The image is a vertical poster with a dark blue background. The top half features a large orange sun at the top center, with several orange lines radiating downwards. Below the sun are several light blue, cylindrical structures representing microalgae production tanks, each containing small blue and purple microalgae cells. The bottom half features a large orange lightbulb in the center, with several orange lines radiating upwards. Inside the lightbulb, the text 'Pieter Cente Oostlander' is written in white. The background of the bottom half is decorated with a pattern of light blue, wavy, U-shaped lines.

Microalgae production for aquaculture hatcheries

Pieter Cente
Oostlander

Propositions

1. Cell count is not a useful method for the determination of biomass concentration – (this thesis)
2. Centralized microalgae production facilities are imperative for commercial success of live microalgae for aqua feed applications – (this thesis)
3. Good scientists do not question everything
4. Innovative solutions are created by restrictions
5. To improve the quality of life, care less
6. The open use of a radio is always detrimental to worker efficiency

Propositions belonging to the thesis entitled:

‘Industrial microalgae production for aquaculture hatcheries’

Pieter C. Oostlander

Wageningen, 29 May 2020

Industrial microalgae production for aquaculture hatcheries

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Industrial microalgae production for aquaculture hatcheries

Pieter C. Oostlander

Thesis

Submitted in fulfilment of the requirements for the degree of doctor

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by the authority of the Rector Magnificus,

Prof. Dr A.P.J. Mol,

in the presence of the

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to be defended in public

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Contents

Chapter 1	Introduction and thesis outline	7
Chapter 2	Microalgae production cost in aquaculture hatcheries	19
Chapter 3	Optimization of <i>Rhodomonas</i> sp. under continuous cultivation for industrial applications in aquaculture	69
Chapter 4	Growth and fatty acid content of <i>Rhodomonas</i> sp. under day:night cycles of light and temperature	93
Chapter 5	Production of <i>Rhodomonas</i> sp. at pilot scale under sunlight conditions	113
Chapter 6	General discussion: Opportunities for industrial microalgae production in aquaculture hatcheries	131
References		149
Summary		161
Samenvatting		167
Nomenclature		177
Acknowledgements		179
About the author		187

Chapter 1

Introduction and thesis outline

1.1 Microalgae for aquaculture

Microalgae are the first trophic level of the aquatic food chain. Microalgae convert sunlight and carbon-dioxide into organic compounds to produce biomass by photosynthesis. In nature, freely swimming marine microalgae are consumed by filter feeders such as bivalves, crustaceans and certain fish [1]. On-shore farming of marine products such as oysters, requires a supply of microalgae to be utilized as feed for the farmed product [2]. As a result, the production of microalgae for aqua feed applications has been a common practice in aquaculture hatcheries for many decades [3-5]. In many applications of microalgae in aquaculture, the use of live microalgae is required. For the production of copepods living and swimming microalgal cells are preferred over processed microalgae products such as paste or dried biomass [6, 7]. For oyster production, studies report the benefit of live microalgae to the water quality compared to the use of formulated microalgal products as these often lead to deteriorated water quality. Better growth is observed with live microalgae compared to other feed products such as bacteria or yeast [8, 9]. As a results of this need for living microalgae, most aquaculture hatcheries maintain an in-house microalgae production facility, typically at small scales, but enough to cover their own biomass requirements [10].

1.2 Production system for microalgae in aquaculture

Small scale microalgae production facilities in aquaculture hatcheries have been around for decades and are typically based on modular microalgae production systems such as bubble-columns, with limited monitoring and control equipment [10]. With the increased interest for microalgae production for markets such as food, feed, cosmetics and bulk chemicals over the last decade, new technologies for microalgae production have emerged [11, 12]. New, more advanced technologies for microalgae production have become readily available at commercial level. The most commonly used systems are scalable photobioreactors such as tubular photobioreactors.

Although microalgae are a crucial part in the food chain of any aquatic production process, very little detail is available on the cost price of microalgae production in aquaculture hatcheries or on the optimization of this process. One study in literature describes microalgae production for aquaculture hatcheries to account for up to 60% of the total hatchery costs [10]. This means that a reduction in microalgae production costs can have a high impact on the cost efficiency of aquaculture products [10]. With the technological advances made over the past decades in the field of microalgae production and the current

commercially available reactor systems, the potential for increased algae production at lower cost for aquaculture hatcheries should be evaluated. Literature describes cost price analysis of bulk chemical production with microalgae on a scale of 100 ha [13, 14]. No detailed study of currently available technology for microalgae production at the small scales as used in aquaculture hatcheries, has ever been performed. A detailed techno-economic analysis will allow to determine the current cost price of microalgae and find the most efficient cost reduction strategies for aquaculture hatcheries. A techno-economic analysis could reveal if the use of the new commercially available scalable production technologies for microalgae production at these small scales is economically feasible, or if the modular systems remain the best approach to supply microalgae to aquaculture hatcheries.

1.3 The quality of microalgae for aquaculture hatcheries

For aquaculture hatcheries it is important to not only focus on the cost price of microalgae but also the nutritional value of the produced microalgae in the feed applications. The quality of microalgae for feed applications is mostly determined by the fatty acid content and composition of the biomass. The need for optimized biomass composition for aquaculture feed application is apparent from the large number of studies and reviews in literature focusing on the nutritional aspect of microalgae applications in aquaculture [15, 16]. A reduction of microalgae cost that results in a lower nutritional value could damage the overall production chain in aquaculture and increase the cost of the final product. Simply focusing on the most cost-efficient biomass production for microalgae could be not the best approach for aquaculture applications.

In most hatchery applications the exact requirement of microalgal biomass composition is still not completely known. Most hatcheries achieve the required dietary composition by mixing multiple microalgal species [7, 17-19], or by finding a strain that performs best in the selected application [20-22]. The nutritional value of microalgae for aquaculture applications is mostly attributed to the content of omega-3 fatty acids. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are generally regarded as the most important fatty acids in microalgae biomass, determining their nutritional value for aquaculture. Literature in microalgae research describes multiple methods to change the biomass content and compositions of fatty acids. Examples include, nitrogen or phosphorous starvation for fatty-acid accumulation in the biomass [23, 24]. Changes in growth parameters such as light and temperature or the implementation of a day:night cycle can also influence the fatty acid content of microalgae [25, 26]. In this study we mostly focus on the effect of

growth conditions (light and temperature) and daily fluctuations of these growth conditions on the content of EPA and DHA in the biomass.

1.4 *Rhodomonas sp.*

Rhodomonas sp. are red single-cell microalgae belonging to the class of cryptophyceae. The freely swimming cells contain 2 unequal flagella (see Figure 1), cells are relatively large with an average diameter of 7-8 μm and an average cell mass of 100-150 pg cell^{-1} [27]. *Rhodomonas sp.* cells do not have a cell-wall, making the cells relatively fragile and sensitive to shear stress. The red color of the cells is caused by the phycobiliprotein, phycoerythrin. This light harvesting pigment protein complex is used for light harvesting for photosynthesis and transportation of light energy to the photosystems [28, 29]. Besides this specific pigment the main positive attribute of *Rhodomonas sp.* biomass is the fatty acid composition containing both EPA and DHA in approximately equal amounts [30, 31]. The unique biomass composition of this strain makes it specifically interesting for aquaculture applications. *Rhodomonas sp.* has been described to be specifically suitable as live feed for the production of copepods [32, 33]. *Rhodomonas sp.* is used, in this study, as an example of microalgae in aquaculture to study the effect of growth conditions on the growth rate, biomass yield on light and the fatty acid content and composition.

Figure 1: Light microscopy picture of *Rhodomonas sp.* cells. Unequal flagella clearly visible for the bottom right cell – magnification 60x

1.5 Thesis aim and outline

The aim of this thesis is to determine the cost price of microalgae for small scale production in aquaculture hatcheries using a techno-economic model and to study cost efficient strategies for cost price reduction, while keeping the microalgae nutritional value. This is done specifically using technologies for microalgae production that are currently commercially available and applied in aquaculture. Reliable inputs for the techno-economic analysis were obtained from commercial aquaculture hatcheries. Accurate values for the biomass yield on light ($Y_{x,ph}$) is imperative for a reliable outcome of the model as this is one of the most important input parameters. This techno-economic model and the resulting cost price of microalgae production in aquaculture hatcheries is described in chapter 2.

Experimental work with *Rhodomonas sp.* is used to determine the effect of growth conditions on the biomass yield on light and the EPA and DHA content of the biomass. Optimization of the growth of *Rhodomonas*

sp. under continuous conditions of light and temperature at different levels is described in detail in Chapter 3. The effect of daily oscillations of temperature and light are studied in Chapter 4. After optimization at lab scale, *Rhodomonas sp.* was cultivated outdoors under sunlight conditions in pilot-scale photobioreactors in chapter 5. An outlook towards cost efficient microalgae production for aquaculture hatcheries is given in chapter 6. Chapter 6 describes the combination of the techno-economic model with the experimental data based on the results of *Rhodomonas sp.* grown at all experimental scale in chapters 3, 4 and 5.

Figure 2: Tubular photobioreactors in a greenhouse located at the HZ University of applied sciences, Vlissingen, The Netherlands (top) and bubble-column reactors using artificial light, located at Roem van Yerseke Hatchery, Yerseke, The Netherlands (bottom) as used for algae production in aquaculture hatcheries.

1.5.1 Chapter 2 - Micro-algae production cost in aquaculture

A techno-economic model to describe the cost price small scale microalgae production in aquaculture hatcheries is developed. Only commercially available reactor systems are incorporated in this model. Scenarios that are likely to be applied in aquaculture industry were modelled using four reference scenarios. We compare the use of two different reactor systems; tubular photobioreactors and bubble-column type reactors.

Tubular photobioreactor represent scalable microalgae production technologies with a high degree of control of the growth conditions. The bubble-column systems represent modular production systems with a low complexity as typically applied in aquaculture hatcheries for microalgae production over the last decades. The two reactors are compared using two types of placement for the reactors; reactors in a climate room utilizing artificial light only and reactors located in a greenhouse utilizing sunlight as the only light source. These two reactor types and placements result in four scenario options, schematically represented in Figure 3A.

The aspects of microalgae production considered in the techno-economic model are represented in Figure 3B. No downstream processing is considered in the model as for aqua feed applications it is assumed all microalgae are directly used in the hatchery facilities. Reference scenarios are created based on currently used technologies in aquaculture. In each of the reference scenarios the cost price is calculated for a production capacity of 125 kgDW year⁻¹. A sensitivity analysis is performed to determine the most efficient cost reduction strategies and make projection on future cost prices of microalgae for aquaculture. The cost price for production of microalgae at a larger scale than presently used, is compared to the reference scenarios to indicate future potential of microalgae for aquaculture utilizing centralized production facilities.

Figure 3A (top): Schematic representation of the four scenarios in the techno-economic model displaying the two reactor types (Tubular photo bioreactor systems – TPBR and bubble-columns BC) utilizing artificial light (AL) or natural sunlight in a greenhouse (GH). Figure 3B (bottom): Schematic representation of the aspects of microalgae production considered in the techno-economic analysis. 1: laboratory, 2: labor, 3: temperature control, 4: light source, 5: nutrients, water and mixing 6: air supply, 7: CO_2 supply, 8: total size of the facility, 9: wastewater treatment and 10: the type and location of the reactor

1.5.2 Chapter 3 - Optimization of *Rhodomonas sp.* under continuous cultivation condition

Without nutrient limitations, the growth rate of microalgae is determined by light and temperature conditions. In chapter 3 the combined effect of light and temperature on the biomass yield on light is studied for *Rhodomonas sp.* in detail. To optimize data output while minimizing the required amount of experimental work a design of experiment approach, specifically a D-optimal design, was used. The D-optimal design used 5 levels for light intensity ($60\text{-}130\text{-}330\text{-}465\text{-}600\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) and 4 levels for temperature ($15\text{-}20\text{-}25\text{-}30\text{ }^{\circ}\text{C}$). All conditions were applied at constant levels for 24 hours day^{-1} using lab-scale photobioreactors with a working volume of 1.8 liter operated in turbidostat mode (Figure 4). In turbidostat operation an optimized biomass concentration is maintained during the experiment by automated dilution of the reactor. Growth rate, biomass yield on light and fatty acid content and composition at the provided light and temperature conditions can be determined accurately in steady-state turbidostat conditions. From the obtained data a model was developed to describe the biomass yield on light as a function of the applied light intensity and cultivation temperature.

Figure 4: *Rhodomonas sp.* growing under continuous cultivation conditions in flat panel photobioreactor at laboratory scale.

1.5.3 Chapter 4 - Fatty-acid content of *Rhodomonas sp.* under day:night cycles of light and temperature.

Microalgae cells that are grown under day:night cycle conditions have a synchronized cell cycle. The synchronized cell cycle results in daily oscillations of biomass components. Specifically components important for energy storage such as starch can fluctuate on a daily basis in synchronized cell cultures [25, 26, 34, 35]. Fatty acids have also been described to fluctuate according to a daily pattern for cells exposed to a day:night cycle [36]. This daily fluctuation in fatty acid content and composition could be of great importance to the biomass composition of *Rhodomonas sp.* when used in aqua feed applications. Namely, the EPA and DHA content of the cells is considered an important nutritional factor [37, 38].

Synchronized cell cycles have shown to result in a higher biomass yield on light for *Acutodesmus obliquus* and *Neochloris oleobundans* [25, 26, 34]. The increase in biomass yield on light by implementation of a day:night cycle could be an important factor for cost reduction of microalgae production in aquaculture hatcheries. Potentially an altered fatty acid composition could be obtained when implementing a day:night cycle. In chapter 4 we investigate the effect of daily oscillations of temperature and light on the biomass yield on light for *Rhodomonas sp.*. In addition, fluctuation in fatty acid content and composition of the biomass throughout one day were investigated.

1.5.4 Chapter 5 - Outdoor cultivation of *Rhodomonas sp.* for aquaculture feed applications

Rhodomonas sp. was grown in three tubular photobioreactors with a working volume of 200 liter each (Figure 5). The reactors are located in a greenhouse utilizing natural sunlight as the only light source. Temperature and pH were maintained within an optimal range as found in the experimental work of chapter 3. Growth experiments were performed for almost 6 months, from February until July 2019 to cover all sunlight conditions that can be experienced under the Dutch climate. This chapter describes the first study to show pilot scale *Rhodomonas sp.* production under sunlight conditions and shows the industrial potential of this strain.

Figure 5: The three tubular photobioreactors operated during the pilot-scale experiments with *Rhodomonas sp.* located in the greenhouse in Vlissingen, The Netherlands.

1.5.5 Chapter 6 - General discussion

The data resulting from lab and pilot scale experiments performed in this thesis with *Rhodomonas sp.* is combined with the techno-economic model in chapter 6. By combining the experimental data (chapter 3 and 4) and the techno-economic model (chapter 2) it is possible to determine the most cost efficient production strategy for *Rhodomonas sp.* with artificial light conditions. The efficiency of implementation of a day:night cycle as a cost reduction strategy is evaluated for *Rhodomonas sp.* production. The effect of the proposed cost reduction strategies on the resulting biomass composition in terms of total fatty acid and EPA+DHA content is evaluated. A comparison between laboratory and pilot-scale experimental results is made to evaluate the potential future improvements for *Rhodomonas sp.*. Suggestions to further determine this potential with future laboratory experiments is discussed. Based on all combined results from experimental data and the techno-economic model an outlook for cost efficient microalgae production in aquaculture hatcheries is provided.

Chapter 2

Microalgae production cost in aquaculture hatcheries

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Microalgae production cost in aquaculture hatcheries

Abstract

Microalgae are a crucial part in many aquaculture feed applications processes, mainly in hatcheries. Many aquaculture hatcheries maintain a small scale microalgae production facility in-house for the production of live feed. Microalgae are usually grown in non-automated bubble-column systems at unknown production costs. Other reactor systems or scenarios utilizing artificial light or sunlight and at different scales could result in a more cost efficient production processes. To determine the cost-price and cost-distribution of microalgae production facilities in Dutch aquaculture industry and identify the most efficient cost reducing strategies a techno-economic analysis for small scale microalgae production facilities (25-1500m²) was developed. Commercially available reactors commonly used in aquaculture were compared; tubular photobioreactors (TPBR) and bubble-columns (BC) in two placement possibilities; using artificial light in an indoor facility (AL) and utilizing sunlight in a greenhouse (GH) under Dutch climate conditions. Data from commercial microalgae facilities in the Netherlands are used to model reference scenarios describing the cost price of microalgae production with state of the art technology in aquaculture for a biomass production capacity of 125kg year⁻¹. The reference cost price for algae biomass (on the basis of dry matter) is calculated at €290 kgDW⁻¹ and € 329 kgDW⁻¹ for tubular reactors under artificial light and a greenhouse, respectively and €587 kgDW⁻¹ and €573 kgDW⁻¹ for bubble-columns under artificial light and a greenhouse, respectively. The addition of more artificial light will significantly reduce production costs (by 33%) in all small-scale systems modelled. Biomass yield on light ($Y_{x,ph}$) showed the largest effect on cost price when not considering a different scale of the production process. Process parameters like temperature control should be aimed at optimizing $Y_{x,ph}$ rather than other forms of cost reduction. The scale of a microalgae production facility has a very large impact on the cost price. With state of the art technologies a cost price reduction of 92% could be achieved by changing the scale from 25m² to 1500m², resulting in a cost price of €43 kgDW⁻¹, producing 3992 kg year⁻¹ for tubular reactors in a greenhouse. The presented techno-economic model gives valuable insights in the cost price distribution of microalgae production in aquaculture. This allows to focus research efforts towards the most promising cost reduction methods and to optimize existing production facilities in aquaculture companies to achieve economically sustainable microalgae production for live feed in hatcheries.

2.1 Introduction

In the past decade the use of microalgae for aquaculture has increased because of the importance of microalgae for the quality aquaculture feed [3, 39-41]. The primary source of nutrition for all stages of filter-feeder bivalves and the larval and juvenile stages of fish is microalgae [42] [1, 43]. The quality of feed is linked to mortality rate of fish larvae, development rate, egg viability and reproductive success of copepods among others [32] [44]. It has been shown that using fresh microalgae as feed for oysters leads to higher product quality than what is achieved with processed algae products such as algae paste or flocculates [9]. In addition, literature describes multiple examples where the positive effect of fresh microalgae is more pronounced when feeding a mix of different algal species. Combining multiple different species of freshly produced algae can create an optimized nutritional composition for the use in aquaculture [42].

For a constant supply of fresh microalgae, aquaculture hatcheries generally have an in-house microalgae production facility, which are generally small in scale, up to 100m². These small-scale production facilities traditionally consist of bubble column type reactors (specifically the SEACAPS reactor systems). Recently, more complex, tubular photobioreactor systems have become readily available and have replaced bubble-column in some aquaculture microalgae production facilities. Maintaining a microalgae production facility has been estimated to account for an average of 30% and up to 60% of the total budget of aquaculture hatcheries and nurseries as found in a study by Coutteau and Sorgeloos [10]. This study estimated the cost of microalgae biomass in aquaculture hatcheries at 50-400 USD kgDW⁻¹ depending on the applied scale. These numbers were estimated based on numbers provided by employees operating microalgae cultivation systems in aquaculture applications in The United States of America through a questionnaire in the early 1990s. Despite the high cost prices, no effort has been described in literature to determine the cost price of microalgae in detail and highlight strategies for cost reduction. Microalgae production systems in aquaculture are typically not selected or optimized for a cost efficient production. The type of production systems used (bubble-columns or tubular reactors) in aquaculture usually represent regional or historical preferences [40]. A reduction of microalgae production cost could improve cost-efficiency and therefore viability of aquaculture companies/hatcheries. Other than the study by Coutteau and Sorgeloos (1992) no studies are found in literature focusing on calculating and reducing microalgae production costs for aquaculture or optimizing cost efficiency of microalgae production in small-scale production facilities. Most studies available in literature on microalgae for aquaculture applications focus on the nutritional value of the algae only.

Although algae have gained interest in research and commercial applications in recent years, a techno-economic analysis of small scale algae production facilities such as the

systems applied in aquaculture is not available. Most techno-economic analysis for microalgae production are performed on much larger scales, focusing on the production of bulk chemicals and commodities such as biofuels [13, 14, 45, 46]. Although these studies show the opportunities and bottlenecks of large scale microalgae production, the costs distribution for small scale aquaculture applications will most likely be very different and cost reduction will require different strategies. In addition to scale, the use of artificial light in a closed environment and/or the use of a greenhouse are common practices in aquaculture but have never been analyzed in previous techno-economic studies, which focus on outdoors production using sunlight.

In this study we aim to evaluate production costs and cost distribution of microalgae production in aquaculture hatcheries and provide guidelines for future cost reduction strategies. This is done by developing a new techno-economic model for microalgae production for the scale and production systems as found in this sector and performing a sensitivity analysis.

