders gebruikt, waarin verschillende niveaus aan schuifspanning zijn getest door de snelheidsgradiënt en de mediumviscositeit te variëren en de gevoeligheid voor schuifspanning van *Chaetoceros muelleri* te bepalen. Schuifspanning hoger dan 1,3 Pa had een negatief effect op de levensvatbaarheid van *Chaetoceros muelleri*. Een plotselinge afname aan levensvatbaarheid tot percentages tussen de 52% en 66% trad op, wanneer *Chaetoceros muelleri* werd blootgesteld aan schuifspanning boven de drempelwaarde. De tijd waarin het effect van schuifspanning zichtbaar werd is vergeleken met de tijd, die doorgaans nodig is voor de algenkweek. Blootstelling aan hoge schuifspanning over een langere periode had geen nadelig effect op de resistente cellen. In de gevoelige cellen moet interne schade zijn opgetreden, omdat externe schade niet was waargenomen.

In **Hoofdstuk 3** is het effect van biomassaconcentratie op de netto volumetrische productiviteit, de opbrengst aan biomassa op licht en de afnamesnelheid van biomassa gedurende de nacht van *Tetraselmis suecica* onderzocht in de tubulaire fotobioreactor op proefschaal, gebruik makend van natuurlijk zonlicht. De uitdaging was om de optimale biomassaconcentratie te vinden waarbij een combinatie van hoge netto productiviteit en een hoge opbrengst op licht werd bereikt. Bij een optimale biomassaconcentratie wordt theoretisch al het beschikbare licht geabsorbeerd door de biomassa. Een te dichte algencultuur veroorzaakt een grotere donkere zone en dit leidt tot verlies aan productiviteit als gevolg van de respiratie. Aan de andere kant zal een te lage biomassaconcentratie tot verlies aan fotonen leiden, met name gedurende periodes met een hoge lichtintensiteit. De resultaten van de experimenten bevestigden deze hypothese. De optimale biomassaconcentratie van *Tetraselmis suecica* gekweekt in een tubulaire fotobioreactor op proefschaal gebruik makend van natuurlijk zonlicht was 0,7 g L^{-1} met respectievelijk de hoogste gemiddelde netto productiviteit en opbrengst op licht van 0,35 ± 0,03 g L^{-1} d^{-1} en 1,19 ± 0,15 g mol^{-1}. De gemiddelde netto productiviteit en opbrengst op licht waren lager voor de kweek bij andere biomassaconcentraties als gevolg van verspilling aan licht in kweken met een lagere biomassaconcentratie. Een verlengde respiratieperiode trad op gedurende periodes met weinig licht voor kweken met hogere biomassaconcentraties. De afnamesnelheid van biomassa in de nacht was het hoogst voor *Tetraselmis suecica* met een optimale biomassaconcentratie. Dit werd veroorzaakt door hogere onderhoudsvereisten voor de microalgen met een hogere groeisnelheid. Toch werd de productiviteit positief beïnvloed door kleinere donkere zones gedurende periodes met weinig licht in vergelijking met hogere biomassaconcentraties en minder verlies aan fotonen gedurende periodes met veel licht vergeleken met een lagere biomassaconcentratie.

De productie van microalgen in een geïntegreerde multitrofische aquacultuur kan economisch en ecologisch duurzamer worden, als afvalwater wordt gebruikt. In het bijzonder kan nutriëntenrijk afvalwater van viskwekerijen gebruikt worden voor de kweek van microalgen. Het afvalwater van de viskwekerij wordt niet alleen gezuiverd door de microalgen, maar het levert ook gratis nutriënten voor de productie van microalgen als voedsel voor bijvoorbeeld schelpdierlarven. Het doel van **Hoofdstuk 4** was daarom het bepalen van de N- en P-zuiveringsrendementen en de productiviteit van *Tetraselmis suecica* in een volledig geautomatiseerde tubulaire fotobioreactor, gebruik makend van afvalwater van een viskwekerij. Wanneer *Tetraselmis suecica* werd gekweekt met een biomassaconcentratie van 0,5 g L^{-1} op puur afvalwater, waren de N- en P-zuiveringsrendementen respectievelijk 49,4%

en 99,0%. Daarbij was de gemiddelde netto volumetrische productiviteit 0,35 g L^{-1} d^{-1}. Omdat fosfaat het limiterende nutriënt was, werd extra P gedoseerd om *Tetraselmis suecica* met hogere biomassaconcentraties te kweken. Dit resulteerde in een 95,7% N-zuiveringsrendement, een 99,7% P-zuiveringsrendement en een significant hogere gemiddelde netto volumetrische productiviteit van 0,52 g L^{-1} d^{-1} bij een biomassaconcentratie van 1,0 g L^{-1}. De resultaten laten zien dat het gebruik van afvalwater als gratis nutriëntenbron voor de productie van microalgen in fotobioreactoren de aquacultuur duurzamer maakt.