First, four commercial reference scenarios were created to closely resemble current microalgae production systems in the Dutch aquaculture industry. These scenarios set the base line for the current production costs of microalgae in this sector and allow pinpointing the parameters with major contribution to the cost price. These scenarios are further referred to as the reference scenarios. Secondly, a sensitivity analysis was performed by changing each input parameter individually and assessing the impact on the production costs and cost distribution. This was done to determine what strategies could result in the highest cost price reduction for aquaculture. Finally, the effect of scale of the production facility is assessed in more detail by comparing nine scenarios with production scales ranging from 25m² to 1500m².

2.2 Materials and methods

A techno-economic model was developed to describe algae production costs in aquaculture hatcheries in the Netherlands. A short description of the techno-economic model is given here. A more detailed description of the model can be found in section 2.5.1.

It is assumed that the produced microalgae are utilized by the facility to directly feed marine organisms such as filter feeders without separating algae from the water stream after leaving the bioreactor. No harvesting or downstream processing steps are therefore considered. The techno-economic model uses a set of input parameters to calculate the production costs and cost distribution, among other outputs. An overview of selected inputs is described in Table 1. The total biomass production is calculated based on the total

available light, photon flux density (PFD – $\text{mol m}^{-2} \text{ day}^{-1}$) and the efficiency of light utilization, i.e. the biomass yield on light ($Y_{x,ph}$ – g mol^{-1}). The total cost for production is calculated using the capital expenditure (CAPEX) and operational expenditure (OPEX) required for purchase and operation of the microalgae production facility, respectively. CAPEX includes the investments for purchasing all major equipment and OPEX contains all operational cost such as labor, electricity and consumables. The total biomass production capacity is calculated in kgDW year^{-1} and the total cost in € year^{-1} resulting in a cost price of biomass in € kgDW^{-1} . Each parameter, as listed in Table 1 affects the biomass production cost by impacting the amount of produced biomass, the total cost or both.

Table 1: Summary of input parameters and their input values as used for the reference scenarios. BC: Bubble-column, TPBR: Tubular photobioreactor.

	Artificial light		Greenhouse	
	BC	TPBR	BC	TPBR
Area of production facility (m^2)	30	20	50	35
Artificial lights Type	TL-D 58W	TL-D 58W	None	None
Total artificial day length (hr day^{-1})	24	24	0	0
Lights bag^{-1} / Units m^{-2}	3	8	0	0
Temperature range ($^{\circ}\text{C}$)	20	20	15-25	15-25
Algae-season Start (day)	1	1	1	1
Algae-season End (day)	365	365	365	365
Lifetime of a culture (days)	75	75	75	75
Biomass yield on light (g mol^{-1})	0.22	0.45	0.32	0.45

2.2.1 Reactor type and placement

The types of reactor systems considered in this study are bubble-columns (BC) and tubular photo bioreactors (TPBR). Both systems are based on commercially available reactors applied in aquaculture; SEACAPs (bubble-columns) and LGem Gemtube™ (tubular photobioreactors). Each reactor systems is modelled at two placement options; an indoor facility using artificial lights only (AL) and a greenhouse facility using only sunlight (GH). This results in a total of four possible scenarios, a schematic representation of these four options is shown in Figure 6. The calculated results are specific to the specified commercially available reactor types used in the techno-economic model.

Figure 6: Schematic representation of the four reference scenarios applied in the model. AL: Artificial light, GH: Greenhouse, TPBR: Tubular photobioreactor and BC: Bubble-column

2.2.2 Reference scenarios

Industrial reference scenarios were established based on input from the producer and users of microalgae production systems in aquaculture. Two scenarios, GH-BC and AL-BC, were monitored for over 1.5 years for the collection of data on $Y_{x,ph}$, labor requirements, produced amount of biomass and energy requirements. The equipment used in these scenarios and data obtained from the monitoring of these systems was used to determine the inputs for the reference scenarios in the techno-economic analysis.

The production capacity of all reference scenarios was set at 125kg year⁻¹ as based on the Dutch aquaculture reference scenarios. This biomass production capacity was achieved for each of the calculated scenarios by adjusting the size of the facility (m²) based on the total available light and biomass yield on light ($Y_{x,ph}$) used in each scenario, see Table 1. The total artificial light available was set at 580 $\mu\text{mol m}^2\text{s}^{-1}$ for the two AL scenarios, based on data from industry. The lifetime of a culture, the production period between starting and cleaning a reactor, was determined from the monitoring data and is different for each scenario. The total available sunlight for GH-scenarios was based on meteorological data for a typical meteorological year for Vlissingen, The Netherlands (Meteonorm 7.3.0). It is assumed that microalgae production is performed all year round for all reference scenarios. The temperature was set at a fixed value of 20°C for the AL-scenarios. A temperature range of 15-25°C was used for GH-scenarios.

2.2.3 Biomass yield on light

BC-systems operated in aquaculture industry were monitored for over 1.5 years to obtain reliable estimates for $Y_{x,ph}$ for the reference scenarios. The average $Y_{x,ph}$ for the BC-systems was 0.22 g mol⁻¹ for AL-BC and 0.32 g mol⁻¹ for greenhouse placement (GH-BC) (for details see section 2.5.1.1). For TPBR reference scenarios the $Y_{x,ph}$ was estimated from data obtained from the AlgaePARC pilot facility data available in literature [47]. The $Y_{x,ph}$ for the reference scenarios using TPBR-systems is set at 0.45g mol⁻¹. The same value was used in both placements (AL and GH) for TPBRs.

2.2.4 Sensitivity analysis

A sensitivity analysis was performed to determine the effect of the different parameters on the cost price of biomass. A total of seven changes were tested on each of the four reference scenarios. The seven changes are: 1 available light, 2 temperature range, 3 biomass yield on light increase ($Y_{x,ph}$), 4 reduced downtime, 5 reduced labor requirements, 6 seasonal production of algae and 7 a combination of scenarios 1-5. A summary of the changed parameters and the applied values can be found in Table 2. To investigate the effect of each parameter individually, each parameter was changed separately while keeping all other parameters identical to the reference scenario. The parameter with the largest impact on cost price (scale) was not included in the main sensitivity analysis but is investigated in more detail separately.

Table 2: Changes made to individual input parameters for each of the seven sensitivity analysis scenarios. AL: Artificial light, GH: Greenhouse, TPBR: Tubular photobioreactor and BC: Bubble-column

	Parameter changed from reference scenario	Artificial Light		Greenhouse	
		BC	TPBR	BC	TPBR
1. Additional AL	Lights bag ⁻¹ / Units m ⁻²	6	16	2	5.5
2. Temperature range	Temperature range (°C)	-	-	10-30	10-30
3. Y _{x,ph} +66%	Biomass yield on light (Y _{x,ph})	0.37	0.75	0.53	0.75
4. Reduced downtime	Lifetime of a culture (days)	150			
5. Labor -33%	-	-33% total labor hours			
6. Seasonal production	Start/End day	60-335			
7. Combined	-	Combined 1-5			

2.2.4.1 Sensitivity analysis inputs

The available light is doubled for the artificial light scenarios from 580μmol/m²/s (reference scenarios) to approximately 1200μmol/m²/s (sensitivity analysis). The available light in greenhouse scenarios is changed by applying additional artificial light in the hours of the day with low sunlight availability. The artificial light is applied to create a total period of light availability of 20h per day. The artificial light is applied from 1.5hours before sunset until 1.5h after sunrise while maintaining a 4hour dark period in between. For GH-BC scenarios 2 TL-D 58W lights were added per BC-reactor, as typically applied in aquaculture industry. For GH-TPBR scenarios, lights are added to reach an equal light intensity per unit floor area as for the GH-BC scenario (399μmol m⁻² s⁻¹). The temperature range for greenhouse scenarios is increased from 15-25°C to 10-30°C. The temperature range was not changed for artificial light scenarios as these indoor scenarios are assumed to operate at a constant temperature in our calculations. The sensitivity analysis related to production season describes a scenario where the algae production facility is not operated during meteorological winter but only during the 60th until the 335th day of the year. The biomass yield on light (Y_{x,ph}) increased by 66% from the reference scenarios. Culture lifetime describes the length (in days) of operation of a reactor between inoculation and shutdown for cleaning procedures. The culture lifetime is doubled, from 75 days to 150 days. Labor is reduced by 33% to assess potential effects of automation on the cost price. Finally a combined scenario was created combining scenarios 1-5.

2.2.5 Effect of scale

The factor with the largest impact on cost price according to literature is the scale of production [13, 48]. The effect of scale on small scale systems for aquaculture is assessed by comparing a total of 9 scenarios (the four reference scenarios the 4 combined scenarios of the sensitivity analysis and a GH-TPBR-NoAL scenario) on 5 different scales each (25-100-250-750 and 1500m²). The cost price of microalgae is evaluated as cost price per unit dry-weight (€ kgDW⁻¹) as a function of the total biomass capacity (kg year⁻¹) of the modelled scenarios to allow for direct comparison between the different systems.

2.3 Results and discussion

2.3.1 Reference scenarios

The results of cost price and biomass capacity for the reference scenarios in the techno-economic analysis are shown in Figure 7. Detailed results and cost distribution can be found in section 2.6. The lowest overall production cost for two placement options (GH and AL) is found with the TPBR-scenarios in both scenarios. A cost price of €290 kgDW⁻¹ is found for the AL-TPBR scenario and €329 kgDW⁻¹ for the GH-TPBR scenario. These results clearly show a cost price advantage for the TPBR-systems over the BC-systems in the reference scenarios. The BC-scenarios result in a cost price of €587 kgDW⁻¹ (AL-BC) and €573 kgDW⁻¹ (GH-BC). These cost prices are in the order of 100x higher than numbers described by Ruiz et al. but describe algae production on a much smaller scale then number described by Ruiz at 100ha scale [13]. The results correlate well with the findings of Sorgeloos who already described this much higher cost price of algal biomass on the small scale of aquaculture hatcheries [10, 13]. Even though the total cost price is similar between placements for each reactor system, the cost price distribution between CAPEX and OPEX shows differences between GH and AL scenarios for one reactor type. In the reference scenarios OPEX always contributes to the largest part of the cost compared to CAPEX; 82%, 74%, 78% and 60% for AL-BC, AL-TPBR, GH-BC and GH-TPBR scenarios respectively. As most of the cost price is attributed to OPEX, there is a large potential for cost-price reduction by reducing these operational costs. Adjusting operational procedures towards more cost efficient operation is considered more feasible than lowering the CAPEX for major equipment. Major equipment cost, contributing to most of the CAPEX, is expected to decrease with increasing microalgae facilities and newer technologies. However, this is not a factor that can be directly influenced by aquaculture or at current day microalgae production systems and it is therefore not considered in the cost price reducing strategies described in our study.

A large cost component for energy is found for the AL-scenarios with 31% and 27% of the total cost price for AL-BC and AL-TBPR attributed to energy, respectively. In the corresponding GH-scenarios the energy component is a much smaller fraction of the total cost price (10% for GH-BC and 3% for GH-TPBR). The higher energy component for the AL-scenarios is the direct result of the electricity consumption of artificial light. Even though the energy use is significantly lower in GH-scenarios, almost the same cost price is obtained for the biomass when comparing the same reactor system in the AL and GH scenario. This is explained by the lower average biomass production rate found for the GH-scenarios due to the lower total amount of available light over a full year, resulting in a larger required reactor capacity for a biomass production of 125 kg year⁻¹. This results in an increased CAPEX for the GH scenarios and an increased OPEX, the latter mainly due to an increase in labor requirements per kg of biomass. These results indicate the economic feasibility of producing microalgae using artificial lights at the small scale for aquaculture whereas the use of artificial light at large scale is typically regarded unfeasible [49].

The lowest cost price for the reference scenarios is found for the TPBR-systems for both reactor placements. Tubular photobioreactors result in 51% and 43% lower production costs than the AL-BC and GH-BC scenarios, respectively. This result is somewhat surprising as TPBRs are generally regarded as more complex and more expensive than the simpler BCs. Our results can be explained by the way these systems are implemented in the aquaculture industry. The initial investment of a BC-facility is, in our model, approximately 2.200 € m⁻³ of reactor volume excluding additional equipment. A TPBR production facility as modelled in the reference scenarios has 1 or 2 reactors with an investment cost of 28.000 € m⁻³. The reference TPBR-scenarios require a total investment of €143.321 and €217.443 in the AL and GH settings, respectively. The initial investment of the reference BC-scenarios is higher for these scenarios (€183.001 and €232.364 respectively). Comparing the investment required per reactor volume the BCs appear much cheaper, but the total investment required for equal biomass capacity, as used in our reference scenarios, results in higher costs for in the BC-scenarios. The total reactor volume required for the AL-BC systems is 24 times larger than for the AL-TPBR application and for the GH-BC scenarios 20 times larger than for the GH-TPBR. This large difference in required volume is the direct effect of the differences in reactor design and is typically overlooked when comparing different reactor systems without applying a techno-economic analysis. In the reference scenarios the BC-scenarios are modelled with a lower $Y_{x,ph}$ (g mol⁻¹) than the TBPR-scenarios, resulting in a lower biomass production rate (g m² day⁻¹) on equal amounts of light (mol m² day⁻¹). This lower $Y_{x,ph}$ is the result of a larger light path, applying lower biomass concentration and the lower level of control in the BC-system. The lower $Y_{x,ph}$ combined with the much larger reactor volume per unit floor area (m³ m⁻²) of BCs compared to TPBRs means that a much larger reactor volume is required for BCs to obtain equal biomass production (kg year⁻¹).

Reference scenarios are compared on equal biomass capacity rather than reactor volume or size of the production facility to allow for a fair direct comparison of the biomass production cost highlighting the difference in required reactor volume for equal production capacities.

Even though the calculations here show that BC-systems a more expensive method for algae production, most aquaculture companies apply these systems. An important reason for aquaculture companies not to opt for TPBR systems might be the flexibility of the systems. Aquaculture often requires multi-species diets for feed applications [42]. A multi-species diet cannot be achieved with a facility of one or two reactors as described in the TPBR reference scenarios. In addition, operating a small number of reactors represents a higher risks of losing the production when one reactor needs to be shut down for maintenance or cleaning. To assess a multi-species production using TPBR-systems a scenario was tested with equal biomass capacity but with more, and therefore smaller, tubular reactors under artificial light. This results in a scenario with 7x 250L reactors compared to the single 750L system applied in the AL-TPBR reference scenario. Results showed a production capacity of 134kg year⁻¹ at €606 kgDW⁻¹. Showing slightly higher cost prices than the BC-system in the reference scenario (121kg year⁻¹ at €587 kgDW⁻¹ for AL-BC) indicating an advantage for BC-systems when a strict multispecies diet is required on this small scale. Moreover, BC-systems have been common practice for algae production in aquaculture hatcheries for many years whereas TPBR-systems have only become commercially available recently.

The price advantage found for the TPBR-systems in the reference scenarios could be partially due to the selected inputs used. The selected biomass yield on light ($Y_{x,ph}$) for TPBR-systems is higher than for the BC-scenarios. To test the impact of the biomass yield on light, the $Y_{x,ph}$ of the AL-TPBR reference scenarios was decreased from 0.45 to 0.22 g mol⁻¹, the lowest value applied for BC-systems. The decrease of $Y_{x,ph}$ was combined with an adjusted floor area (from 25m² to 40m²) to result in an equal total biomass capacity as the reference scenarios (124kg year⁻¹). A scenario with one 1500L reactor shows a biomass production cost of €401 kgDW⁻¹ whereas a scenario of 2x 750L reactors results in a cost price of €438 kgDW⁻¹ at this low value for $Y_{x,ph}$. When operating a fully controlled TPBR in a commercial setting the low value for $Y_{x,ph}$ (0.22 g mol⁻¹) is unlikely, especially in a highly controlled setting as described in the AL- scenarios. Nevertheless, this comparison shows that at equal biomass production capacity (124 and 121kg year⁻¹) and equal $Y_{x,ph}$ (0.22g mol⁻¹) the cost price of biomass in the TPBR-systems is lower than for BC-scenarios. This shows that even when operating at low $Y_{x,ph}$ the TPBRs yield the most cost efficient production strategy even though the disadvantage of not having a multispecies diet remains.

Figure 7: Cost price of microalgae production, divided over 10 categories. Left half: four reference scenarios. Right half: combined scenarios from the sensitivity analysis excluding the effect of scale (bars – primary axis – € kgDW⁻¹). Total biomass capacity of each scenario (kg year⁻¹) is represented by the diamonds (secondary axis). At scales of 20-50m². AL: Artificial light, GH: Greenhouse, BC: Bubble-column and TPBR: Tubular photobioreactor.

2.3.2 Sensitivity analysis

The results of the sensitivity analysis display the effect of each input parameter on the cost price. The results of cost price reduction (in % from the reference scenarios) are presented in Figure 8, with more detailed values for the combined scenarios (7) available in Figure 7 and section 2.6.2.

2.3.2.1 Additional artificial light

The results in Figure 8 show that additional artificial light has a large impact on the cost price for all systems. A cost price reduction in biomass production is found for all four scenarios with additional artificial light. Doubling the artificial light in AL-scenarios doubles the total biomass production per year while adding costs for the lights in both CAPEX and OPEX. This results in a total cost reduction of 36% for AL-BC ($\text{€}375 \text{ kgDW}^{-1}$) and 35% for AL-TPBR ($\text{€}188 \text{ kgDW}^{-1}$). An equal cost price reduction is found for the GH-scenarios with 36% for GH-BC ($\text{€}364 \text{ kgDW}^{-1}$) and 35% GH-TPBR ($\text{€}212 \text{ kgDW}^{-1}$). For the GH-scenarios the total biomass output is increased by 70% and 72% for the GH-BC and GH-TPBR scenarios, respectively, instead of doubled as for the AL-scenarios. Even though the biomass output is not doubled the same percentage cost price reduction is found.

Although algae production utilizing artificial light has been described in literature to not be cost efficient [49], the addition of extra artificial light on the small scales modelled here reduces the overall cost price for all scenarios. Even though the additional artificial light increases the total CAPEX and OPEX, a cost price reduction is obtained due to the large increase in total biomass output. The additional cost for the added artificial light is low compared to cost price of biomass in the reference scenario. The OPEX of artificial light per unit biomass is a function of the biomass yield on light ($Y_{x,ph} - \text{g mol}^{-1}$), the PAR efficiency of the light source ($\mu\text{mol W}^{-1}$) and the price of electricity (€ kWh^{-1}). The CAPEX of artificial light per unit biomass is a function of the cost price of the light source (€ unit^{-1}), the biomass yield on light ($Y_{x,ph} - \text{g mol}^{-1}$) and the lifetime of the light source (hours) [49]. In our study the lights applied are TL-D 58W fluorescent lamps with a PAR efficiency of $1.25 \mu\text{mol W}^{-1}$ and a lifetime of 15.000 hours and using the $Y_{x,ph}$ of the reference scenarios ($0.22\text{-}0.45 \text{ g mol}^{-1}$). This results in a cost price for artificial lights between $\text{€}86$ and $\text{€}42 \text{ kgDW}^{-1}$ biomass for the tested scenarios. The cost of artificial light is significantly more than the numbers described by Blanken et al. (2013) who describes additional cost of $\text{\$}26.7 \text{ kgDW}^{-1}$ (approximately $\text{€}21 \text{ kgDW}^{-1}$ in 2013). The difference can be explained by the differences in assumptions used. Blanken used an average PAR efficiency of $1.67 \mu\text{mol W}^{-1}$, a lifetime of 50.000 hours for LED lights and a $Y_{x,ph}$ of 1.0 g mol^{-1} (compared to $0.22\text{-}0.45 \text{ g mol}^{-1}$ in our work) resulting in a lower price of artificial light per unit biomass.

Blanken described that the use of artificial lights is too expensive for microalgae production from a perspective of bulk chemical production. The added price of artificial light in aquaculture applications however does result in a cost reduction for all reference scenarios, due to the high initial biomass production cost price on the small scale applied. The addition of artificial light can be regarded as an easy to implement and cost effective method for cost price reduction in existing microalgae production facilities in aquaculture if the reference cost price is higher than the cost of additional light. The use of highly efficient light sources such as LEDs, combined with higher a $Y_{x,ph}$ will make the use of artificial light even more economically interesting for small scale algae production than shown in our results.

In the scenario with additional light it was assumed that the $Y_{x,ph}$ remains unchanged from the reference scenarios. However, literature describes a negative correlation between light intensity and biomass yield on light. Increased light intensities typically result in a lower photosynthetic efficiency as was shown by results of pilot-scale systems by de Vree [47, 50]. On the other hand the very controlled method of light application in an artificial light scenarios allows for better control of the culture and potential higher $Y_{x,ph}$. In reality the cost reduction by additional artificial lights could therefore be smaller than shown in the modelled AL-scenarios. Assuming a 25% decrease of $Y_{x,ph}$ for doubled light intensity scenarios shows a cost price decrease of 14-15% in the modelled scenarios, still showing the positive effect of more light. This effect will be different on GH-scenarios as there the additional artificial light is added during dark periods and therefore does not double the incident light intensity on the algae cells.

2.3.2.2 Temperature range

The temperature range was only tested for the GH-scenarios. In AL-scenarios a constant temperature of the indoor facility is assumed and cooling requirements are based on heat production by the artificial lights. For the GH-BC scenario, reactors are not actively controlled for temperature and a change in the temperature range does therefore not affect the cost price. In the GH-TPBR scenario the temperature of the systems is actively controlled. The larger temperature range results in a reduced cost price, but only by a very small percentage at only 0.36% (€328 kgDW⁻¹). This result seems contradicting with literature as Ruiz et al. showed that temperature was one of the main influencing parameter on the cost price of biomass [13]. Ruiz describes a 12% cost price decrease for a scenario with increased temperature tolerance (30-45°C) for flat-panel PBRs located in the South of Spain. The cost price reduction for our calculations with increased temperature range is €1.20 kgDW⁻¹ from a base price of €329 kgDW⁻¹ (0.36%) where Ruiz described a cost price reduction of €0.40 kgDW⁻¹ on a base cost price of €3.40 kgDW⁻¹ (11.7%). The cost price

reductions in € kgDW⁻¹ is of comparable order of magnitude between the different scenarios and the difference could be attributed to actual temperature range differences, location (solar input, wind speeds, etc), electricity cost, reactor geometry and more. The cost reduction in our calculations is smaller due to the much higher cost price for biomass used as reference. Algae growth at temperatures other than the optimal growth temperatures will result in a negative effect on the biomass on light [51]. It is therefore concluded to be more cost efficient on these small scales to optimize temperature control towards optimized $Y_{x,ph}$ rather than energy reduction.

2.3.2.3 Biomass yield on light

Biomass yield on light ($Y_{x,ph}$) has a large effect on the cost price as this directly impacts the total amount of biomass produced. The same effect of $Y_{x,ph}$ is found for all systems: by increasing the biomass yield on light, the total amount of biomass produced is increased without adding costs in our calculations. These results show the importance of operating a microalgae facility using optimized growth parameters and maximizing $Y_{x,ph}$. The $Y_{x,ph}$ found for the monitored systems (0.22-0.32 g mol⁻¹) is much lower than values available in literature. Examples in literature describe 0.75-0.80 g mol⁻¹ on experimental reactors [52], 0.79 g mol⁻¹ in pilot-scale TPBRs in Spain [45], and values in the range of 0.2-1.0 g mol⁻¹ in pilot-scale TPBRs in the Netherlands depending on reactor operation, dilution rate, biomass concentration and the photon flux density [47, 50]. The most relevant numbers for comparison are those described by de Vree et al on a pilot plant TPBR-system under the Dutch climate. The systems described by de Vree are more advanced and were better controlled than the commercial BC-systems used for data collection, explaining part of the difference in $Y_{x,ph}$ described. De Vree describes data produced in reactor systems operated in chemostat or turbidostat at constant pH, monitored and controlled by computer systems. The commercial BC-systems operate without pH control in chemostat mode with a dilution rate that is manually adjusted per reactor. Nutrients are equal for all strains and adjustments are made manually rather than computer controlled. The gap between $Y_{x,ph}$ from literature and the monitoring data of commercial aquaculture systems indicates a large potential for improvement of the aquaculture production systems.

Literature describes how optimizing growth parameters can increase biomass productivity and biomass yield on light [53, 54]. Optimizing growth parameters such as light and temperature for the species in use or adjusting the harvesting regime and nutrient addition can result in higher $Y_{x,ph}$ with limited or no additional cost. Operating at optimized growth conditions requires controllable reactor systems, favoring the AL-TPBR scenario. In such scenarios, each reactor can be operated at a different temperature, light intensity, pH,

nutrient concentration and harvesting regime optimizing growth parameters for different species in one facility.

It has to be noted that solely aiming for the highest $Y_{x,ph}$ would not yield the most cost efficient system. Maximum $Y_{x,ph}$ is typically found at low light levels as was indicated in the data by de Vree [47]. Biomass production cost is a function of the total biomass capacity (kg) and total cost (€) of a production facility. Increasing $Y_{x,ph}$ should only be the aim if it leads to a larger total biomass production without substantially increasing operational cost. For each change to any scenario a breakeven for added cost (CAPEX and OPEX) vs additional biomass produced could be determined. Such breakeven scenarios requires complete understanding and quantification of the biological impact of any changes for the produced species. This biological information coupled to the techno-economic model could allow to determine the effect of specific changes of the operation parameters on the cost price in detail. Research focused on understanding the algal species used in aquaculture application and optimizing the applied growth parameters for most cost efficient production is therefore one of the most promising cost reduction methods for small scale microalgae production but requires more effort than other cost reduction strategies.

2.3.2.4 Reduced downtime

The lifetime of a culture describes the production period that can be obtained between starting a new reactor and stopping the system to be cleaned. This parameter is only relevant for continuous systems as described in our modelled scenarios and does not apply to batch operation. Maintaining a culture for longer periods without cleaning the reactor systems reduces production cost price due to reduced labor requirements with increased biomass production as a result of the lower downtime. The results of a doubled culture lifetime (75 to 150 days) show a reduced production cost by 1.8% and 3.0% for the AL-BC and GH-BC scenario and 3.8% and 4.1% for the AL-TPBR and GH-TPBR scenarios, respectively. The largest impact of culture lifetime is found for TPBR-systems as the downtime for cleaning and restarting a new culture in TPBR-system is much larger than for BC-systems.

The relatively small effect of reduced downtime on the production cost can be explained by the relatively long culture life assumed in the reference scenarios. The reference of 75 days was determined as the average from the monitoring data of commercial AL-BC and GH-BC systems. The data showed a large variation for culture lifetimes ranging from 10 to 200 days for the same system between different reactor runs – this behavior was observed for both the AL-BC and GH-BC reactors systems. With the large fluctuations in culture lifetime observed in the commercial systems used in our input data this parameter is more

important that initially suggested by the modelled scenarios. In commercial applications it is found that a reactor usually significantly decreases in productivity and observed $Y_{x,ph}$ before a reactor is stopped for cleaning. This reduction of efficiency was also described by de Vree et al who suggests inline cleaning of TPBR-systems could help prevent this loss of efficiency [47]. Our monitoring data showed that cultures that were recorded with shorter and longer than average culture lifetimes were linked to lower than average values for $Y_{x,ph}$, indicating a trade-off between maintaining a culture longer at a lower $Y_{x,ph}$ and cleaning a reactor to restart a new culture.

2.3.2.5 Labor

Labor is the largest cost price component for all reference scenarios, representing between 46-65% of total cost. The results of the sensitivity analysis with labor requirements reduced by 33% show a cost price reduction of 16.6% and 22.2% for the AL-BC and GH-BC scenarios and 15.6% and 19.4% for the AL-TPBR and GH-TPBR scenarios. The largest effect of reduced labor is found for the GH-scenarios as these scenarios describe systems containing a larger number of total reactors to reach the total biomass capacity of the reference scenario than their AL counterparts (80 reactors for AL-BC and 134 for GH-BC and 1 vs 2 TPBRs for AL-TPBR and GH-TPBR). More reactor capacity results in a larger labor requirement and therefore a larger effect of labor reduction on these scenarios is found. In practical applications reduced labor costs would typically be obtained by the implementation of more automation for example to harvesting, nutrient addition and artificial light systems, among others, typically linked to higher CAPEX which has not been regarded in the above calculations for cost reduction. A scenario with highly automated production facilities is more feasible for the TPBR-systems than for BC-system. TPBR-systems already have an automated pH and temperature control integrated but lack automatic harvesting. Applying an automated harvesting system to operate the reactors in turbidostat or chemostat mode would create more constant culture conditions, especially under artificial light conditions potentially also resulting in an increased $Y_{x,ph}$. A breakeven point between additional automation and labor reduction could be determined using the presented model, but would be different for every scenario.

2.3.2.6 Seasonal production

The scenarios in greenhouses under the Dutch climate show a very low biomass productivity in winter months due to the low light availability. The scenarios simulated with no production in winter show a reduction of the microalgae production costs of 12.6% and 7.9% for the GH-BC and GH-TPBR scenarios, compared to production during all days of the year. For AL-

scenarios a cost price increase (negative effect on cost price) is found as lower total biomass capacity results in a larger cost component for the depreciation of major equipment per unit of biomass produced. The results for GH-scenarios do show a cost price reduction indicating that production costs during winter are significantly higher than the rest of the year. The costs for operation do not outweigh the small amount of biomass produced in these months. The modelled scenarios without production in winter shows a reduction of only 7.2% of the total biomass capacity compared to the reference scenarios (9 kg for GH-BC and 8 kg GH-TPBR) while reducing the operational time by 24.6% . In practice, microalgae are required all year round, most commercial application in aquaculture therefore apply artificial light when low levels of sunlight are available. The results of GH-scenarios utilizing additional artificial light are described in more detail in the artificial light section of the sensitivity analysis (2.3.2.1) and showed a total cost price reduction of almost 40% while maintaining year round production.

2

Figure 8: Results of the sensitivity analysis; the effect on cost price, as a % in cost reduction from the reference scenarios for changes made to the scenarios. AL = Artificial light, GH = Greenhouse, BC = Bubble-column and TPBR = Tubular photobioreactor

2.3.2.7 Combined scenario

A scenario described a combination of additional artificial light, temperature range (where applicable), increased $Y_{x,ph}$, reduced downtime and reduced labor serve as an outlook on possible cost reductions for small scale aquaculture microalgae production facilities in the nearby future. The parameter seasonal production did not show a cost price reduction when combined with other parameter changes and is therefore excluded from the combined scenario. A total cost reduction of 67-70% compared to present practice can be obtained (see Figure 7 and section 2.6.2). This future outlook on small scale commercial systems shows a cost price of €96 kgDW⁻¹ for AL-TPBR and €101 kgDW⁻¹ for the GH-TPBR scenarios and €195 kgDW⁻¹ and €170 kgDW⁻¹ for AL-BC and GH-BC, respectively. Part of the cost price reduction is achieved by methods that increase the total biomass productivity of the system while other parameters directly impact the total cost of operation on these systems as described in their individual chapters. The results show that a very large cost price reduction could be achieved with the combined parameter optimization. It has to be noted that the combination of all these cost reduction methods combined might be difficult to obtain as combining more light, with lower labor requirement and an increased temperature range while achieving a higher $Y_{x,ph}$ could be challenging without additional costs.

2.3.2.8 Scale

The scale of an algae production facility has a large impact on the cost price. The total effect of scale on the cost price for each scenario is compared by comparing scenarios at equal floor area. The results for the effect of scale are summarized in Figure 9, showing the cost price as a function of total biomass capacity (kg year⁻¹) for each of the scenarios. Detailed values for biomass production cost and biomass capacity for each scenario can be found in section 2.6.4.

The largest impact on the biomass production cost is found for TPRB-systems whereas the results related to scale are highly dependent on the selected inputs from the starting reference scenario. Therefore, and because it is a parameter with a high impact, the effect of scale was analyzed in more detail for a total of 9 scenarios. This analysis shows the potential cost price reduction that could be achieved when producing algae for multiple aquaculture facilities in one centralized algae production facility. Four scenarios show the cost price at different scales for the reference scenarios (solid lines in Figure 9). The scenarios with combined optimization of the sensitivity analysis (1-5) are used for a future outlook on biomass cost price at larger scales (dotted lines in Figure 9). Additionally a GH-TPBR scenario using the combined cost reduction methods from the sensitivity analysis is

modelled without the addition of artificial light (GH-TPBR-NoAL) in order to indicate the breakeven point for the use of artificial light in a GH-TPBR scenario.

A large difference in scalability of the two reactor types is found in the results of Figure 9. The scalability is mostly dependent on the reactor type (BC vs TPBR) and not reactor placement (AL vs GH). Both reactor types show a different effect of scalability on the cost price. For the BC-reactors an effect of scalability is observed in the smaller sizes but this reaches a minimum cost price for the applied scenario and barely decreases with increased scale above 100m². The TPBR-scenarios keep decreasing the biomass cost price for each subsequent larger scales. For the BC-scenarios the total number of reactors increases linearly with increased ground area.

Figure 9: Cost price of biomass (€ kgDW⁻¹ y-axis, logarithmic scale) represented over the total biomass production (kg year⁻¹ x-axis) of 9 different scenarios for 6 scales (25-100-250-500-750-1500m²), each subsequent marker represents the next scale size. Solid lines represent reference scenarios, dotted lines show scenarios described as combined scenarios of the sensitivity analysis. BC = bubble-columns (circles), TPBR = Tubular photobioreactors (squares), AL = artificial light (black), GH = Greenhouse (grey). GH-TPBR-NoAL represents the same scenarios as GH-TPBR with combined scenarios of the sensitivity analysis but without the addition of artificial light. For combined scenarios some scales produce more biomass than the maximum represented 10.000kg year⁻¹; detailed numbers can be found in the supplementary files.

The BC-reactors have an investment cost of 2.200 € m⁻³ and this does not change with increased scale. The TPBR-systems are commercially available in different sizes with a steep decrease in investment per volume unit (€ m⁻³) when moving to larger systems. The TPBR-systems considered in this model include reactor sizes of 250, 750, 1.500 and 18.000 liter culture volume. Investment cost per volume unit of these systems ranges from 120.000 € m⁻³ for the 250L-systems to 8.000 € m⁻³ for the 18.000L reactors.

Labor requirements scale differently for the reactor systems as a result of the way the reactor types scale up. Labor requirements for the BC-systems are defined to have a decreased average labor requirement per reactor scaling up to 160 reactors in our study. For more than 160 BC-reactors the labor requirements per reactor remain the same at 0.014 h reactor⁻¹ day⁻¹.

This amount of labor only includes normal culture maintenance, labor requirements for cleaning and culture replacement is calculated separately depending on culture lifetime. Labor requirements for the TPBR-systems is based on a fixed amount of hours of labor required reactor⁻¹ day⁻¹. TPBRs at the 250-1500L scales require 0.25 h reactor⁻¹ day⁻¹ whereas the 18.000L system requires 0.5 h reactor⁻¹ day⁻¹ for normal operation. The labor requirements for cleaning procedures for the 250-1500L systems was set at 1.5 h reactor⁻¹ per clean and for the 18.000L system at 3.0 h reactor⁻¹.

The largest effect of scale for BC-systems is found for the smallest production facilities. A strong price decrease is found for scales increasing up to about 500 kg year⁻¹ (25-250m²). After an initial price decrease the BC-systems do not show a significant scale effect. It has to be noted that for the BC-systems large scale scenarios are not considered practically feasible and are only calculated to show the effect of scale. In real world applications it is not advised to operate a facility with more than 160 BC-reactors of the type specified in this study, due to practical difficulties and the high cost price associated to this system at large scale compared to other production methods available, as also shown in the results. The BC-systems, used in this study, are primarily designed as small scale production systems for use in aquaculture.

For TPBR-systems, scale-up significantly decreases biomass production cost. Comparison of all TPBR-scenarios at an equal biomass capacity of 4000 kg year⁻¹ shows the lowest cost price at the reference scenario for tubular reactors (GH-TPBR-Base in Figure 9) at the largest tested scale (1500m²). This GH-TPBR-Base-1500m² scenario describes 2x 18.000L reactors to produce biomass at €43 kgDW⁻¹ using state of the art technology. The AL-TPBR scenario with combined optimization obtains an equal biomass capacity at the 500m² scale. This smaller scenario contains 14x 1500L reactors at a biomass production cost price of €45 kgDW⁻¹. The comparison between these two scenarios shows the large impact scale has on

the cost price in TPBR-systems, without optimization with current day technology the lower cost prices can be obtained at a larger scale.

The results show that all TPBR scenarios on the 1500m² scale utilizing the 18.000L reactors result in a large cost price reduction compared to the previous scales with 1500L systems. The same pattern is found between scenarios utilizing the 750L and 1500L systems and all other steps with a change of reactor scale. This is the result of the steep decrease in investment cost per unit reactor volume and the decrease in labor requirement between operation 2 or 14 reactors.

The overall lowest biomass production cost for the scenarios studied is found for the GH-TPBR scenarios not utilizing artificial lights (GH-TPBR-NoAL) on the largest scale (1500m²) producing 6.892 kg year⁻¹ at €23,47 kgDW⁻¹. The addition of artificial lights on a larger scale under more optimized conditions yields higher biomass cost price while producing much more biomass (11.822 kg year⁻¹ at €26,35 kgDW⁻¹ for the GH-TPBR scenario). This shows that when moving to larger scales the addition of artificial light will not always result in a cost price reduction as it does for the small scale. At the 1500m² scale the cost of artificial light exceeds the relatively low cost price of biomass, supporting the results as described for the sensitivity analysis on additional artificial light.

Optimizing the scale of a microalgae production facility shows a big potential for cost reduction. The results show that for the reference scenarios the cost price could be reduced by 21-24% for BC-scenarios and 55-65% for TPBR scenarios by simply increasing the operation from the reference scenarios of 25-40m² to 250m² and much further when moving to 1500m². Most aquaculture applications do not require the amount of biomass that is produced at these scales. A specialized algae facility providing biomass for multiple aquaculture applications could produce at a larger scale, reaching much lower production prices than the smaller individual systems. The scenarios describing the lowest biomass production cost in this comparison, GH-TPBR-NoAL at €23,47 kgDW⁻¹ and GH-TPBR €26,35 kgDW⁻¹, produce 55-100x the biomass capacity as described in the reference scenarios for aquaculture. More realistic near term prediction can be found in the result for GH-TPBR-Base at 250m². This scenario describes a facility producing 762 kg year⁻¹ at €123 kgDW⁻¹ without further improvements of state of the art technologies other than a scale increase.

2.4 Conclusion

The cost price of small scale industrial microalgae production facilities as presently used in the aquaculture industry was evaluated using a techno-economic analysis. Modelled scenarios based on current commercial standards showed that microalgae biomass cost prices are currently between €587 kgDW⁻¹ and €290 kgDW⁻¹. Two of the most applied commercial systems were compared (bubble columns vs tubular reactor systems) showing a significant price advantage for the tubular reactor systems in all tested scenarios. The potential for cost price reduction based on state of the art technology and operation can be significant, up to 90% cost reduction can be achieved from the reference scenarios. From the tested scenarios, the addition of artificial light, increasing $Y_{x,ph}$ and reducing labor requirements showed the largest potential for cost price reduction when not changing the scale of the production facility. For existing production facilities the easiest strategy for cost price reduction is the addition of artificial light, yielding a cost reduction of 35-36% for all tested scenarios. Other cost price reducing strategies should focus towards higher efficiency of the available production methods, thereby increasing the $Y_{x,ph}$. This could be achieved by optimizing growth parameters for the produced strains by optimizing temperature, light, pH, nutrient availability and harvesting strategies in the applied systems. Changes in temperature should focus towards improved growth rather than cost reduction for energy consumption.

The size of the facility shows the largest impact on the cost price of microalgae, but a larger scale could be difficult to implement in most existing facilities due to a lack of biomass requirements. A centralized microalgae production facility providing high quality microalgae for multiple aquaculture applications could decrease the cost price by 60-80% when producing 762 kg year⁻¹ at €123 kgDW⁻¹ on a 250m² scale, compared to the 125 kg year⁻¹ in the reference scenarios. This larger scale is still considered to be a very small production facility, but does show a large reduction of overall biomass production cost. On an even larger scale (1500m²) a cost price of €43 kgDW⁻¹ is feasible with state of the art technology and future improvements can reduce this to €26,40 kgDW⁻¹ on the large 1500m² scale utilizing TPBR-systems. The large cost reduction that could be achieved in such a facility could have a large impact on aquaculture production processes in the future.

2.5 Supplementary files

2.5.1 Techno-economic model.

A techno-economic model was developed for microalgae biomass production for small commercial scale, specific for the aquaculture industry (50-250m²). The model is based on available production data from commercially available reactor systems used in Dutch aquaculture industry. Where applicable assumptions specific to aquaculture hatcheries applications are based on these production facilities, these assumptions are referred to as industrial standards or as based on aquaculture industry. Where needed, assumptions as described by Ruiz et al were used [13]. The techno-economic model is set up in a way that allows projection of scenarios for either a greenhouse (GH) using sunlight only or with additional artificial light or an indoor setting with artificial lights only (AL). The two commercially available reactors systems selected are: tubular reactor systems (TPBR – LGem Gemtube™ PBRs) and bubble-column reactor systems as commonly applied in aquaculture (BC – SEACAPs reactor systems).

The model uses selected input parameters which are used to calculate the total amount of available light per unit ground area ($\mu\text{mol m}^{-2} \text{s}^{-1}$) and the total reactor volume per unit ground area ($\text{m}^3 \text{m}^{-2}$). The input value for biomass yield on light ($Y_{x,ph}$ in g mol^{-1}) is used to convert the available sunlight to biomass.