In **Hoofdstuk 5** is het effect van koeling in de nacht op de 24-uurs productiviteit en veranderingen van de biochemische samenstelling van *Tetraselmis suecica* gekweekt in een tubulaire fotobioreactor bepaald. Deze studie is gedaan om te onderzoeken of de netto productiviteit verhoogd kan worden door het biomassaverlies ten gevolge van nachtelijke respiratie te verminderen. De hypothese was dat koeling in de nacht de respiratie zou verminderen met minder verlies aan biomassa gedurende de nacht als resultaat. Omdat de netto productiviteit de resultante is van zowel fotosynthese als respiratie, werd er een hogere netto productiviteit verwacht. Onze resultaten laten echter zien dat koeling in de nacht niet leidt tot een vermindering van de respiratie 's nachts en een verhoging van de netto productiviteit. De netto volumetrische productiviteit en biochemische samenstelling werden alleen beïnvloed door de dagelijkse lichthoeveelheid. Meer licht resulteerde in cellen met een hoger koolhydratengehalte en een lager eiwitgehalte. Gedurende daglicht nam het gehalte aan koolhydraten toe, die dan 's nachts werden gebruikt voor de eiwitsynthese. Het vetzuurgehalte bleef over de gehele dag constant, behalve voor het gehalte aan EPA. Het EPA-gehalte nam af gedurende daglicht en nam 's nachts toe. Dit geeft de functie van EPA weer als een structuurvetzuur aanwezig in de polaire fosfolipide membranen. Verder was het koolhydratenverlies in de nacht lineair gerelateerd aan de specifieke groeisnelheid en daardoor aan de lichtcondities gedurende daglicht.
Hoewel koeling in de nacht de productiviteit niet laat toenemen of de biochemische samenstelling beïnvloedt, kan energie worden gespaard door de temperatuurregulatie 's nachts uit te zetten.

Uit literatuuronderzoek bleek dat een gelimiteerd aantal algensoorten, die worden gebruikt in de aquacultuur in de praktijk, succesvol worden gekweekt in tubulaire fotobioreactoren met centrifugale pompen. Dit suggereert dat de hydrodynamische krachten in fotobioreactoren en recirculatiepompen te hoog zijn voor algensoorten die gevoelig zijn voor schuifspanning. De drempelwaardes van schuifspanning voor *Tetraselmis suecica*, *Isochrysis galbana* en *Skeletonema costatum* zijn bepaald met

de shear cilinders, die ook gebruikt zijn voor *Chaetoceros muelleri* in **Hoofdstuk 2**. Het vermogen van deze vier soorten om te groeien in een tubulaire fotobioreactor met een centrifugale pomp is onderzocht om de relatie tussen gevoeligheid voor schuifspanning van de algensoorten en de toegepaste niveaus van schuifspanning in tubulaire fotobioreactoren te bepalen (**Hoofdstuk 6**).
Uit dit onderzoek bleek dat *Tetraselmis suecica* tolerant was voor schuifspanning. Deze alg kan een maximale aangebrachte schuifspanning van 88 Pa weerstaan zonder verlies van levensvatbaarheid. *Tetraselmis suecica* werd ook succesvol gekweekt in de tubulaire fotobioreactor aangedreven door een centrifugale pomp. Alleen wanneer *Tetraselmis suecica* werd gerecirculeerd met de hoogste pompsnelheid, trad stressgerelateerde aangroei op.
Isochrysis galbana, *Skeletonema costatum* en *Chaetoceros muelleri* waren allen veel gevoeliger voor schuifspanning. Cellen werden beschadigd wanneer ze werden blootgesteld aan schuifspanning tussen 1,2 en 5,4 Pa. *Isochrysis galbana*, *Skeletonema costatum* en *Chaetoceros muelleri* bleken ook niet te kunnen groeien in de tubulaire fotobioreactor, zelfs niet als de laagste pompsnelheid werd toegepast. In de buizen en aan de drukzijde van de pomp werden de microalgen blootgesteld aan schuifspanningen van respectievelijk 0,57 en 1,82 Pa. Deze schuifspanningen zijn in de orde grootte van de maximale tolerantie voor schuifspanning van deze drie gevoelige soorten. Hogere schuifspanningen treden waarschijnlijk op in de recirculatiepomp, wat verklaart waarom algen die gevoelig zijn voor schuifspanning niet succesvol gekweekt kunnen worden in een tubulaire fotobioreactor met een centrifugale pomp. Toekomstig ontwerp van fotobioreactoren voor de kweek van schuifspanningsgevoelige algensoorten zou daarom rekening moeten houden met schuifspanning.