The input parameters used for the amount of available light include: ground area (m^2), location (GH or AL and the geographical location), selected artificial light (amount and type of light) and light hours of the artificial light applied per day ($\text{h } 24\text{h}^{-1}$). The parameters influencing the reactor volume include: ground area (m^2) and the type of reactors used (TPBR or BC). Other parameters affecting the model output include the mode of reactor operation (chemostat or turbidostat), the method and set points for temperature control, the production season for algae biomass (days year^{-1}) and lifetime of a culture (days between cleaning a reactor system). Finally the input for biomass yield on light ($Y_{x,ph}$) is used to calculate the total amount of biomass produced. The total biomass production cost is represented as the cost price of biomass in € kgDW^{-1} and is obtained by dividing the total cost of the facility (both capital investment and operational costs) by the total yearly biomass production. Every change in input parameter affects either the totals costs, the total biomass production capacity or both outputs.

A schematic representation of all factors of algae production taken into account in the model are represented in Figure 10.

Figure 10: Schematic representation of the aspects of algae production considered in the techno-economic analysis

The symbols represent: 1; Laboratory for maintenance of microalgae cultures and analysis of biomass, 2; Labor requirements for both laboratory work and reactor maintenance, 3; Temperature control of the reactor systems, 4; The selected light source(s) 5; (from top to bottom) water pre-treatment by sterilization, the addition of nutrients and pumping requirements for the water and, 6; pump requirements for air, 7; CO₂ consumption, 8; the required ground area / ground space and costs associated to the selected building type (building not shown), 9; the costs of wastewater treatment and finally 10; the costs of the applied reactor systems, here depicted with a schematic drawing of a TPBR. Note that in our techno-economic analysis we do not consider any downstream processing as it is assumed that produced algal biomass is used directly as live feed at the aquaculture facility where the algae are produced. The algae are produced in natural seawater equal to the water of the organisms consuming the microalgae and therefore no other treatment of the water is required and no harvesting is performed.

Table 3 shows a list of all cost categories and subcategories for costs specified and used in the techno-economic model for the calculation of the cost price of the biomass. Every

category and its associated assumptions are explained in more detail below. The costs can be divided in capital expenditure (CAPEX) and the operating expenditure (OPEX). The CAPEX contains all cost for major equipment (MEC), the location, laboratory and construction costs (see Table 3). OPEX contains all costs required for operation of the reactor systems including the costs specified as "others", the nutrient consumption, consumables for reactor operation, energy requirements of all equipment, costs for water consumption and wastewater removal and labor costs.

Table 3: Cost distribution in categories and subcategories specified for each reactor type. ¹⁻ See Table 4 for cost detailed cost of Major equipment, ²⁻ See corresponding text segment, ³⁻ Based on Ruiz et al. 2016 ⁴⁻ Based on Acién, et al. 2012

Category	Subcategory	Bubble-column systems	Tubular reactor systems
CAPEX	Major Equipment Cost (MEC)	Pumps	included in reactors ¹
		Sterilization equipment	
		Air Blowers	
		Cooling and heating	based on temperature calc ¹
		Reactors	SEACAPs ¹ LGem tubular reactor ¹
		Artificial Lights	As selected for scenario ¹
	Location	Land	No additional cost
		Building	Depend on building type ¹
	Laboratory	Costs for lab equipment	equal for all scenarios ¹
	Construction	Electrical installation	10 % of MEC ^{3,4}
		Plumbing (heating/cooling etc)	20% of MEC ^{3,4}
		Installation and instrumentation	20% of MEC ³ included in reactors ²
		Construction expenses	Assumed to be included in the building costs ²
		Engineering and supervision	30% of MEC ³
		Contractors fee	Assumed to be included in the building costs ²
OPEX	Others	Operating supplies	0.4% of Raw materials + energy + water ^{3,4}
		Contingency	15% of MEC ^{3,4}
		Maintenance	4% of MEC ^{3,5}
		General plant overhead	0% assumed to be part of normal Aquaculture facility ²
	Nutrients	Nutrients and CO2	Based on biomass production ²
	Consumables	€1.50 per bag	N.A.
	Energy	Heating and cooling energy	From MEC consumption ¹
		Energy consumption of reactors	N.A. From MEC consumption ¹
		Energy consumption of pumps	N.A.
		Energy consumption sterilization	From MEC consumption ¹ N.A.
		Energy consumption Blowers	N.A.
		Energy consumption Artificial lights	From MEC consumption 1
	Water Usage	Seawater	Calculated from liquid flow - 0.33 €/m ³
		Wastewater treatment	Calculated from liquid flow - 0.00 €/m ³
	Labour	Regular maintenance labour	see equation ² 0.25-0.5 h/reactor/d ¹
		labour requirements cleaning	0.125 h/bag 1.5-3 h/reactor ¹

Many inputs and assumptions are required to create realistic scenarios for the model. Not only the inputs for the scenarios, but also underlying assumption of the functioning of the selected reactor systems are required. As the model specifically describes situations applicable to aquaculture all underlying assumptions for the selected reactor systems were set in collaboration with commercial users of these systems in aquaculture facilities. Assumptions or choices further referred to as commercial standards or as based on Dutch aquaculture industry are a result of the collaboration with the aquaculture industry with microalgae production systems. Besides setting choices and assumptions the commercial facilities also provided the data to calculate other input parameters such as the biomass yield on light ($Y_{x,ph}$). Values obtained from this data are referred to as numbers obtained from the monitoring data from Dutch aquaculture industry.

2.5.1.1 Biomass production

The total biomass production capacity of a given scenario (kg year^{-1}) is calculated as a function of the total available light ($\text{mol m}^{-2} \text{ year}^{-1}$), the total illuminated ground area for the reactors (m^2) and the efficiency of light use (g mol^{-1}). The efficiency of light use is described as the biomass yield on light ($Y_{x,ph}$) and was obtained from monitoring data of commercial microalgae production facilities in Dutch aquaculture industry.

Available light

The total available light is calculated based on the climate data and the availability of artificial light for each scenario. The available sunlight for all GH-scenarios is based on a meteorological typical year and data was obtained from the MeteoNorm 7 software package for Vlissingen, The Netherlands. The typical year corresponds to the long term mean and is therefore the reference for assessments and planning purposes. The typical year data contains values for irradiance, ambient temperature and wind speed for 365 days year^{-1} and 24 hours day^{-1} . Biomass production rates and other weather dependent data is calculated on a per hour basis.

The total available artificial light is based on the amount of light units per m^2 and the conversion of electrical consumption (W) in to light energy ($\mu\text{mol m}^{-2} \text{ s}^{-1}$), this conversion is kept constant at $1.25 \mu\text{mol W}^{-1}$ for the lifetime of the lights used in our model. The orientation of the light sources is not considered in the model. It is assumed that light sources are positioned in the most optimal method for the applied reactor type. Values given for total artificial light in the results are represented as $\mu\text{mol m}^{-2}_{\text{ground}} \text{ s}^{-1}$, assuming all the produced light is projected directly perpendicular to the ground surface. These values

therefore do not represent the average light intensity on the reactor surface. The reactor geometry, light orientation and placement relative to the reactor surface would determine the true light intensity on the reactor surface. The reactors systems have a total of 6.0m² and 3.5m² reactor surface area per m² of ground area for the AL-BC and AL-TPBR scenarios, respectively. Light not reaching the reactor surface is assumed to be corrected for in the value for biomass yield on light used as the inputs. For the AL-BC and GH-BC scenarios the values for biomass yield on light are determined based on the same assumptions.

AL-scenarios are assumed to apply the artificial lights for 24 hours day⁻¹ without adding a night phase. GH-scenarios with additional artificial light are assumed to require a dark phase of 4 hours per day for legal reasons. It is therefore assumed that GH-scenarios with artificial light apply artificial light to achieve a total day-length of 20 hours day⁻¹ where the artificial lights are turned on during the hours of the day with low light levels. These low light level hours are selected as the first 1,5 hours after sunrise and the last 1,5 of the day before sunset. The hours of artificial light are defined per day and are based on the meteorological defined hours for sunset and sunrise in Vlissingen, The Netherlands.

Ground area

Total illuminated ground area is based on the selected reactor type and the total ground area given as input. Only the ground area containing reactor systems is considered in the calculations for productivity of biomass, for tubular reactors this means only the tubular section, and the ground area between two separate reactor systems, of the reactors is considered in the illuminated ground area. For BC-systems the ground area used as walkways between the reactor systems is also included in the illuminated ground area.

Biomass yield on light and monitoring data

The biomass yield on light for the BC-reference scenarios is calculated from data of two commercial applications for biomass production in aquaculture. These two scenarios (GH-BC and AL-BC) were both monitored by their operators for over one year and the collected data was used for calculations for biomass yield on light. The values found for biomass yield on light are used as input in the reference scenarios for the techno-economic model.

Each production site monitored 2 reactors in detail, measuring the biomass concentration and dilution rate of these reactors. Data was collected when possible creating a data set with productivity information. The final data sets contained 93 data points over a period of 147 days for the GH-BC scenario and 104 Data points over 376 days for the AL-BC scenarios representing a total of 7 and 10 reactor runs, respectively. For days with no available data it was assumed that the biomass concentration is the average between the two surrounding

data points and the dilution rate was unchanged from the previous measurement in time. Cell count was used as a measure for biomass concentration for every data-point as dry-weight was determined less-often. Cell count was converted to a biomass concentration (in g l^{-1}) using the average cell mass as found in the available data of the dry-weight determinations in the dataset. The results showed an average value of $Y_{x,ph}$ 0.22 g mol^{-1} and 0.32 g mol^{-1} for AL-BC and GH-BC, respectively.

For TPBR-scenarios no operational commercial systems were available for the collection of long term monitoring data. Data from literature was used to estimate a value for $Y_{x,ph}$ for these systems. The selected values are based on AlgaePARC data, an experimental pilot scale facility for microalgae production located in Wageningen, The Netherlands. The AlgaePARC data contains information for a vertically stacked horizontal tubular photobioreactor located outdoor under Dutch climate conditions without the use of artificial lights. Even though the geometry and method of operation of this experimental system is different from the commercially available systems the data is assumed to represent achievable values for commercial production in TPBR-systems. The value for $Y_{x,ph}$ was set a 2% photosynthetic efficiency, or 0.45 g mol^{-1} for both TPBR-reference scenarios based on results of de Vree et al. [47, 50]

2.5.1.2 Detailed cost factors

All cost factors are summarized in Table 3. This table shows the distribution of CAPEX and OPEX. The majority of equipment applied influences both CAPEX and OPEX e.g. pumps have an associated investment cost and an energy consumption. All categories are explained in detail below in the order of appearance in Table 3.

Major equipment cost

Major equipment cost (MEC) contains 6 subcategories. Each of these categories contains different sizes of equipment with associated capacity and power requirements (see details in Table 4). The required capacity for equipment is based on calculations for the day of maximum capacity requirement, it is assumed that equipment will not operate at more than 90% of the listed capacity. For most major equipment (Pumps, air blowers, and temperature control) three units with increasing capacity are used in the model. The largest unit within the required capacity is applied, if more capacity is required this is filled up with the next largest unit that fits the required capacity. For some scales of MEX the CAPEX is calculated based on cost prices of available equipment using Equation 2.1 as used by Ruiz et al. and

Acien et al. This approach is used for the CAPEX of temperature control equipment and for the cost of buildings (greenhouse and indoor facilities).

$$\text{Equation 2.1: } CostB = CostA * \left(\frac{SizeB}{SizeA} \right)^{0.85}$$

The yearly cost for CAPEX of MEC is based on the depreciation and purchase price. The depreciation of most MEC was set at 15 years with exception of the air blowers for which the depreciation was set at 10 years and the artificial light of which the depreciation is based on the hours of operation with a lifetime of 15.000 hours.

Some cost factors do not follow directly from CAPEX and/or OPEX but are added to the total cost calculation by the application of Lang-factors. These Lang-factors are based on values that are applied in techno-economic studies as published by Ruiz et al and Acien et al [13, 45]. Lang-factors are applied in the category Construction and the category Others as seen in Table 3.

The OPEX of all major equipment is based on the capacity and the electrical consumption of the equipment.

Liquid pumps

The required pump capacity is calculated based on the total liquid flow for the scenario. The total liquid flow is based on the selected dilution rate of the reactor systems and the total reactor volume. It is assumed that all liquid is only pumped once; into the reactor. Both reactor types work with air circulation systems for mixing of the culture, no further liquid pumps are required when the water has reached the reactor systems. As both applied reactor types work with an overflow systems no pumping capacity is required to extract the culture from the reactors. Pump requirements to bring the culture to the destined feed location is not considered as these requirements depend on the application of the algae in aquaculture which is out of the scope of our techno-economic model. Major equipment costs for pumps are based on numbers from Ruiz et al, see Table 4 (Ruiz et al. 2016).

Table 4: Cost price of Major equipment used in the techno-economic model per unit size or ¹-prices per m² of ground area.

Subcategory	Version	Capacity	Cost	Power consumption	Labour req	
		Unit	€ unit ⁻¹	Unit	Maintenance	Cleaning (h/clean)
Liquid pumps	Small	2 m ³ h ⁻¹	€ 455	0.18 kW		
	medium	4 m ³ h ⁻¹	€ 1,035	0.4 kW		
	large	15 m ³ h ⁻¹	€ 3,528	5.5 kW		
Sterilization equipment	Custom build	1 m ³ h ⁻¹	€ -	See text		
Air Blowers	Small	250 m ³ h ⁻¹	€ 1,000	1.3 kW		
	medium	1000 m ³ h ⁻¹	€ 5,653	3.96 kW		
	large	2499 m ³ h ⁻¹	€ 11,182	11.15 kW		
Cooling and heating	Heatpump small	16.6 kW	€ 21,590			
	Heatpump medium	49.8 kW	€ 54,930			
	Heatpump large	149.4 kW	€ 139,752			
	Aircoditioning small	10 kW	€ 3,595			
	Aircoditioning medium	25 kW	€ 7,833			
	Aircoditioning large	50 kW	€ 14,120			
Reactors	LGem RD1-250 (GLASS)	0.21 m ³	€ 30,000	0.03 kW	0.25	1.5
	LGem MK2-750 (GLASS)	0.68 m ³	€ 29,000	0.10 kW	0.25	1.5
	LGem MK1-750 (GLASS)	0.67 m ³	€ 22,500	0.10 kW	0.25	1.5
	LGem MK1-1500 (GLASS)	0.97 m ³	€ 36,500	0.15 kW	0.25	1.5
	LGem MK1-18000 (GLASS)	13.27 m ³	€ 160,000	1.99 kW	0.5	3
	SEACAPs	0.226 m ³	€ 500	-	See equation	0.125
Artificial Lights	Philips TL-D 58W	72.5 μmol/s	€ 2.12	58 W		
Building	Greenhouse ¹	100 m ²	€ 250	-		
		500 m ²	€ 196	-		
		1000 m ²	€ 177	-		
		2500 m ²	€ 154	-		
	Indoor facility ¹	100 m ²	€ 325	-		
		500 m ²	€ 255	-		
		1000 m ²	€ 230	-		
		2500 m ²	€ 207	-		
Costs for lab equipment	Basic lab facility	-	€ 28,886	-		

Water pre-treatment (sterilization equipment).

Pre-treatment of the water flow is performed differently for the two reactor types used in the techno-economic model, this is based on common practice in commercial microalgae applications in Dutch aquaculture industry. For the BC-systems the water is pre-treated by pasteurization. For the TPBR-scenarios filter systems that come as a part of the reactor systems are used to filter the water at 2.0 and 0.2 μm. The cost price of a pasteurizer for bubble-column systems could not be quantified and is therefore neglected – only the energy requirement for these systems is taken into account. The energy requirement for heat sterilization of seawater is based on measurements of commercial systems used to obtain the monitoring data and was set at 22 kWh m⁻³. The total energy capacity is based on the total liquid flow through the systems which is based on the total reactor volume and the dilution rate. All tubing for the bubble-column applications are steam sterilized once a week. The cost of these systems is also based on monitoring data from industrial facilities. Electrical consumption for steam cleaning was set at 0.05 kWh bubble-column⁻¹ week⁻¹.

Capital investment for the steam cleaners was set at 1000 € kW⁻¹ and the steamer capacity was determined based on 0.1kW bubble-column⁻¹.

Air blowers

Air blowers are used for the air flow required for BC- systems. The TPBR-systems do not required input of air flow as air pumps are integrated in the reactor design and their energy consumption and CAPEX are included in the reactor systems. The air flow for BC-systems is based on a standard airflow of 0.25 vvm for bubble-columns and is therefore correlated to the total reactor volume. Cost prices of air blowers are taken from Ruiz et al and based on the commercial installations used for the collection of monitoring data (Ruiz et al. 2016).

Cooling and heating

The method of temperature control of a reactor system and the surrounding air is depending on the selected scenario (reactor type and placement). Temperature control for GH-TPBR-systems is based on the use of a heat pump for the production of hot/cold water. The water is then circulated over a heat exchanger. CAPEX for a heat pump is based on the heat pump installation (16.6kW installation) in the lab facilities at the HZ University of Applied Sciences, Vlissingen, The Netherlands and adjusted for scale according to equation 2.1 as based on Acien et al. (Acien et al. 2012). Final prices applied of heat pump systems can be found in Table 4.

For AL-scenarios it is assumed that the only requirement for temperature control is the cooling of the indoor facility to remove the heat produced by the applied artificial lights. The method of temperature control for the AL-scenarios of both reactor types is an air-conditioning system. CAPEX is based on the air-conditioning installation for air temperature control at the HZ University of Applied Sciences, Vlissingen, The Netherlands, adjusted for different scales as based on equation 2.1. The OPEX of temperature control is described in more detail below.

Reactor systems

In our study the bubble-column scenario describes Seasalter Continuous Algal Production Systems, or SeaCAPS in short, as typically used in aquaculture. CAPEX for 1 single SeaCAPS system is set at €500 per bag system. This price includes support structure and the glass- and silicone-tubing for a basic system. The cost price of consumable plastic bags is set at €1.50 per plastic bag as based on Dutch aquaculture industry.

The tubular reactor system (TPBR) cost prices are obtained from the Dutch manufacturer of tubular photobioreactor LGem and is based on the standard available LGem GemTubes™ photobioreactor systems. Tubular reactor systems are limited to the glass-tube variant of these TPBRs. Even though previous PVC TPBRs were a popular choice due to the price advantage of PVC, with state of the art technology for glass tubing this is no longer the case. Reactor models 250, 750, 1500 and 18.000 are considered in the techno-economic model. The CAPEX for TPBR systems is assumed to include the following parts of the reactors: tubular reactor system including air and liquid pumps for circulation, a measuring and control unit for temperature and pH control and water filters for sterilization of the inflow of fresh water. Excluded from these systems are the temperature control unit (cold water), CO₂ source for pH control, pumps for water inflow etc. TPBR-systems are assumed to be delivered and constructed on site and the CAPEX for these systems includes installation of the system, excluding the installation of the additional equipment required for operation. As a result of this some of the Lang-Factors, used to calculate additional costs such as installation and instrumentation, have been adapted from assumptions as typically used in literature for an algae facility (Ruiz et al. 2016; Acién et al. 2012)

Artificial lights

The artificial light applied in the techno-economic model is based on the standard use of fluorescent tubes in aquaculture hatcheries. The same type of lights was used for all reactor systems for a fair comparison and is based on standard Philips TL-D 58W fluorescent tubes. Electrical consumption of the lights set at 85 W with an efficiency of 1.25μmol W⁻¹ (Blanken et al. 2013). Depreciation is based on operational hours and is set at a time of 15.000 hours, no time dependent loss in efficiency was assumed. The fixture for the lights is assumed to have a depreciation of 15years as with all other equipment.

Location

The cost of the location for an algae production facility of a hatchery is simplified to only the cost of the building required for the facility. This has been done as the cost of land can vary greatly between location and the amount of land required. It is assumed that an aquaculture facility will apply the algae facility as part of the larger operation on an already owned piece of land. The cost of the selected building is determined for the smallest scale of a facility up to 100m². The other scales are calculated based on equation 2.1. The cost price of the selected building is represented in cost per m² of the facility, the cost price of the location/facility include costs for the described building type including the construction of the building and the construction costs. The two building types are based on a normal

greenhouse or an indoor facility based on commercial scale warehouse type facility buildings. The Lang-factors for the categories building and construction costs have therefore been reduced to 0% as compared to Ruiz [13]. The cost price of buildings are set at €250 m⁻² and €325 m⁻² for GH and AL locations at the smallest scale up to 100m² and decrease with increasing ground area (see Table 4).

Laboratory

The laboratory equipment required for basic operation of a functional microalgae facility was selected based on the equipment typically found in small scale algae labs of microalgae facilities in aquaculture industry. A basic lab as included in the techno-economic model has the equipment to produce medium and maintain stock cultures of the algae cultures. The basic lab includes a scale, pH-meter, refrigerator, freeze, standard glassware, magnetic stirrer, microscope, milliQ dispenser and basic lab furniture. The CAPEX of the major equipment was determined from the equipment as purchased as part of the construction of the AlgaePARC pilot facilities at Wageningen University and Research. As the lab is expected to be used for stock culture maintenance and small scale medium preparation the lab CAPEX of the lab facilities does not change with the scale of microalgae production facility in our calculations. The standard cost price of a lab as considered in the model is set at €28.886 of total CAPEX or €1.926 year⁻¹. No OPEX is considered for the lab.

Construction and Others

Lang-factors are used for the estimation of the category Construction costs and Other costs in the techno-economic model. The Lang factors applied in our model are a combination of the factors used in the publications of Ruiz et al. and Acien et al. [13, 45]. There is a difference in the method of application of the Lang-Factors for the two reactor types as the systems are built differently. The Lang factor for Installation and instrumentation is not included for the tubular reactor systems. The cost for installation and instrumentation for these systems is assumed to be included in the CAPEX of the reactor systems as the systems are delivered to be almost plug and play. Any additional costs are assumed to be sufficiently covered by the subcategories electrical installation and plumbing for the tubular reactor systems. As CAPEX of both reactor systems includes the construction of these systems and the CAPEX for buildings is assumed to include the construction of these buildings the Lang-factor for the subcategories "Construction expenses" and "Contractors fee" are set to 0% in our calculations.

Nutrients

Nutrient supply in our calculations is limited to the main nutrients required for microalgae production; Nitrogen, Phosphorus and CO₂. The total required nutrient amount is calculated as a function of biomass productivity and is corrected for the assumed nutrient uptake efficiency as measured by Ación et al. Nitrogen consumption efficiency was set at 88.2%, Phosphorus at 65.8% and CO₂ at 74.5% [45]. The price of Nitrogen and Phosphorus is equal for all scales and was set at €2.00 kg⁻¹ and €2.71 kg⁻¹ respectively. The price of CO₂ depends on the scale of consumption of CO₂. Two different systems are considered in the calculations; a single CO₂ bottle of 37.5kg or a cartridge of 12-cylinders containing a total of 450kg CO₂ with a price of €0.67 kg⁻¹ at the smaller scale and €0.43 kg⁻¹ for the larger capacity system.

Consumables

Only the plastic bags of the bubble-column systems are included in the consumables. Every time a bubble-column is cleaned/restarted one plastic bag is consumed. A plastic bag has a cost price of €1.50 per bag. No consumable equipment is considered for the laboratory or the tubular reactors.

2.5.1.3 Energy requirements and temperature calculations

The total energy requirements of all Major equipment is calculated based on the capacity of the equipment and the electrical consumption of the selected equipment. The capacity of the equipment follows from the calculation of biomass production and reactor specifications as explained for Major equipment cost. The cost of electricity was set at €0.082 kWh⁻¹.

Heating and cooling energy

Energy requirements for temperature control is dependent on the scenario and the temperature boundaries. A schematic representation of the heat flows considered in the energy calculation for a GH-TPBR scenario is given in Figure 11. This figure represents the most complex scenario for the temperature calculations as for other scenarios some/most heat transfers are not considered due to assumptions for the temperature calculations in these scenarios.

The four different scenarios (GH-TPBR, AL-TPBR, GH-BC and AL-BC) have different methods defined for temperature control and some of the heat transfers shown in Figure 11 do not apply to all scenarios.

For the AL-scenarios it is assumed that the reactors are placed indoors where no heat transfer occurs from the indoors location to/from the outside environment. The AL-building is assumed to be 100% isolated from the surrounding area. In these AL-scenarios a climate room approach is considered with the assumption that only the heat produced by the artificial lights has to be cooled away. It is assumed that the temperature in the climate room is maintained using an air-conditioning with a capacity based on the electrical heat input generated by the selected amount of artificial lights. The reactors are assumed to maintain the same temperature as the surrounding air as a result of sufficient air-flow around the reactor systems. In aquaculture commercial application the artificial lights are typically placed in an enclosure separating the lights from the reactors using glass or plastic panels. This enclosure separates the air flow around the light from the air flow around the reactor systems, limiting heat from the artificial lights to reach the reactors, making temperature control somewhat more efficient. This type of light placement is not considered in our calculations.

For GH-scenarios, the temperature control for both reactor types is defined differently than in the AL-scenarios. In the GH-TPBR scenarios, reactors are actively controlled for temperature and are assumed to be heated and cooled using a heat pump circulating water over a heat exchanger located inside the reactor system. The capacity of the heat pump is calculated based on all heat transfers as described below.

Temperature control of a GH-BC scenario is assumed to be performed by controlling the air temperature in the GH-location. No active temperature control is possible on the BC-reactor systems. For BC-systems this type of temperature control is often sufficient due to the large culture volume to surface area ratio of these systems which provides a very large temperature buffer.

Figure 11: Simplified schematic representation of heat fluxes considered in temperature calculations for the GH-TPBR scenario.

The temperature of the reactor system ($T_{reactor}$) can be determined from the energy balance of the reactor systems. Heun's method is used to find the results of the temperature balance over the reactor system. The balance over the reactor systems is solved for time steps of 1 hour as the climate data used contains data per hour.

The temperature of the reactor is defined as:

$$T_{reactor}(n+1) = T_{reactor}(n) + \Delta T$$

where ΔT is the final approximation of ΔT after applying Heun's method for the initial approximation and final approximation and ΔT calculated as described below.

$$\Delta T = (Q_{total} / (V_{culture} * \rho C_{water}) * 3600)$$

Where Q_{total} is defined as the sum of all heat fluxes over the reactor system as follows from the energy balance over the reactor system.

$$Q_{total} = Q_{irradiance} - Q_{convection} - Q_{radiation}$$

$Q_{\text{irradiance}}$ is the heat input from sunlight into the reactor and is calculated the heat input from the sun. Climate data for a typical meteorological year for the location of Vlissingen, The Netherlands is used for the sunlight data.

$Q_{\text{convection}}$ is the convective heat transfer from the reactor to the surrounding air and is calculated from the difference in temperature between the reactor and the surrounding air and the heat transfer coefficient for radiation α_{rg} which is mainly dependent on the wind speed.

$Q_{\text{radiation}}$ is the heat transfer from the reactor to the surrounding air by direct radiation and is calculated from the difference in temperature between the reactor and the surrounding air and the heat transfer coefficient α_c .

$$Q_{\text{irradiance}} = A_{\text{ground}} * I_{\text{irradiance}}$$

$$Q_{\text{convection}} = A_{\text{tubes}} * \Delta T_{(\text{reactor-air})} * \alpha_{rg}$$

$$Q_{\text{radiation}} = A_{\text{tubes}} * \Delta T_{(\text{reactor-air})} * \alpha_c$$

With the $I_{\text{irradiance}}$ the total solar energy input (W m^{-2}) determined from the same climate dataset used for T_{ambient} and v_{ambient} (the outdoor windspeed), A_{ground} the total ground area of the algae production facility, A_{tubes} the total outside surface are of the reactor tubes $\Delta T_{(\text{reactor-air})}$ the temperature difference between the reactor and the surrounding air ($T_{\text{greenhouse}}$).

The temperature of the greenhouse ($T_{\text{greenhouse}}$ or T_{gh}) can be calculated from the energy balance of the air of the greenhouse and is defined as follows:

$$T_{\text{greenhouse}(n+1)} = \frac{(u_{rg} * T_r) + (u_{ga} * T_a) + (u_{gc} * T_c)}{(u_{rg} + u_{ga} + u_{gc})} + \Delta T_{\text{sun}}$$

Where ΔT_{sun} is the air temperature difference as a result of solar input and is defined as:

$$\Delta T_{\text{sun}} = \frac{Q_{\text{irr-floor}}}{(\rho C_{\text{air}} * V_{\text{air}})}$$

$Q_{\text{irr-ground}}$ is the heatflux entering the greenhouse by sunlight not hitting the reactors but the greenhouse ground. It is assumed that $Q_{\text{irr-ground}}$ contributes completely to heating the greenhouse air and no energy is taken up by the ground itself; the ground is assumed to maintain a constant temperature and not influence the greenhouse temperature in any way.

T_{cover} follows from the energy balance over the cover and is defined as:

$$T_{\text{cover}(n+1)} = \frac{(u_{gc} * T_{gh(n+1)}) + (u_{rc} * T_r) + (u_{ca} * T_{\text{ambient}}) + (u_{c-sky} * T_{\text{ambient}})}{(u_{gc} + u_{rc} + u_{ca} + u_{c-sky})}$$

All temperatures can be calculated with the u 's and heat transfer coefficients (α with unit: $\text{J m}^{-2} \text{K}^{-1}$) given or calculated using the following details:

$$U_{rg} = \alpha_{rg}$$

$$U_{ga} = \Phi_{vent} * \rho C_{air}$$

$$U_{gc} = \alpha_{gc} * A_{glass}/A_{ground}$$

$$U_{rc} = \alpha_{rc} * A_{tubes}/A_{glass}$$

$$U_{ca} = \alpha_{ca} * A_{glass}/A_{ground}$$

$$U_{c-sky} = \alpha_{c-sky} * A_{glass}/A_{ground}$$

$$\alpha_{rg} = 2.8 + (1.2 * v_{indoor}) \text{ with } v_{indoor} = \text{wind speed indoor} - \text{set at } 1 \text{ m s}^{-1}$$

$$\alpha_{rg} = 2.8$$

$$\alpha_{gc} = 2.8$$

$$\alpha_{ca} = 2.8 + (1.2 * v_{ambient}) \text{ with } v_{ambient} = \text{wind speed of the outside wind given in climate data}$$

$$\alpha_{rc} = 4 * \sigma * a_1 * ((T_{reactor} + T_{air})/2)^3$$

$$\alpha_{c-sky} = 5$$

$$\Phi_{vent} = 0.1 * 4/3600 \text{ (m}^3\text{s}^{-1}\text{)}$$

$$\rho C_{air} = 1.2E3 \text{ (Jm}^{-3}\text{K}^{-1}\text{)}$$

$$\rho C_{water} = 1.2E3 \text{ (Jm}^{-3}\text{K}^{-1}\text{)}$$

$$C_{Pair} = 1006 \text{ (J}\cdot\text{kg}^{-1}\cdot\text{K}^{-1}\text{)}$$

$$\rho_{air} = 1.203 \text{ (kg}\cdot\text{m}^{-3}\text{)}$$

$$\sigma = 5.6703e-8 \text{ (W m}^{-2} \text{ K}^{-4}\text{)}$$

$$a_1 = 1$$

$$V_{culture} = \text{total culture volume of all reactor systems combined}$$

$$V_{air} = \text{total air volume of the indoor location}$$

A_{glass}/A_{ground} is the ratio of outside glass of the greenhouse per m^2 of ground area of the total scenario. This ratio therefore depends on the size of the scenario applied as this changes with a changed wall:roof ratio for the greenhouse applied.

A_{tubes}/A_{glass} the ratio of outside tubes surface area of the reactor to the glass surface area of the greenhouse

When the calculated temperature of the reactor system ($T_{reactor}$) is not within the defined set points for $T_{reactor}$ in the techno-economic model, the reactors are cooled or heated. The sum of all heat required for heating and cooling over the full year is used to calculate the total energy requirements for temperature control whereas the hour with maximum ΔT

requirements for cooling and heating is used to determine the capacity of the heat pump required for temperature control in each GH-TPBR scenario.

Energy consumption reactors

Energy consumption of the reactors is only considered for the TPBR reactor and is set at a fixed value of 0.15 kW m^{-3} of total reactor volume. This energy capacity is required for the air pumps integrated in the reactor design that allow for air flow in the tubes.

Energy consumption pumps

Liquid pumps are assumed to have a fixed energy requirement of 0.1 kW m^{-3} . The energy requirements for the pumps are based on the actual amount of water displaced rather than the amount of pumps and their operating hours.

Energy consumption water pre-treatment

Water pre-treatment is performed differently for the different reactor systems. For TPBR-systems the water is pre-treated using the filter setup that is part of the reactor systems. No additional cost for energy-consumption for water pre-treatment is therefore applied for TPBR systems. The BC-systems apply pasteurization heat-treatment to the water and an energy consumption is set at 22 kWh m^{-3} .

Energy consumption artificial light

Total artificial light energy consumption is based on the hours of operation of the light. Standard lights used are TL-D 58W fluorescent tube lights with an electrical consumption of 58W per unit and light output based on $1.25 \mu\text{mol W}^{-1}$.

Water usage

The consumption of water is based on the dilution rates of the reactors and includes the costs for the supply of the seawater and the disposal of wastewater back into the sea. The cost price of seawater is based on the assumption that an aquaculture facility will have a supply of seawater available at the location of the microalgae production facility. No costs are taken into account for water treatment equipment or other equipment required to collect seawater. The only cost considered is the cost of extra water consumption from the existing

system. For Dutch aquaculture the average cost of water is set at €0.33 m⁻³. Waste water treatment is assumed to not be required.

Labor requirements

The total amount of labor is generally based on the total number of reactors in operation and the hours required to maintain stock cultures and perform lab work. Numbers of average labor requirement are based on Dutch aquaculture applications.

For TPBR scenarios it is considered that 1h day⁻¹ is required to maintain stock cultures, perform lab work and maintain inoculum to start new reactors independently of the amount of reactors in operation. For BC systems stock culture maintenance was added in the total amount of hours per reactor as no distinction could be made between hours spend on culture maintenance or reactor work from the available data. As a result the amount of labor for BC-scenarios was given a non-linear labor requirement described by equation 2.2. This function is applied for systems up to 160 reactors. It is assumed that for facilities larger than 160 bubble-columns a standard of 8hours per week of labor is required to maintain the facility with an additional 0.1hrs per week for every bubble-column added, see equation 2.3.

$$\text{Equation 2.2 (\#reactors} < 160\text{): } h/\text{day} = 1.4955 * \#reactors^{0.5469}$$

$$\text{Equation 2.3 (\#reactors} > 160\text{): } h/\text{day} = \frac{((0.1 * (\#reactors - 160)) + 8)}{7}$$

The total labor costs are calculated based the labor requirements for the labs and the reactor plus 10% for supervision of work. Labor is set at 3x minimal wage for operators and 4.3x minimal wage for a supervisor according to Ruiz et al. The minimal wage is €18.619,20 year⁻¹ as based on numbers of Dutch law in 2018.

2.6 Detailed results for calculated scenarios

2.6.1 Industrial reference scenarios

Four industrial reference scenarios were created; AL-BC, AL-TPBR, GH-BC and GH-TPBR. These four scenarios were created based on the commercial applications of these systems in aquaculture and were designed to allow comparison of biomass production costs at an equal biomass production capacity (125 kg year⁻¹). The input for Y_{x,ph} was used as a fixed value in the design of the scenarios and the total size of the production facility was adjusted

to obtain equal biomass production capacity. An overview of the final inputs applied for these scenarios is given in Table 5.

Table 5: Selected inputs for all four reference scenarios. BC: Bubble-columns, TPBR: Tubular photobioreactor

	Artificial Light		Greenhouse	
	BC	TPBR	BC	TPBR
Area of production facility (m ²)	30	20	50	35
Artificial light type	TL-D 58W	TL-D 58W	None	None
Total artificial day length (hr day ⁻¹)	24	24	0	0
Lights bag ⁻¹ / Units m ⁻²	3	8	0	0
Temperature range (°C)	20	20	15-25	15-25
Algae-season Start (day)	1	1	1	1
Algae-season End (day)	365	365	365	365
Culture lifetime (days)	75	75	75	75
Biomass yield on light ($Y_{x,ph}$ - g mol ⁻¹)	0.22	0.45	0.32	0.45

Area of production facility

The area of the production facility is used to change the scenario to a scenario with a total biomass production capacity of 125 kg year⁻¹ to allow for direct comparison of biomass cost price. The 125 kg year⁻¹ biomass capacity is based on the two commercial applications for BC-reactor systems in aquaculture used for the collection of data for the values of $Y_{x,ph}$ for the two BC scenarios.

By selecting the ground surface area the total amount of reactors changes for the BC-scenarios whereas for the TPBR scenario this also changes the type/size of reactor applied. Specifically for the GH-TPBR scenario multiple scenarios could be created to achieve approximately 125 kg year⁻¹ biomass capacity. The selected scenario for GH-TPBR utilizes two reactors of 750L capacity producing biomass at a price of €329 kgDW⁻¹ producing a total 109 kg year⁻¹. Equal biomass capacity could be achieved by one 1500L reactor system showing a cost price of €286 kgDW⁻¹ producing 109 kg year⁻¹, or a scenario with four 750L reactors producing 145 kg year⁻¹ at a cost price of €360 kgDW⁻¹ is also possible. The scenario

with four reactors represents reactors of slightly different geometry (shorter but higher reactors) and therefore represents an inefficient use of artificial lights with the given inputs of total available light. From these three options the scenario with two 750L reactors was selected as most representative for production of microalgae at this scale. It is neither the cheapest nor the most expensive available option for the required biomass capacity of the reference scenario.

Artificial lights and total artificial day length

The artificial lights on the BC and TPBR systems are defined differently, resulting in very different values as input for the amount of lights used. For BC-scenarios the amount of TL-D units placed on/around one reactor are used as input value whereas for TPBR scenarios the number of TL-D units placed per m^2 of ground area is given as input. Only the illuminated ground area is considered for artificial light with TPBR systems and not the total ground area of the facility.

The total amount of lights of the TPBR systems was set at 8 corresponding with the total amount of TL-D light typically supplied by the supplier of the TPBR systems. Resulting in a total light intensity of $580 \mu\text{mol m}_{\text{ground}}^{-2} \text{s}^{-1}$ assuming all light is projected perpendicular to the ground surface. According to the supplier of this reactor, this corresponds to about $190 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the tube surface area.

The amount of lamps applied for the AL-BC scenario is based on the commercial applications of these systems and was set at 3 TL-D lights per bubble-column reactor system. In these scenarios this corresponds to a total light intensity of $586 \mu\text{mol m}^{-2} \text{s}^{-1}$ assuming all light is projected perpendicular to the ground surface. All artificial light in AL-scenarios is applied during 24hours day^{-1}

Temperature range

The temperature range of AL-scenarios is not set as the temperature range does not influence the calculations for energy consumption in these scenarios.

Algae-season start and end

These two parameters give the day of the year that algae production is active. For all reference scenarios algae production is considered to be applied all days of the year. In the sensitivity analysis these values are changed to exclude the winter months.

Lifetime of a culture

The lifetime of the culture describes the total run time of a reactor before it requires to be cleaned and re-started. This value is set equal for all reactor systems and is based on data from commercial applications of BC-reactors and was determined at an average value of 75 days per reactor run.

Biomass yield on light (PAR)

The biomass yield on light was set as described in section 2.5.1.1.

2.6.2 Results reference scenarios and improved scenarios

Table 6: Detailed cost price of microalgae production for the four reference scenarios and the four scenarios with the combined improvements from the sensitivity analysis. AL: Artificial light, GH: Greenhouse, BC: Bubble-column, TPBR: Tubular photobioreactor

	Reference scenarios				Combined sensitivity scenarios			
	AL		GH		AL		GH	
	BC	TPBR	BC	TPBR	BC	TPBR	BC	TPBR
Techno-economic model results								
Average biomass production $\text{r g m}^{-2} \text{d}^{-1}$	11.1	22.6	6.9	9.7	37.0	75.2	19.4	27.7
Total biomass capacity kg year^{-1}	121	127	125	109	401	438	353	322
Total investment costs €	183,001	143,321	232,364	217,443	202,139	157,072	241,810	225,393
Total cost per year € year^{-1}	70,921	36,710	71,666	35,837	78,097	42,155	60,197	32,726
CAPEX € year^{-1}	12,579	9,640	15,532	14,503	14,198	10,639	16,357	15,021
OPEX € year^{-1}	58,342	27,070	56,134	21,334	63,898	31,516	43,840	17,705
Biomass costs € kg^{-1}	587	290	573	329	195	96	170	102
Detailed cost distribution results								
Major Equipment € kg^{-1}	38.76	31.91	49.18	61.52	13.61	10.47	18.57	21.65
Location € kg^{-1}	10.35	3.42	10.65	5.36	3.12	0.99	3.77	1.81
Laboratory € kg^{-1}	15.95	15.19	15.40	17.69	4.80	4.40	5.45	5.97
Construction and fixed costs € kg^{-1}	31.01	19.15	39.35	36.91	10.89	6.28	14.85	12.99
Others € kg^{-1}	8.13	6.39	9.61	11.72	3.00	2.18	3.68	4.17
Nutrients € kg^{-1}	1.99	1.99	1.99	1.99	1.38	1.38	1.38	1.38
Consumables € kg^{-1}	5.32	-	8.64	-	0.80	-	1.53	-
Energy € kg^{-1}	184.11	78.67	56.85	6.16	100.36	45.41	34.27	12.16
Water usage € kg^{-1}	4.51	0.20	7.30	0.47	1.36	0.06	2.59	0.16
Labour € kg^{-1}	287.35	132.70	374.07	187.32	55.52	25.17	84.40	41.21

2.6.3 Sensitivity analysis

For the sensitivity analysis parameters are changed one at a time while maintaining all other parameters equal to the reference scenario. An overview of specific changes made to the reference scenarios is given in Table 7.