In **Hoofdstuk 7** worden de mogelijkheden voor het realiseren van een kosteneffectieve en duurzame productiefaciliteit van microalgen voor hatchery's aan de orde gesteld. Dit is nodig om tegemoet te komen aan de behoefte voor een stabiele productie van microalgen met een constant kwaliteit. Volledig geautomatiseerde gesloten fotobioreactoren voorzien van pompen met lage schuifspanning worden bepleit om te voorkomen dat de algenkweek vroegtijdig moet worden gestaakt, ten gevolge van besmetting, en om lage schuifspanningscondities te creëren voor een stabiele productie.
De kosten voor de productie van algen in volledig geautomatiseerde fotobioreactoren zijn berekend voor de kweek van algen in tubulaire fotobioreactor systemen op een schaal van 0,1 ha en 1 ha. Dit resulteerde in een geschatte kostprijs van €53,70 per kg drooggewicht aan algen op 0.1 ha en €30,90 op 1 ha schaal. Het is duidelijk dat

arbeidskosten enorm afnemen door de productieschaal te verhogen. Een andere manier om de kosten te verlagen is door de algenproductie in het concept van geïntegreerde multitrofische aquacultuur op te nemen, waarin afvalwater in plaats van meststoffen gebruikt wordt. Andere belangrijke kostenreducerende methodes zijn het uitzetten van de temperatuurregulatie ‘s nachts of door gebruik te maken van afvalwarmte van warmte-krachtkoppeling (WKK) installaties voor verwarming en grondwater te gebruiken voor de koeling. Als deze kostenverlagende methodes worden toegepast, kunnen de kosten worden gereduceerd van respectievelijk €53,70 en €30,90 tot €36,20 en €13,40 per kg drooggewicht aan algen op een schaal van 0,1 ha en 1 ha.

Gebaseerd op deze resultaten wordt vervolgonderzoek aanbevolen om een economisch en ecologisch duurzamere productiefaciliteit van algen voor aquacultuur te ontwikkelen en implementeren.

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First of all, I want to thank René for his guidance throughout the PhD project. René was always able to keep me on track giving clear suggestions.

Not long after the first meeting where we discussed my plan to do a PhD research, I was accepted to do a PhD research for which I also needed the approval of the HZ University of Applied Sciences. Thanks to the big help and confidence of Anja, who was then the lector of Sustainability and Water, I was able to start the PhD research on part-time basis next to my work at the HZ University of Applied Sciences. The research was a part of the Zeeland Sole project and Jan was the person who always had a confidence in a good outcome.

My first co-promotor was Niels-Henrik, who helped me a lot at the start of the first experiment about shear stress, which was actually a totally new subject to me. Thanks to Atze Jan, I started to understand this topic. Then, we had to design a pilot-scale horizontal tubular photobioreactor (PBR), which was built by Paques and installed in the SEA Lab in Vlissingen. The first year working with the PBR, I faced many start-up problems, but Fred was always willing to help me out. Fred often drove to Vlissingen in that year and he made it possible to have the PBR working fine. In that year, Marian became my co-promotor and she helped me to stay positive. Marian was the best co-promotor I could have hoped for. Marian, thank you for all the work you did for me.

Carsten became my best algae friend, who let me stay at his place when I was in Wageningen. We also had a course, several conferences and the PhD trip to the USA together, where we got to know each other even better. I have spent many hours with Carsten discussing all kinds of aspects of life. During that time, it was not difficult to become friends with Ana as well. I was very glad that you also came to Zeeland, when we had a great time.

My first success with the PBR was when I ‘accidently’ inoculated the PBR with *Tetraselmis suecica* instead of the shear stress sensitive species *Chaetoceros muelleri*. Therefore, I also want to thank the nice growing flagellate *Tetraselmis suecica*. The experiments with these algae were done in the PBR in a greenhouse. So the algae were growing on sunlight. Therefore, the light intensity was different during the day and season. Ellen helped me a lot on how to interpret the light data, next to many other aspects of the PhD research she helped me with.

The idea of growing algae on wastewater of a fish farm was realized with the help of Mitra, who was my colleague and at that time student as well. We managed to grow the algae on wastewater for 130 days without any problems, while measuring everything every day, also during all the weekends and 'holidays'. Mitra, I am very proud of you and our results, which can lead to a more sustainable microalgal cultivation.

Javier, at that time a PhD student of the University of Almería, came to Vlissingen to work together with me on an experiment about cooling at night. Next to the normal measurements, we also had six 24 hour sampling days, for which we had to stay over and sleep in the SEA Lab between the sampling periods. The cooperation was perfect and we always knew what we had to do without asking what the other was going to do. Javier often played the trumpet outside the SEA Lab bringing a Spanish atmosphere into a Dutch harbor.

Although I did most of my work in Vlissingen, I always felt at home when I was in Wageningen. Many people of the Bioprocess Engineering group, Food Process Engineering group and related groups helped me in some way during these years and I want to thank them as well: Wendy, Giulia, Jeroen, David, Emre, Anne, Lenny, Lenneke, Guido, Tim, Sina, Nadine, Packo, Maria C, Maria B, Kiira, Kim, Dorinde, Kanjana, Sarah, Klaske, Claudia, Marieke, Petra, Richard, Ilse, Rouke, Ward, Stefan, Edwin, João, Agnes, Marjon, Christa, Marcel, Dirk, Mathieu, Marina, Hans T, Michel, Arjen, Hans R, Brenda, Remko, Karin, Jarno, Jos, Maurice, Martin, Martha, Anna, Catarina, Jacqueline, Kasia, Ton and Farnoosh.
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Michiel

ABOUT THE AUTHOR

Michiel Henricus Aloysius Michels was born in Helmond, the Netherlands, on the 20th of August 1967. He went to secondary school at the St.-Willibrord Gymnasium in Deurne. After that, he started to study Environmental Sciences (Milieuhygiëne) at Wageningen University and graduated in 1992 (MSc). After one year of voluntary work for Friends of the Earth (Vereniging Milieudefensie) in Amsterdam, he continued to study at the Utrecht University to obtain his Master's degree as a chemistry teacher at preparatory higher education level (Universitaire Lerarenopleiding Scheikunde 1e graads) in 1994 and became a chemistry and physics teacher at several secondary schools. From 1997 till present, he is working as a senior lecturer in the study program Aquatic Ecotechnology at the HZ University of Applied Sciences. He is involved in teaching several courses, like water treatment, ecotoxicology, river basin management, etc. Furthermore, he organizes and supervises research projects for the research group Aquaculture in Delta Areas. In 2014, he started to organize projects for the Centre of Expertise Biobased Economy in close cooperation with the Avans University of Applied Sciences.
His PhD research at the Bioprocess Engineering group of Wageningen University started in 2008 on part-time basis next to his work for the HZ University of Applied Sciences. The results of his PhD research are described in this thesis.

PUBLICATIONS

Doelman P, Jansen E, Michels M, Van Til M (1994) Effects of heavy metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameters. Biol Fertil Soils 17:177–184.

Michels MHA, Van Der Goot AJ, Norsker NH, Wijffels RH (2010) Effects of shear stress on the microalgae *Chaetoceros muelleri*. Bioproc Biosyst Eng 33(8): 921-927.

Michels MHA, Slegers PM, Vermuë MH, Wijffels RH (2014) Effect of biomass concentration on the productivity of *Tetraselmis suecica* in a pilot-scale tubular photobioreactor using natural sunlight. Algal Res 4: 12-18.

Michels MHA, Vaskoska M, Vermuë MH, Wijffels RH (2014) Growth of *Tetraselmis suecica* in a tubular photobioreactor on wastewater from a fish farm. Water Res 65: 290-296.

Michels MHA, Camacho-Rodríguez J, Vermuë MH, Wijffels RH (2014) Effect of cooling in the night on the productivity and biochemical composition of *Tetraselmis suecica*. Algal Res 6, Part B: 145-151.

Michels MHA, Van Der Goot AJ, Vermuë MH, Wijffels RH. Cultivation of shear stress sensitive and tolerant microalgal species. Submitted for publication.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses

Bioreactor design and operation (Wageningen, 2008)
Course Mathlab, Mathworks (Vlissingen, 2009)

Conferences

1e Algencongres 'Waarheden en onwaarheden over algen' (Dronten, 2008)
Seminar 'Toekomst voor aquacultuur' (Yerseke, 2008)
Dag van de Zeeuwse Visserij (Vlissingen, 2008)[1]
Science & Technology Summit (Amsterdam, 2008)[1]
International Algae Congress (Amsterdam, 2008)
13th Netherlands Biotechnology Congress (Ede, 2010)[1]
8th European Workshop Biotechnology of Microalgae (Nuthetal, Germany, 2010)[1]
1st Young Algaeneers Symposium (Wageningen, 2012)[2]
3rd International Conference on Algal Biomass, Biofuels and Bioproducts (Toronto, Canada, 2013)[1]
2nd Young Algaeneers Symposium (Montpellier and Narbonne, France, 2014)[1]

General courses

VLAG PhD Week (Bergeijk, 2008)
Information literacy, including introduction Endnote (Wageningen, 2009)
Science, the press and the general public: communication and interaction (Wageningen, 2009)

Optional

Preparation project proposal (2008)
Excursion to IGV GmbH and Salata Gmbh, Germany (2009)[3]
PhD trip to USA (2010)[1,2]
PhD trip to Spain (2012)[1,2]

[1]Oral presentation
[2]Poster presentation
[3]Organization

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