Table 7: Overview of changes made to reference scenario inputs for the sensitivity analysis. AL: Artificial light, GH: Greenhouse, BC: Bubble-column, TPBR: Tubular photobioreactor

	Parameter changed from reference scenario	AL		GH	
		BC	TPBR	BC	TPBR
1. Additional AL	Lights bag ⁻¹ / Units m ⁻²	6	16	2	5.5
2. Temperature range	Temperature range	-	-	10-30	10-30
3. Y _{x,ph} +66%	Biomass yield on light (Y _{x,ph})	0.37	0.75	0.53	0.75
4. Reduced downtime	Lifetime of a culture	150			
5. Labour -33%	-	-33% total labour hours			
6. Seasonal production	Algae-season Start/End	60-335			
7. Combined	-	Combined 1-5			

For the first sensitivity analysis scenario, additional artificial light, the total artificial light is doubled for the original AL-scenarios. For the GH scenarios extra light is added to the scenario during the hours of the day in which low light levels are available. These low light levels are defined as the first 1.5 hours after sunrise and the last 1.5 hours of the day before sunset. During these hours artificial light is applied in these scenarios to result in a final total day-length of 20 hours. In the GH-BC scenario a total of 2 TL-D lights are added per bubble-column based on common practice in aquaculture. The GH-TPBR is supplied with an equal amount of artificial lights to yield approximately 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by artificial lights assuming all light is projected perpendicular to the ground surface.

2.6.4 The effect of scale

The effect of scale was assessed in detail by determining the cost price for 9 different scenarios at 6 different scales each. Details for the total biomass cost price and total biomass capacity of each scenarios are given in Table 8 and Table 9.

Table 8: Biomass cost price for the effect of scale scenarios

	Biomass cost price € kg ⁻¹					
	25m ²	100 m ²	250 m ²	500 m ²	750 m ²	1500 m ²
AL-BC	€ 198	€ 167	€ 162	€ 160	€ 159	€ 158
GH-BC	€ 209	€ 151	€ 141	€ 137	€ 135	€ 134
AL-TPBR	€ 96	€ 67	€ 61	€ 60	€ 60	€ 53
GH-TPBR	€ 152	€ 68	€ 48	€ 45	€ 43	€ 26
AL-BC-Base	€ 613	€ 471	€ 444	€ 434	€ 431	€ 428
GH-BC-Base	€ 721	€ 495	€ 453	€ 438	€ 433	€ 427
AL-TPBR-Base	€ 286	€ 160	€ 131	€ 126	€ 124	€ 96
GH-TPBR-Base	€ 510	€ 195	€ 123	€ 111	€ 104	€ 43
GH-TPBR-NoAL	€ 239	€ 94	€ 61	€ 56	€ 52	€ 23

Table 9: Total biomass capacity for the effect of scale scenarios

	Biomass capacity kg year ⁻¹					
	25m ²	100 m ²	250 m ²	500 m ²	750 m ²	1500 m ²
AL-BC	340	1365	3416	6832	10253	20506
GH-BC	176	707	1769	3539	5310	10621
AL-TPBR	583	1750	6127	12253	18380	32091
GH-TPBR	215	645	2257	4514	6771	11822
AL-BC-Base	101	406	1016	2031	3048	6096
GH-BC-Base	63	251	628	1256	1885	3770
AL-TPBR-Base	169	507	1775	3549	5324	9295
GH-TPBR-Base	73	218	762	1524	2287	3992
GH-TPBR-NoAL	125	376	1316	2631	3947	6892

Chapter 3

Optimization of *Rhodomonas sp.* under continuous cultivation for industrial applications in aquaculture

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Optimization of *Rhodomonas sp.* under continuous cultivation for industrial applications in aquaculture

Abstract

The microalgae species *Rhodomonas sp.* is a commonly used in aquaculture for its high nutritional value due to the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content. Understanding the effect of cultivation parameters on biomass production rate and composition is presently limited, however essential in further commercialization of this strain. Under nutrient replete conditions, light intensity and temperature are the main factors determining biomass growth and composition. Therefore, the combined effect of light and temperature on the biomass production rate and biomass composition of *Rhodomonas sp.* was studied using a statistical Design of Experiment approach. *Rhodomonas sp.* was cultivated under continuous (turbidostat) conditions in lab-scale reactor systems (1.8L) under different temperature (15-20-25-30 °C) and light conditions (60-195-330-465-600 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Stable biomass production was observed under all conditions except experiments performed at 30 °C, which led to cell death. Under optimized growth conditions, high growth rates ($>1.0\text{d}^{-1}$) and high biomass production rates, up to $1.5 \text{ g l}^{-1} \text{d}^{-1}$ were obtained in this study. The biomass production rate reported here is more than 10-fold higher than values reported in literature on *Rhodomonas sp.*. The optimal temperature for maximal growth was found at $T=22-24 \text{ °C}$ under all light conditions. The maximum biomass yield on light ($Y_{x,ph} = 0.87 \text{ g mol}^{-1}$) was found at light levels between $110-220 \mu\text{mol m}^{-2} \text{s}^{-1}$. The fatty acid profile was only significantly influenced by temperature, with higher EPA and DHA contents at lower temperatures (15 °C). A total fatty acid (TFA) content of 8-10% of the total dry-weight was found for all tested conditions. The EPA content fluctuated between 9 - 16% of TFA and DHA content between 6 - 9% of TFA, only affected by temperature. A maximum EPA+DHA production rate of $114 \text{ mg l}^{-1} \text{d}^{-1}$ was obtained at 20°C and high light ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$) conditions.

3.1 Introduction

The microalgae *Rhodomonas sp.* is an important species in the aquaculture industry. It is used mainly as feed for copepod production, a live feed in aquaculture. *Rhodomonas sp.* has been shown to increase egg production, growth and survival rate of copepods [32, 55]. The biomass composition and specifically the DHA and EPA content of *Rhodomonas sp.* are often mentioned as key factors for using this algae in aquaculture applications [17, 27]. Despite the importance of *Rhodomonas sp.* for aquaculture, the growth parameters have not been well characterized or optimized. *Rhodomonas sp.* is described as difficult to maintain and unpredictable in mass cultivation [32].

A better understanding of the strain and the production process could greatly increase the use of *Rhodomonas sp.* in aquaculture. A significant improvement of biomass production rate and robustness is the first step towards more stable production of *Rhodomonas sp.* for aqua feed applications. This requires a better understanding of the effect of cultivation parameters (light and temperature) on the biomass yield on light, biomass production rate and the biomass composition, specifically the fatty acid composition.

Large-scale algae production aims at light limited production of algae while optimizing the biomass yield on light for maximum biomass production rates. In a light limited situation, the cultivation temperature and the available light will determine the maximum growth rate. Temperature and light are therefore the most relevant parameters to investigate for industrial cultivation of microalgae, assuming non-limiting conditions for all other growth parameters. It is therefore required that no nutrients are limiting during cultivation. This means that all macronutrients (nitrogen, phosphorus and CO₂) as well as trace-minerals are supplied in sufficient concentrations at all times of a given cultivation.

Although the effect of light and temperature on the growth rate of *Rhodomonas sp.* has been described in literature before [54, 56-60], most literature does not describe biomass production rates or biomass yield on light. In addition, the growth rates reported in literature are very different for similar temperatures and light intensities. This might be due to the fact that most studies are performed in shake-flask batch experiments with very limited or no control of cultivation parameters such as nutrients and pH (CO₂). Often these experiments are performed in the nutrient limited f/2 culture medium. Moreover, these studies are performed under very low light conditions with over 80% of the data found in literature working with light intensities lower than 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ [59-61]. The temperatures described in literature range from 5-35°C [56, 58, 62] with most work focusing at 19-20°C [33, 57, 63-65]. Evaluation of available data on *Rhodomonas sp.* in literature indicates a high probability that light is often not the limiting growth factor in these studies. Growth rates for experiments described at a temperature of 20°C and a light

intensity of $100 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ vary between 0.23 d^{-1} and 0.72 d^{-1} [54, 58, 60, 65, 66]. The large spread of growth rates, yields uncertainty for further implementation at large scale. The large fluctuations of data could as well partially be due to a result of the use of different strains as not all studies use the same strain. A standard for *Rhodomonas sp.* in research has not been established. However two studies comparing up to five different strains under identical conditions per study showed no differences in growth between difference strains of *Rhodomonas sp.* [54, 67].

Batch experiments in shake flasks are difficult to translate to industrial applications where most production is done using photobioreactors under continuous operation and controlled conditions. Data on continuous production of *Rhodomonas sp.* in photobioreactors is very limited in literature. Only two recent studies describe *Rhodomonas sp.* in larger scale reactor systems under continuous conditions in highly controlled artificial light conditions [67, 68] and one study reports a lab scale system with continuous conditions [69]. Thoisen et al [67] describe a tubular photobioreactor setup (200L) operating continuously with fixed biomass concentrations between $0.5\text{-}2.0 \times 10^6 \text{ cells ml}^{-1}$ at growth rates of 0.28 and 0.52 d^{-1} producing approximately $0.02\text{-}0.08 \text{ g l}^{-1} \text{ d}^{-1}$. Vu et al [68] describe a bubble-column (84L) for *Rhodomonas* production with a concentration of $2.4 \times 10^6 \text{ cells ml}^{-1}$ with a dilution rate of 0.46 d^{-1} achieving an average production rate of about $0.11 \text{ g l}^{-1} \text{ d}^{-1}$ under continuous operation. Studies with other algal species grown under continuous operation at pilot scale outdoors show much higher biomass production rates. Data of de Vree for chemostat and turbidostat operation of tubular reactors showed an average biomass production rate of $0.57 \text{ g l}^{-1} \text{ d}^{-1}$ up to $0.90 \text{ g l}^{-1} \text{ d}^{-1}$ using *Nannochloropsis sp.* under Dutch climate conditions [47, 50]. A pilot-scale experiment in Spain described in a study by Acién et al [45] showed a biomass production rate of $0.3 - 0.7 \text{ g l}^{-1} \text{ d}^{-1}$ depending on the solar irradiance variations over a year for *Scenedesmus sp.*. The large difference in volumetric biomass production rate between algae found in literature and currently available data for *Rhodomonas sp.* could indicate a large potential for improvement of the production of *Rhodomonas sp.* in industrial settings with optimized growth conditions.

This study aims to determine the combined effect of light intensity and cultivation temperature under continuous operation of *Rhodomonas sp.* using a larger range of conditions than described in literature. To characterize the effect of these growth parameters of *Rhodomonas sp.*, the alga was grown in highly- controlled lab-scale flat-plate photobioreactors under continuous (turbidostat) conditions. A Design of Experiment approach was applied using a D-optimal design for the selection of experimental conditions. The design space was selected to contain light conditions between $60\text{-}600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperatures between $15\text{-}30^\circ\text{C}$. The growth rate, biomass production rate and biomass yield on light as well as the fatty acid composition are determined for each combination of temperature and light.

3.2 Materials and Methods

3.2.1 Strains, cultivation medium and pre-culture conditions

Rhodomonas sp. was provided by the Dutch aquaculture industry, as being one of the strains used in commercial applications. The strain was characterized by 18S sequencing and confirmed to be *Rhodomonas sp.* (data not shown). Pre-cultures of *Rhodomonas sp.* were maintained in 250 ml Erlenmeyer flasks with 100 ml culture volume. Flasks were placed in an orbital shaker (100 rpm) maintained at 25 °C and low light conditions ($50 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$) applied 24 hours per day. The headspace was enriched with 2.5 % CO_2 for pH control maintaining a pH of 8.0 ± 0.5 . Cultures were used for the inoculation of experimental reactors after 10 days of batch cultivation in flasks. The growth media used was based on the L1-medium with an adjusted iron source [70]. Artificial seawater was used in all experiments. All nutrients were added in higher concentration than the L1-media to prevent limitations. FeCl_3 was substituted by lower concentrations ($27.8 \mu\text{mol l}^{-1}$) of NaFeEDTA to prevent precipitation. The complete growth medium and the artificial seawater contained final concentrations of: NaCl 420 mM; NaNO_3 17.6 mM; Na_2SO_4 22.5 mM; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 48.2 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 3.6 mM; K_2SO_4 4.9 mM;; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.7mM; NaFeEDTA 27.8 μM ; Na_2EDTA 0.30 mM; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 14.3 μM ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5 μM ; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.8 μM ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.2 μM ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 1.6 μM ; $\text{H}_2\text{SeO}_3 \cdot 2\text{H}_2\text{O}$ 0.2 μM ; $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ 0.2 μM ; Na_3VO_4 0.2 μM ; K_2CrO_4 0.2 μM ; Vitamin B1 7.5 μM ; Vitamin H 0.04 μM ; Vitamin B12 0.1 μM . The same growth media was used in reactor experiments and shake flasks with the exception of 20 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) and 10 mM NaHCO_3 , which were added as a pH buffer to flask cultures only. The pH of the growth medium was adjusted with NaOH to pH 7.5 and the media was filter sterilized prior to use (Sartobran 300 – pore size 0.2 μm).

3.2.2 Photobioreactor operation and experimental setup

Experiments were performed in an aseptic, heat-sterilized, flat-panel, photobioreactor (Labfors 5 Lux, Infors HT, Switzerland) with a total culture volume of 1.8 L. Mixing was applied by aeration of the culture with 900 mL min^{-1} filtered air (0.2 μm). CO_2 was injected on-demand to the airflow for pH control, with the pH maintained at pH 7.5. Culture temperature was controlled at the selected value for each experiment (15-20-25-30 °C) by recirculation of water through a water jacket in direct contact with the cultivation chamber of the reactor. The incident light intensity was provided by the integrated LED-light system of the Labfors 5 Lux. The 260 LED lights, evenly distributed over the reactor were calibrated

and the incident light intensity was set at the selected values for each experiment and supplied 24 hours per day (60-195-330-495-600 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Reactors were inoculated at a starting OD₇₅₀ of 0.15 ± 0.02 with *Rhodomonas* cultures from shake flasks. Reactor operation started with a batch phase, operated with a luminostat for light control, until the desired biomass concentration and light level were achieved for continuous operation. Not every experiment started from flask cultures, some experiments were started from a steady-state of a previous experiment by changing the reactor settings to new conditions for temperature and light. During continuous operations in turbidostat mode, on-demand dilution was applied to maintain the light at the backside of the reactor at the set-point (15 $\mu\text{mol m}^{-2} \text{s}^{-1}$) measured by the integrated light sensor (LI-250, Licor, USA). Under continuous dilution and constant conditions, the growth rate of the microalgae is equal to the dilution rate of the reactor under steady state. Reactors were sampled daily and operated until a steady-state was obtained under the turbidostat conditions before sampling for analysis of the fatty acid composition. Steady state was defined when the total dilution volume was equal to three times the reactor volume with unchanged biomass concentration and dilution rate over the time required to reach this dilution volume. Depending on the experimental settings, a steady-state was achieved between 10-15 days after the start of an experiment and required 3-6 days of stable conditions in the reactor system.

3.2.3 Experimental design

A Design of Experiment approach was used to select the experiments to be performed within the selected design space. The design space consists of a 2-factor design using 5 levels for each factor. The first factor is light intensity with values between 60-600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The second factor is cultivation temperature (15-30 °C). A D-optimal design was selected and it was created using the Umetrics MODDE 9.1 software package. This method was applied to minimize the required reactor runs while maximizing data output. The D-optimal design consist of a total of 15 experiments. The center point of the D-optimal design ($I_{ph} = 330 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $T=20 \text{ }^{\circ}\text{C}$) was run in 3 separate experiments. One of the experimental settings was run in duplicate ($I_{ph} = 60 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $T=15 \text{ }^{\circ}\text{C}$), with all other experimental settings only performed once, according to the D-optimal design. Using this approach, a total of 12 different experimental conditions were measured. A list of all experiments and corresponding settings for temperature and light can be found in Table 10. With no growth at 30 °C (see section 3.3) the use of 27.5 °C was tested.

3.2.4 Measurements (Growth, biomass production rate, biomass yield on light and fatty acid composition)

Daily samples were taken from each reactor to monitor culture growth. Biomass concentration, growth rate and biomass yield on light were determined daily. Data of four experiments in steady-state is presented in section 3.5.2.

Biomass concentration was determined by OD-measurements and cell-counts. Dry-weight was only determined at steady-state. A 2.0 ml sample was taken daily directly from the reactor volume. The optical density was determined, in duplicate, at 750nm using a UV-VIS spectrophotometer (Hach Lange DR-6000, light path 1cm). Cell count was determined, in duplicate, with a MultiSizer II (Beckman Coulter) using a 50 μm aperture tube after diluting the sample using the original growth medium. Growth medium was applied rather than Isotone as the latter influences cell size for *Rhodomonas sp.* (data not shown).

In steady-state the growth rate is equal to the dilution rate and is described by equation 3.1. The daily dilution rate (D) was determined by the mass decrease of the fresh medium vessel for inflow of medium between two measurements (V_{dilution}) and the total reactor volume (V_{reactor}) [35]. The vessel was placed on a balance and logged continuously.

$$\text{Equation 3.1: } \mu = D = \frac{V_{\text{dilution}}}{V_{\text{reactor}}}$$

The biomass yield on light ($Y_{x,ph}$ in g mol^{-1}) is calculated based on the daily dilution rate (D in d^{-1}), the biomass concentration in the reactor system (C_x in g l^{-1}) and the volumetric photon supply rate (r_{ph} in $\text{mol}_{ph} \text{ l}^{-1} \text{ d}^{-1}$) according to equation 3.2 [26].

$$\text{Equation 3.2: } Y_{x,ph} = \frac{D * C_x}{r_{ph}}$$

Rhodomonas sp. does not contain a cell wall making the cells too fragile for conventional dry-weight methods where cells are filtered and washed with ammonium formate on a filter. An adapted dry-weight protocol was established for dry-weight determination of *Rhodomonas sp.* in this study. The dry weight of the biomass was determined in triplicate by collecting a 10.0ml sample in a pre-dried and weighed glass tube. Cells were collected by centrifugation (10 minutes at 1200 rcf), the supernatant was stored for nutrient analysis and the pellet was washed by re-suspending in ammonium-formate (0.55M). Cell were collected by centrifugation (10 minutes at 1200 rcf) and supernatant was discarded. The glass tube containing the washed cell-pellet was dried, for at least 24hours, to a stable weight in a stove at 100°C to determine the final dry weight. A correlation between OD_{750} and the measured dry weight for all experiments was used for the estimation of dry weight at other time-points during the experiments. The OD-DW correlation is given in equation

3.3 (also see section 3.5.1) were DW is the dry-weight of the sample in g l⁻¹ and OD₇₅₀ is the optical density at 750nm measured according to the above described method.

$$\text{Equation 3.3: } DW = 0.7236 * OD_{750} - 0.0205$$

3.2.5 Fatty acid analysis

Samples for fatty acid analysis were collected and prepared according to Breuer et al using sample preparation method 1.1 and stored at -20 °C until extraction [71]. Fatty acid extraction and quantification was performed in duplicate for each reactor as based on the method as described by Breuer et al. [71]. The method was slightly adjusted as *Rhodomonas sp.* did not require extended cell disruption steps. In short: cells are disrupted by bead-beating and transferred to a glass tube using chloroform containing C₁₅-TAG internal standard. Chloroform was evaporated from the tube by a nitrogen gas stream. Fatty acids were methylated by incubation with methanol/H₂SO₄ (5%) at 70 °C for 3 hours and extracted using hexane. The fatty acids in the hexane phase were identified and quantified by gas chromatography (GC-FID) as described by Breuer et al. with the exception that the oven temperature was maintained at 200 °C for 54min to allow for EPA and DHA separation and detection [71].

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3.2.6 Data analysis

Data processing and visualization for growth rate, biomass production rate and biomass yield on light was performed in Sigmaplot V10.0. Contour plots are created to depict the results in 2D, showing the trends of each of the results as a function of temperature and light intensity. Only data of successful steady-state experiments are used for data analysis. For biomass yield on light data; Sigmaplot V10.0 was used to plot a regression and describe biomass yield on light as a function of the cultivation temperature and light intensity. All data used for regressions follow a normal distribution. The presented trends for growth rate, biomass yield on light and fatty acid composition cannot be extrapolated beyond the upper temperature limit given in the graphs. Observed trends might be extrapolated for higher light levels or lower temperatures, but have not been tested in our study. Statistical analysis of fatty acid data was performed using a two-tailed two-sample equal variance Student's T-test in Microsoft Excel.

3.3 Results and Discussion

All experiments showed stable and reproducible results under all conditions except for the experiments performed at 30 °C. No biofilm or fouling was observed for any of the experiments under steady state conditions. For experiments performed at 30 °C growth decreased daily until no growth was observed. As a result, no steady state could be obtained for conditions at 30 °C, similar behavior was found for reactors operated at 27.5 °C as for 30 °C (data not shown). All other temperature conditions (15-20-25 °C did show good growth and resulted in stable turbidostat operations. It is therefore concluded that *Rhodomonas sp.* cannot be cultivated under continuous conditions at 27.5 °C or above. The maximum temperature for *Rhodomonas sp.* should be between 25.0 and 27.5 °C but was not determined in this study. This inability to grow at higher temperatures is an unexpected result as literature describes growth studies with *Rhodomonas sp.* at 29 °C up to 33 °C [31, 54, 56, 72]. In our results, no immediate cell death was observed but the growth rate decreased daily over a 4-7 day period until growth stopped completely. All studies in literature reporting growth rates at higher temperatures were performed under batch culture conditions. With multiple days of a daily decreasing growth rate, a measurement for growth rate is possible under batch culture conditions when only measured at two time points but this growth behavior does not allow for a steady-state measurement under continuous conditions.

The center point of the D-optimal design ($T=20\text{ °C}$ and $I_{ph}=330\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) shows highly reproducible results as indicated by the small deviation found between the three biological replicates. For the three biological replicates, a biomass concentration of $1.05 \pm 0.02\text{ g l}^{-1}$ was found with a growth rate of $0.98 \pm 0.02\text{ d}^{-1}$ resulting in a biomass production rate of $1.03 \pm 0.02\text{ g l}^{-1}\text{ d}^{-1}$ and a biomass yield on light of $0.76 \pm 0.02\text{ g mol}^{-1}$. The duplicate experiment ($T=15\text{ °C}$ and $I_{ph}=60\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) shows slightly larger deviations between the two duplicate experiments at steady-states. This larger deviation is attributed to technical challenges associated to the very low incident light levels ($60\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) in the turbidostat mode. A biomass concentration of $0.18 \pm 0.02\text{ g l}^{-1}$ was obtained with a growth rate of $0.57 \pm 0.02\text{ d}^{-1}$ during steady state. This resulted in a biomass production rate of $0.11 \pm 0.01\text{ g l}^{-1}\text{ d}^{-1}$ with a biomass yield on light of $0.55 \pm 0.06\text{ g mol}^{-1}$. The results of the replicate experiments of the center point and indicated good reproducibility of all tested conditions also for conditions further away from the center of the experimental design.

3.3.1 Biomass concentration, Growth rate and Biomass production rate

A contour plot of the biomass concentration is shown in Figure 12A. The results show that the biomass concentration is almost exclusively affected by light intensity showing a positive correlation between light intensity and biomass concentration. This correlation is the direct result of the turbidostat settings as applied in the experiments. A higher incident light intensity with equal outgoing light in all experiments results in a higher biomass concentration.

Figure 12: Experimental results for biomass concentration (Figure 12A - g l⁻¹), growth rate (Figure 12B - d⁻¹) and biomass production rate (Figure 12C - g l⁻¹ d⁻¹) as a function of temperature (°C) and light intensity (μmol m⁻² s⁻¹) represented as 2D-countour plots.

The contour plot of Figure 12B shows the growth rate. It can be seen that both temperature and light intensity have a large effect on the observed growth rate. An optimum growth rate seems to be within the tested limits for the two conditions for light at 300-500 μmol m⁻² s⁻¹ and a temperature of 23-25 °C. The experiments closest to this optimum were performed at 25 °C with 330 and 600 μmol m⁻² s⁻¹ with growth rates of 1.10 and 1.02 d⁻¹, respectively. The effect of temperature on growth is clearly observed when comparing data of experiments at all different temperatures for high light conditions (600 μmol m⁻² s⁻¹). An increased growth rate is found for increased temperatures from 15 °C (0.55 d⁻¹) to 20 °C (0.94 d⁻¹) and 25 °C (1.02 d⁻¹). The maximum growth rate for experiments at 15 °C did not exceed 0.65 d⁻¹ regardless of the applied light intensity. All experiments at 15 °C showed very similar growth rates of 0.60 ± 0.05 d⁻¹ with almost no effect of light intensity. This indicates that under lower temperature conditions the growth of *Rhodomonas sp.* is limited by temperature rather than by light intensity. A clear effect of light intensity on the observed growth rate is found for higher temperature conditions at 25 °C with the maximum observed growth rate of 1.10 d⁻¹ under higher light conditions (> 330 μmol m⁻² s⁻¹) compared to the

lower values found under lower light conditions (0.43 d^{-1} at $60 \mu\text{mol m}^{-2} \text{ s}^{-1}$). It has to be noted that even though the data could suggest higher growth rates at even higher temperatures, this is not the case as *Rhodomonas sp.* did not show stable growth at 27.5°C and 30°C in our experiments. The maximum growth rate found in our study is higher than most values described in literature. Only batch cultivations are described in literature, which means that other factors than light and temperature might be limiting the growth rate. Most studies, including very recent work, on *Rhodomonas sp.* describe maximum observed growth rates between $0.5\text{--}0.75 \text{ d}^{-1}$ [57, 61, 65, 66]. Higher growth rates, similar to the maximum value found in our study are reported in some studies but only for a short period (1 or 3 days) in batch cultivation under very dilute culture conditions [59, 60]. The consistent high growth rates under continuous cultivation conditions has never been described before.

The biomass production rate (r_x in $\text{g l}^{-1} \text{ d}^{-1}$) was calculated from the growth rate (μ) and biomass concentration (C_x) and is shown in Figure 12C. The biomass concentration is mostly affected by light intensity, growth rate is positively affected by both light and temperature. Therefore, the biomass production rate also shows a positive influence from both temperature and light. Higher temperatures and higher light conditions result in higher biomass production rates within the given boundaries for temperature. The effect of temperature is mostly observed at higher light intensities ($>300 \mu\text{mol m}^{-2} \text{ s}^{-1}$) where a 65% higher biomass production rate is found for the $T=25^\circ\text{C}$ $I_{ph}=600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ($1.44 \text{ g l}^{-1} \text{ d}^{-1}$) compared to $T=15^\circ\text{C}$ $I_{ph}=600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ($0.87 \text{ g l}^{-1} \text{ d}^{-1}$), in similarity to the results for growth rate. No optimum is found for biomass production rate within the tested levels of light and temperature. Light conditions higher than those tested here will result in increased biomass production rate until photo inhibition occurs. Photo inhibition was not observed within the tested conditions in this study.

Very limited literature on *Rhodomonas sp.* under continuous cultivation conditions is available. Biomass production rates available in literature range between 0.02 and $0.11 \text{ g l}^{-1} \text{ d}^{-1}$ but were all obtained in large pilot-scale systems, which are more difficult to control. Patil et al [69] did describe a 1.8L lab-scale reactor with a production rate of $0.09 \text{ g l}^{-1} \text{ d}^{-1}$ in turbidostat mode under low light conditions. These results show that we were able to achieve a more than 10-fold increase, with $1.4 \text{ g l}^{-1} \text{ d}^{-1}$, for *Rhodomonas sp.* under continuous cultivation conditions. This large increase in biomass production rate compared to common practice found in literature is a very significant improvement for *Rhodomonas sp.* as a production strain for aquaculture.

The production rate of some other well-known microalgae strains for aquaculture application such as *Nannochloropsis sp.* and *Tetraselmis sp.* have been described in literature before. Pilot scale studies of *Nannochloropsis sp.* under outdoor conditions showed production rates

of $0.60 \text{ g l}^{-1} \text{ d}^{-1}$ and $0.90 \text{ g l}^{-1} \text{ d}^{-1}$ [47, 50]. A lab scale screening study of multiple species of *Nannochloropsis* described biomass production rates up to $0.766 \text{ g l}^{-1} \text{ d}^{-1}$ [73] *Tetraselmis sp.* has a described production rate of around $0.50 \text{ g l}^{-1} \text{ d}^{-1}$ under outdoor pilot-scale production [74]. *Phaeodactylum tricornutum* in literature has been described with biomass production at pilot scale of about $0.20 \text{ g l}^{-1} \text{ d}^{-1}$ using Norwegian sunlight conditions [75] and up to $1.38 \text{ g l}^{-1} \text{ d}^{-1}$ in a different study [76]. At lab scale *P. tricornutum* showed a biomass production rate $0.68 \text{ g l}^{-1} \text{ d}^{-1}$ at relatively low light conditions ($260 \mu\text{mol m}^{-2} \text{ s}^{-1}$) [77]. *Isochrysis sp.* was described with production rates of about $0.50 \text{ g l}^{-1} \text{ d}^{-1}$ under semi continuous lab experiments [78]. A literature study performed under very similar culture conditions (30°C and $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$) in the same lab scale reactor as described in our study showed a biomass production rate of $1.3 \text{ g l}^{-1} \text{ d}^{-1}$ compared to $1.4 \text{ g l}^{-1} \text{ d}^{-1}$ for *Rhodomonas sp.* at 25°C and $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ using *Neochloris oleoabundance* [25]. The results with *Rhodomonas sp.* in our study show that the biomass production rate of *Rhodomonas sp.* can be equal to other microalgae species for aquaculture under optimized growth conditions.

3.3.2 Biomass yield on light

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The biomass yield on light represents the efficiency of light conversion into biomass. Figure 13 shows the biomass yield on light (z-axis) as a function of the temperature (x-axis) and light intensity (y-axis). The experimental data is displayed as black dots. A Gaussian regression showed the best fit through all data-points and is depicted with the 3D-field in Figure 13. The regression has an R^2 of 0.97 passing all statistical tests. From this regression, the optimal conditions and maximum values for biomass yield on light were calculated. The predicted maximum biomass yield on light for *Rhodomonas sp.* is calculated at 0.87 g mol^{-1} for temperatures between $22\text{--}24^\circ \text{C}$ with light intensities between $110\text{--}220 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The results for biomass yield on light clearly show a negative correlation between the biomass yield on light and light intensity. This correlation is expected and has been described in literature before at different scales [25, 47, 50].

Comparison of the biomass yield on light for *Rhodomonas sp.* from this study with other, more commonly used, algae species in literature shows that *Rhodomonas sp.* performs very similarly to other algae strains. De Winter found a biomass yield on light of 0.66 g mol^{-1} for *Neochloris oleoabundance* at 30°C and $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ using the same lab scale reactor [25]. The regression shows a predicted biomass yield on light for *Rhodomonas sp.* of 0.65 g mol^{-1} at equal light conditions at 25°C .

Figure 13: Biomass yield on light (g mol⁻¹) as a function of light intensity (μmol m⁻² s⁻¹) and temperature (°C). Black dots: measurements of individual experiments. Grey field: Gaussian regression through all datapoints.

The negative impact of lower temperatures on *Rhodomonas sp.* as found for growth rate and biomass production rate is also clearly found for biomass yield on light. A predicted 33–40% reduction of biomass yield on light was calculated at 15 °C when compared to 24 °C, at equal light conditions. The effect of temperature on biomass yield on light increases with increasing light intensities, indicating the need to grow *Rhodomonas sp.* at 22–24 °C for maximum biomass yield on light. The optimum light intensity for biomass yield on light is higher (110–220 μmol m⁻² s⁻¹) than what is used in most literature (mostly 20–80 μmol m⁻² s⁻¹) on *Rhodomonas sp.*, suggesting that studies performed under these low light conditions are performed under sub-optimal light conditions. This could explain the low growth rates described for *Rhodomonas sp.* in literature [32, 79, 80]. It has to be noted that although the highest biomass yield on light is found at 110–220 μmol m⁻² s⁻¹, these conditions will not lead to the most optimized *Rhodomonas* production at large scale due to the relatively low biomass production rates found at these light levels as can be seen from data for biomass production rate (Figure 12C).

3.3.3 Fatty acid composition

The fatty acid composition of *Rhodomonas* sp. in all experimental conditions is summarized in Figure 14, detailed results of total fatty acid composition are shown in Table 11. The total fatty acid content (TFA) of *Rhodomonas* sp. in all experiments fluctuates between 78 and 95 mgFA gDW⁻¹. A significant effect of the cultivation temperature on the total fatty acid content ($P < 0.05$) and composition ($P < 0.05$) was found whereas light intensity did not show a significant effect on the fatty acid content or composition. Figure 14 represents the average fatty acid composition and profile of *Rhodomonas* sp. for all experiments performed under equal temperatures. The error bars depict the standard deviation of all experimental conditions (varying light intensities) with the given cultivation

Figure 14: Fatty acid composition of *Rhodomonas* sp. as grouped in 4 categories, SFA: Saturated fatty acids, MUFA: mono-unsaturated fatty acids, PUFA: poly unsaturated fatty acids excluding EPA and DHA and EPA+DHA. Values depict the average over all experiments of the same temperature but performed under different light intensities (15°C n=4, 20°C n=5, 25°C n=3) error bars show the deviation between samples of different light intensities.

temperature. The fatty acid profiles are summarized in four categories of fatty acids; saturated fatty acids (SFA) mono-unsaturated fatty acids (MUFA) poly unsaturated fatty acids (PUFA) excluding eicosapentaenoic acid (C20:5 - EPA) and docosahexaenoic acid (C22:6 - DHA) and EPA+DHA as a separate category (EPA+DHA). The fatty acid profiles show a high degree of unsaturation for all temperatures with a total 72-80% of TFA found as PUFAs and EPA+DHA. The content of EPA ranges between 9 - 16% of TFA and the DHA between 6 - 9% of TFA for the different temperatures. No significant changes in SFA, MUFA and PUFA are found between different temperatures or light intensities between any of the tested conditions ($P > 0.05$). The temperature shows a significant effect on the EPA+DHA fraction only ($P < 0.05$) but light does not significantly influence this fraction ($P > 0.05$). The effect on total fatty acid content between the different cultivation temperatures is therefore exclusively the result of the change in EPA+DHA content at different temperatures. Figure 15 summarizes the correlation for both EPA and DHA content in the biomass (mgFA gDW⁻¹) as a function of the cultivation temperature with linear regressions. The regressions have an R^2 of 0.89 and 0.90 for EPA and DHA, respectively ($P < 0.01$). A very similar distribution

of the fatty acid classes, with a strong effect of temperature on the total PUFA fraction in *Rhodomonas* sp. has been described in literature [68, 79-81]. However, the total fatty acid content in literature fluctuates greatly depending on culture conditions but is typically higher ($150 - 250 \text{ mgFA gDW}^{-1}$) than the average of 83 mgFA gDW^{-1} found in our study. Fernandes et al [79] describes a very comparable fatty acid composition and content and TFA content (71 mgFA gDW^{-1}) under nutrient replete conditions but shows a large effect of reduced

nutrients conditions on the TFA content increasing to $150 \text{ mgFA gDW}^{-1}$ of dry weight. The relatively low TFA-content found in our study could therefore be explained by the fast growing cells under continuous cultivation conditions compared to the TFA content found for potentially nutrient limited batch cultures described in literature [79, 82]. Studies that show comparable TFA content to this study also report similar EPA and DHA content in %TFA [67, 68, 79, 81].

A large change in PUFA composition occurs with changing temperatures (detailed results found in Table 11). Experimental conditions at 15°C show a higher degree of unsaturation in the PUFA fraction, mainly represented in the C18:2, C18:3 and C18:4 fractions. With C18:2 increasing from $5.2 \pm 0.8\%$ to $25.4 \pm 1.0\%$ of total PUFAs from 15°C to 25°C experiments respectively, compared to C18:4 which decreases from $32.1 \pm 2.4\%$ of total PUFAs at 15°C to $19.5 \pm 2.3\%$ under 25°C conditions. Similar correlations between cultivation temperature and the fatty acid composition has been described in literature for other algae strains and cyanobacteria [83-85]. The effect of temperature on the degree of saturation has been found in multiple studies for other aquaculture related algae species such as *Isochrysis* sp. [86, 87]. Aussant et al [30] describe a similar trend for the saturation of fatty acids as a result of growth under non-optimal temperatures for multiple algal species including *Rhodomonas salina*. Hoffmann et al [88] describe that the increase of TFA and degree of saturation under lower temperatures could be the result of temperature induced limited nitrogen uptake. It has also been suggested that the increased content of PUFAs at low temperatures compensates for reduced membrane fluidity under lower temperatures, even though this was never proven in detail [89].

Figure 15: Regression lines for the EPA (black circles) and DHA (white circles) content as a function of temperature. Circles show individual measurements of all experimental conditions. Solid line is the regression and dotted lines indicated the 95% confidence interval.

3.3.4 Total fatty acid and EPA+DHA production rate

The production rates ($\text{mg l}^{-1} \text{d}^{-1}$) of total fatty acids and EPA+DHA are presented in Figure 16. The TFA-production rate (Figure 16A) follows the same pattern as the biomass production rate with an effect of both temperature and light intensity. The highest TFA-production rate ($114 \text{ mg l}^{-1} \text{d}^{-1}$) is found at the highest temperature and light condition tested ($T=25 \text{ }^{\circ}\text{C}$ and $I_{\text{ph}}=600 \text{ } \mu\text{mol m}^{-2} \text{s}^{-1}$). The EPA+DHA production rate shows an optimum at around $20 \text{ }^{\circ}\text{C}$, with the effect of temperature only clear at higher light intensities. The maximum EPA+DHA production rate measured in our experiments was $24 \text{ mg l}^{-1} \text{d}^{-1}$ at $T=20 \text{ }^{\circ}\text{C}$ and $I_{\text{ph}}=600 \text{ } \mu\text{mol m}^{-2} \text{s}^{-1}$. Even though the maximum EPA+DHA content is observed at the lowest experimental temperature of $15 \text{ }^{\circ}\text{C}$ (Figure 15) the EPA+DHA production rate decreases at lower temperatures as a result of the lower growth rates for these temperatures (Figure 12B). The decrease of EPA+DHA production rate at higher temperatures ($25 \text{ }^{\circ}\text{C}$) is the result of higher growth rates with a decreased EPA+DHA content in the biomass. Values for the EPA or DHA content of *Rhodomonas sp.* are available in literature but production rates under continuous cultivation conditions and the clear effect of temperature on EPA+DHA production rate have not been described before. The effect of temperature on fatty acid content and production rate described in our study could be used to effectively select culture conditions for highest nutritional value of the biomass in aqua feed applications while achieving maximum production.

Figure 16: TFA production rate (Figure 16A - left) and EPA+DHA production rate (Figure 16B - right) in $\text{mg l}^{-1} \text{d}^{-1}$ plotted as a function of Temperature (x-axis $^{\circ}\text{C}$) and light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)

3.4 Conclusion

Rhodomonas sp. was successfully cultivated under continuous conditions with stable biomass production rates and high growth rates ($>1.0 \text{ d}^{-1}$) under optimal conditions. The obtained biomass production rates ($0.5\text{-}1.5 \text{ g l}^{-1} \text{ d}^{-1}$) show more than 10-fold increase from values currently available in literature for *Rhodomonas sp.*. This improved biomass production rate was possible due to optimization of both light and temperature without any other limitations to the culture. This optimization could be a significant step towards industrial implementation of *Rhodomonas sp.* production for aquaculture feed applications. The production rates obtainable with *Rhodomonas sp.* are comparable with more commonly used algal strains in aquaculture such as *Nannochloropsis sp.* and *Tetraselmis sp.* in similar reactor systems. The highest biomass production rate is found at temperatures of $25 \text{ }^{\circ}\text{C}$ and high light conditions but no stable cultivation of *Rhodomonas sp.* was obtained at higher temperatures in the tested conditions. For all tested temperatures, the total biomass production rate increases with increasing light intensities, showing no indications of light inhibition of *Rhodomonas sp.* within the tested light levels ($60\text{-}600 \mu\text{mol m}^{-2} \text{ s}^{-1}$). The fatty acid composition and content of the biomass is significantly influenced by temperature only and not by light intensity. Fatty acid composition shows a strong correlation between cultivation temperature and degree of saturation with a higher degree of saturation found at higher temperatures. The total-fatty acid production rate follows the same pattern as the biomass production rate with a maximum at higher temperatures and high light conditions. The EPA+DHA content, however, has an optimum at $20 \text{ }^{\circ}\text{C}$ at all tested light intensities. These results show that stable production of *Rhodomonas sp.* at high production rates and with high EPA and DHA content is possible under lab scale conditions. The results provide valuable information moving towards large-scale production of this species for aqua feed applications.

Table 10: Experimental conditions and results on the growth of *Rhodomonas* sp. under different combinations of temperature and light intensities. All values represent measurements during steady-state. Biomass concentration represented the calculated biomass concentration as based on the OD-DW ratio given in equation 3.3.

Temperature	Light intensity	Biomass concentration		Growth rate	Biomass production rate	Biomass yield on light
°C	$\mu\text{mol m}^{-2} \text{s}^{-1}$	g l^{-1}	cells ml^{-1} (million)	$\mu \text{ (d}^{-1}\text{)}$	Rx ($\text{g l}^{-1} \text{d}^{-1}\text{)}$	$Y_{x,ph} \text{ (g mol}^{-1}\text{)}$
15.0	60	0.19	1.65	0.59	0.11	0.59
15.0	60	0.18	1.53	0.56	0.10	0.51
15.0	465	1.37	10.54	0.65	0.87	0.45
15.0	600	1.59	12.18	0.55	0.87	0.34
20.0	195	0.75	5.78	0.90	0.68	0.88
20.0	330	1.06	8.32	0.97	1.03	0.76
20.0	330	1.03	7.83	1.01	1.06	0.78
20.0	330	1.04	7.82	0.97	1.01	0.74
20.0	600	1.41	11.32	0.94	1.35	0.53
25.0	60	0.36	2.98	0.43	0.15	0.79
25.0	330	0.99	7.64	1.10	1.10	0.81
25.0	600	1.41	11.25	1.02	1.44	0.57
27.5	60-195-330	-	-	-	-	-
30.0	60-195-465	-	-	-	-	-

Table 11: Fatty acid content of Rhodomonas sp. cultivated under different combined conditions for temperature (°C) and light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$). Fatty acids are represented as mgFA gDW⁻¹ and fatty acids classes give as %TFA (SFA: Saturate Fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Poly-unsaturated fatty acids with EPA+DHA as a separate class).

T (°C)	15	15	15	15	20	20	20	20	20	25	25	25
Iph ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	60	60	465	600	195	330	330	330	600	60	330	600
C12:0	0.4	0.3	0.0	0.2	0.0	0.0	0.0	0.0	0.2	0.0	0.3	0.3
C14:0	7.6	6.0	5.1	5.4	5.7	4.5	5.5	4.2	4.8	6.1	4.8	4.6
C16:0	7.0	6.3	9.8	8.8	7.5	9.5	9.1	8.3	9.3	7.5	9.3	10.6
C16:1	1.3	1.1	0.8	0.9	1.1	1.0	1.0	1.0	0.9	1.0	1.0	0.8
C16:2	0.8	0.7	0.4	0.2	1.1	0.6	0.7	0.1	0.2	2.4	0.5	0.4
C16:3	0.3	0.6	0.2	0.4	0.3	0.3	0.4	0.4	0.4	0.0	0.3	0.4
C18:0	0.0	0.0	0.1	0.1	0.0	0.1	0.1	0.0	0.1	0.0	0.1	0.1
C18:1	5.4	4.2	2.5	1.8	4.1	4.8	4.6	4.2	3.9	2.1	3.6	3.6
C18:2	3.9	2.7	4.3	3.9	5.7	6.1	6.2	5.6	5.1	15.5	14.2	15.4
C18:3	23.8	25.0	20.6	19.9	20.0	19.7	19.7	19.1	22.3	21.5	15.8	14.5
C18:4	24.0	24.4	20.2	21.6	18.7	18.3	18.2	20.3	18.6	9.8	13.0	11.9
C20:1	0.0	0.0	0.6	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C20:3-n3	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.8	0.0	0.0	2.3	2.5
C20:5-n3 - EPA	13.1	13.7	12.2	13.4	9.3	9.9	9.6	9.7	10.7	5.4	7.2	8.6
C22:6 - DHA	8.4	7.5	7.4	7.7	6.0	6.6	6.5	6.3	6.9	5.5	5.1	5.5
TFA (mg gDW-1)	95.8	92.3	84.2	84.6	79.4	82.7	81.5	80.0	83.4	76.9	77.4	79.1
Σ SFA (%TFA)	16	13	16	15	14	15	15	13	15	14	15	16
Σ MUFA (%TFA)	7	5	4	3	5	6	6	5	5	3	5	5
Σ PUFA excl. EPA+DHA (%TFA)	55	56	48	48	48	48	47	48	49	51	48	47
Σ EPA+DHA (%TFA)	22	22	20	22	16	17	17	17	18	11	13	15

3.5 Supplementary files

3.5.1 OD750 – DryWeight correlation of *Rhodomonas sp.*

Figure 17: Plot of data for the OD-DW correlations for *Rhodomonas sp.*, representing data points from steady-state experiments.

3.5.2 Raw-data from four steady-state reactors

Figure 18: Data from four of the steady-state reactors with daily measurements of three experimental conditions. OD_{750} (top left), cell-count (top right), growth rate (bottom left) and calculated biomass yield on light from these values (bottom right). Two centre point experiments are represented with one experiment started from a batch luminostat process (DOE #1) and a steady-state after a previous experiment (DOE #13). One experimental dataset of an experiment at higher light conditions ($I_{ph} = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $T = 20^\circ\text{C}$ - DOE #6) and one experiment at a high temperature ($I_{ph} = 195 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $T = 30^\circ\text{C}$ - DOE #7).

Chapter 4

Growth and fatty acid content of *Rhodomonas* sp. under day:night cycles of light and temperature

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Abstract

The biomass composition of microalgae used in aqua feed is of great importance for the nutritional value of the biomass. Day:night cycles of light and temperature could influence the growth and biomass composition of microalgae. To study the effect of these cycles on *Rhodomonas sp.* the algae were grown under 16:8 day:night cycles in lab-scale photobioreactors running as turbidostat. Different temperature and light intensities were applied during the light phase. Synchronized cell cycles were observed for *Rhodomonas sp.* under day:night conditions with oscillating cell size, cell number, biomass concentration and fatty acid content and composition. Cells increase in size during the light phase, storing energy, with cell division scheduled in the dark phase.

The introduction of a 16:8 day:night cycle did not affect the biomass yield on light, when operating at optimal conditions of light ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$) and temperature (21°C). However, under high light ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$) or temperature (25°C), an increased biomass yield on light of up to 22% was found under day:night cycles in comparison to continuous conditions under equal light and temperature levels. Implementation of a day:night cycle increased the maximum daytime temperature for *Rhodomonas sp.* from 25°C to 30°C . The fatty acid content and composition was influenced by the implementation of day:night cycles. Daily fluctuations in total fatty acid content from $76 \pm 2 \text{ mg gDW}^{-1}$ at the end of the light phase to $94 \pm 2 \text{ mg gDW}^{-1}$ in the first hours of the light phase are found. The eicosapentaenoic acid and docosahexaenoic acid content fluctuated by 30% ($12.1 - 16.1 \text{ mgEPA+DHA gDW}^{-1}$) on a daily basis. These daily fluctuations can be exploited in aqua feed applications by selecting a specific time of the day to harvest algae.

4.1 Introduction

Rhodomonas sp. is an important microalga for aquaculture as it is often used in live feed applications, specifically for copepods [32, 90]. Applications of *Rhodomonas sp.* as copepod feed require a lower minimal feed concentration ($1.13 \mu\text{g DW ml}^{-1}$) than other algae species such as *Isochrysis sp.* ($2.02 \mu\text{g DW ml}^{-1}$) and *Pavlova salina* ($1.86 \mu\text{g DW ml}^{-1}$) [32]. A higher development index of the copepods is reached when feeding *Rhodomonas sp.* compared to any other algae species tested [32], whereas copepods grown on *Tetraselmis sp.* show fast growth but also development of growth anomalies [32, 91]. The positive effects of *Rhodomonas sp.* on copepod development is mainly due to the high requirements of fatty acid content in copepods during transformation and egg-production. *Rhodomonas sp.* is used as feed source in copepod production processes because they contain high levels of the essential fatty acids for copepod development; both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [20, 55, 92, 93]. These fatty acids are specifically essential for fecundity, egg-production and copepod transformation [94]. Furthermore the biomass composition and therefore nutritional value of copepods can be manipulated by the microalgae diet [20]. Matching the nutritional value of *Rhodomonas sp.* with nutritional requirements of copepods is therefore of great importance for copepod production [7, 95]. Optimization of *Rhodomonas sp.* cultivation towards favorable fatty acid composition could benefit copepod production. *Rhodomonas sp.* shows a high content for both EPA and DHA whereas other strains contain only EPA or almost exclusively DHA [30]. A study comparing the fatty acid content of eight strains for aquaculture showed only three were able to produce DHA. *I. galbana* showed a higher DHA content ($15\text{--}20 \text{ mgDHA gDW}^{-1}$) than *Rhodomonas salina* ($5\text{--}15 \text{ mgDHA gDW}^{-1}$) but with very low EPA concentration in *I. galbana* ($<2 \text{ mgEPA gDW}^{-1}$) compared to *Rhodomonas salina* ($15\text{--}20 \text{ mgEPA gDW}^{-1}$) [30]. This EPA and DHA content for *Isochrysis* is confirmed in multiple other studies [31, 79, 82]. Other microalgae strains used in aquaculture such as *Nannochloropsis sp.*, *Tetraselmis sp.* and *Chaetoceros sp.* were shown to produce only EPA and no DHA [30, 31, 79, 82].

For industrial production of microalgae, productivity, biomass yield on light and fatty acid composition are influenced by light intensity and cultivation temperature. The combined effect of these two parameters under continuous conditions, with both light and temperature at a constant level for 24h day^{-1} , has recently been described in detail [96]. Daily fluctuation of these parameters could influence the biomass composition significantly as cell components are used for energy storage [26, 34, 36]. When algae are produced at large scale under sunlight conditions a day:night cycle for light intensity occurs and potentially temperature oscillations will also occur, depending on the cultivation system and location.

A day:night cycle of light has a positive effect on the biomass yield on light for multiple microalgae strains. León-Saiki & Remmers et al showed an increase of the biomass yield on light of approximately 20% under day:night cycles compared to continuous light cultivation for *Acutodesmus obliquus* [26]. De Winter et al showed a 10-15% increase in biomass yield on light for *Neochloris oleobundans* grown under a day:night cycles compared to continuous light conditions [25, 35]. The increase of the biomass yield on light is the result of a lower efficiency of light use during cell division than during other parts of the cell cycle. By synchronizing the cell division with the dark cycle, the overall biomass yield on light increases compared to continuous conditions [97]. Furthermore, it has been shown that during a day:night cycle the biomass composition of strains described in literature fluctuates significantly as the cells synchronize the cellular processes [26, 98]. Cells under day:night cycles store energy rich components during the light period and utilize energy stored in these components during the dark phase when energy intensive and light sensitive processes like cell division take place [25, 26, 36, 99, 100]. The best described example of this process of diurnal energy storage is the use of starch in *Acutodesmus obliquus* [26]. An identical daily fluctuation of starch is found in *Chlorella sp.*, with contents varying between 13-45% of the biomass dry-weight depending on the time of the day [100]. Another study describes that content of other cell components, such as fatty acids, could be closely related to cell division processes. The fatty acid content has shown a daily fluctuation in *Nannochloropsis oceanica* [36]. A study of *Neochloris oleobundans* with synchronized cell cycles showed a daily fluctuation in starch, fatty acid and protein content even under continuous illumination [34]. The fatty acid content of *Isochrysis sp.* was dynamic under day:night cycles [99]. It is therefore hypothesized that the implementation of a day:night cycle for *Rhodomonas sp.* could affect the nutritional value of the biomass significantly. Understanding the effect of daily fluctuations of the biomass composition over a day:night cycle could result in improved nutritional value of the produced microalgae, namely for the essential feed ingredients, EPA and DHA content. Therefore, in this study we evaluate the effect of day:night cycles of both light and temperature on *Rhodomonas sp.*, focusing on the biomass yield on light, biomass production rate and fatty acid composition; specifically, the EPA and DHA content of the cells.

4.2 Materials and Methods

Rhodomonas sp. was grown in a 16:8 day:night cycle for different light and temperature oscillations. A day:night cycle (for both light and temperature) is compared with a growth experiment without any fluctuations, the baseline experiment, and compared to cells experiencing a day:night cycle for temperature or light only. The effect of the day:night

cycle is investigated at more extreme conditions with higher light ($600\mu\text{mol m}^{-2} \text{s}^{-1}$) and higher daytime temperature conditions (25°C and 30°C). Finally one full day:night cycle was measured in detail to obtain insight in the daily fluctuations of the biomass composition. A detailed list of all experimental conditions is given in Table 12.

4.2.1 Strain, cultivation medium and culture conditions

Rhodomonas sp. was obtained from commercial production in Dutch aquaculture and characterized by 18S sequencing as *Rhodomonas sp.*. Cultures were maintained in Erlenmeyer flasks placed in an orbital shaker (100rpm) maintained at 25°C and 2.5% CO_2 enriched air with low light conditions ($80 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$) under a 16:8 day:night cycle. Cultures were used for the inoculation of experimental reactors after 7-10 days of batch cultivation in flasks. The growth medium for reactor experiments was based on the L1-medium with an adjusted iron source and used in combination with artificial seawater [70]. Changes to the medium and concentration of the nutrients was done as described by Oostlander et al. [96].

4.2.2 Photobioreactor operation and experimental setup

Reactor experiments were performed in a heat-sterilized, flat-panel, photobioreactor (Labfors 5 Lux, Infors HT, Switzerland) with a total working volume of approximately 1.8L. Aeration of the culture with 0.9 L min^{-1} filtered air ($0.2\mu\text{m}$) was applied for mixing and CO_2 was injected on-demand to the airflow for pH control. The pH was maintained at 7.5. Cultivation temperature was controlled through a water jacket in direct contact with the cultivation chamber of the reactor with temperature profile controlled by the control computer. The incident light intensity was provided by the integrated LED-light system of the Labfors 5 Lux. A shroud between the reactor and light panel combined with a dark cover at the backside of the reactor protected the culture from any environment light entering the reactor. Light was applied 24hours per day or as 16:8 day:night cycles with a block pattern as described in section 4.2.3. *Rhodomonas sp.* was inoculated from flask cultures to a starting OD_{750} of 0.15 ± 0.05 .

Reactor operation started with a batch phase for biomass production followed by turbidostat operation under the experimental conditions to be tested, a summary of all experimental conditions is given in Table 12. During turbidostat operation, on-demand dilution with fresh culture medium is applied to maintain the light leaving the reactor at $15 \mu\text{mol m}^{-2} \text{s}^{-1}$, measured by the integrated light sensor (LI-250, Licor, USA). Turbidostat operation was only applied during the light hours of the experiments. Daily samples were taken and

measured at sunset ($t=16$ hours after sunrise) and at a fixed time of the day if continuous light was applied. Daily measurements consisted of determination of biomass concentration and daily dilution rate. Daily measurements were continued until steady-state was reached. A reactor was defined to be in steady-state when a stable dilution rate was observed with a stable biomass concentration for the duration of at least 3 hydraulic retention times. Depending on the experimental settings, steady-state was achieved between 7-20 days after the start of the experiment. At steady-state the biomass concentration dry-weight and fatty acid composition of the biomass were determined.

4.2.3 Experimental design

Eight different combinations of growth parameters were applied in the reactor experiments. Each experimental condition was performed in duplicate. The first four experiments compare day:night (DN) oscillations of only temperature (Base-Temp, BT), only light (Base-Light, BL) and both light and temperature (DN-BT-BL) with the baseline experiment (Baseline) in which no oscillations are applied. The growth conditions of the

Figure 19: Schematic representation of the light and temperature oscillations applied during reactor operation.

baseline experiments were set at $I_{ph} = 150 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $T = 21.0^\circ\text{C}$, previously determined as the values for maximum biomass yield on light for *Rhodomonas sp.* under continuous conditions [96]. A schematic representation of the light and temperature profile in the reactors for oscillation situations is given in Figure 19. The solid line shows the daily oscillations of the light level. Light was applied in a block form (on/off) with a 16:8 day:night period and at baseline level ($BL = 150 \mu\text{mol m}^{-2} \text{s}^{-1}$) or at high light ($HL = 600 \mu\text{mol m}^{-2} \text{s}^{-1}$). Temperature oscillations (dotted line Figure 19) maintain a day and night temperature at fixed levels according to the experimental settings and the depicted pattern. For all temperature oscillation experiments the night temperature is maintained at 15°C with a day temperature at three different experimental levels. Baseline temperature ($BT = 21^\circ\text{C}$), High temperature ($HT = 25^\circ\text{C}$) or at extreme temperature levels ($XT = 30^\circ\text{C}$). These temperature levels were selected based on previous data that showed optimized biomass yield on light for baseline temperature. The upper temperature limit previously showing successful growth is set as the high temperature (25°C). Extreme temperature conditions

(30°C) for *Rhodomonas sp.* were proven as unviable growth conditions when applied continuously. The XT-experiments test the hypothesis that tolerance to these higher temperatures increased under day:night cycles. A gradual increase/decrease of temperature between the temperature during the day and night temperature was applied. This temperature transitioning period was kept constant (2 hours) for all experimental conditions, regardless of the ΔT between the two temperature level, as depicted in Figure 19.

Table 12: Growth conditions of all eight experimental conditions and experiment names. Experimental names represent the baseline experiment and deviations from the baseline values for light (BL) as high light values (HL) and from the base temperature (BT) as high temperature (HT) and extreme temperature (XT)

Experiment name	Light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Temperature day (°C)	Photon supply rate r_{ph} ($\text{mol}_{\text{ph}} \text{l}^{-1} \text{d}^{-1}$)
Baseline	150	21	0.58
BL	150	21	0.39
BT	150	21	0.58
DN-BL-BT	150	21	0.39
DN-HL-BT	600	21	1.68
DN-HL-HT	600	25	1.68
DN-BL-XT	150	30	0.39
DN-HL-XT	600	30	1.68

4.2.4 Detailed 24-hour cycle

A detail analysis of the daily fluctuations of the fatty acid composition was performed using the growth parameters of the experiment with high light ($I_{\text{ph}}=600 \mu\text{mol m}^{-2} \text{s}^{-1}$) and high temperature (25°C). These settings allowed for the largest oscillations in both growth parameters and are expected to show the largest fluctuations in biomass composition. For practical reasons two reactors were operated with day:night cycles set out of synchronization by 12 hours rather than one reactor where the full cycle was measured over 24hours. Both reactors were sampled every two hours during a 14hour time period. Sampling was performed for two separate days during steady-state in both reactor systems. A two-sample overlap, sample $t=11\text{h}$ and $t=23\text{h}$, between the two reactor systems shows the reproducibility between the two systems. All samples were analyzed for biomass concentration in OD_{750} , dry-weight, cell-count, cell-size and fatty acid content and composition.

4.2.5 Measurements and calculations

Daily measurements for biomass concentration and calculations for growth rate and biomass yield on light were performed. A 2.0 ml sample was taken directly from the reactor to measure optical density, in duplicate, at 750nm using a UV-VIS spectrophotometer (Hach Lange DR-6000, light path 1cm). The cell count was determined, in duplicate, with a MultiSizer II (Beckman Coulter) using a 50µm aperture tube after diluting the sample using the original growth medium. The daily dilution rate was calculated from the difference in mass of the medium vessel used for dilution of the reactor, during one day, and the total reactor volume, given in equation 4.1.

$$\text{equation 4.1: } D_{24h} = \frac{V_{\text{harvest } 24h}}{V_{\text{reactor}}}$$

The biomass concentration and fatty acid content were determined at steady-state. Dry-weight was measured as described by Oostlander et al [96]. Fatty acid extraction and quantification were performed using GC-FID analysis based on the method described by Breuer et al with samples collected according to method 1.1 [71]. The extraction method was adjusted for *Rhodomonas sp.* according to Oostlander et al. as no extended cell disruption is required [96].

The biomass yield on light ($Y_{x,ph}$) was calculated using the dilution rate (D_{24h}), the biomass concentration in the reactor system as measured at the time of sampling in steady-state (C_x) and the photon supply rate (r_{ph}), as described by Leon-Saiki & Remmers et al. and equation 4.2 [26]. Values for biomass yield on light are given as average over all days within the defined steady-state.

$$\text{Equation 4.2 : } Y_{x,ph} = \frac{D_{24h} * C_x}{r_{ph}}$$

4.2.6 Statistical analysis

Statistical analysis is performed by one way ANOVA tests and two sample T-test with equal or unequal variance with the variance tested using an F-test by Microsoft Excel. The standard error of the mean or standard deviations are shown in all graphs, depending on the total sample size (see figure captions).

4.3 Results and Discussion

4.3.1 Effect of day:night cycle on biomass yield on light

The results of the measured biomass yield on light are given in Figure 20. Values represent the average of all days under steady state as measured in duplicate reactors with the standard deviation over all measurements. The biomass yield on light for the baseline experiment with continuous growth conditions for both light and temperature showed a biomass yield on light of $0.87 \pm 0.03 \text{ g mol}^{-1}$. This correlates exactly to the expected value as described in previous work [96]. A day:night cycle for only light, but with a constant temperature (BL), only temperature fluctuations but with continuous light (BT) or a full day:night cycle for both temperature and light (DN-BL-BT) did not show a significant difference for the biomass yield on light compared to the Baseline experiment ($P>0.05$). The biomass yield on light of the full day:night cycle experiment (DN-BL-BT) shows a slight but not significant increase from $0.87 \pm 0.03 \text{ g mol}^{-1}$ to $0.91 \pm 0.07 \text{ g mol}^{-1}$

Figure 20: biomass yield on light results for *Rhodomonas* sp. grown under different day:night cycle conditions for light and temperature. Values show average values, error bars depict standard deviation ($n=8$ or $n=10$). Means with the same letter are not significantly different ($P>0.05$)

($P>0.05$). This increase is not significant as a result of the relatively large standard deviation found on the experimental results. The results of these four experiments performed under baseline light ($150 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and temperature (21°C) conditions show no effect of a day:night cycle when operating at the optimum conditions for biomass yield on light. It is suggested that under these light conditions there is no negative effect of the light intensity on the cell division processes or other light sensitive processes in the cell. Therefore, the addition of a dark cycle and the synchronization of the cellular processes does not result in an increase of the biomass yield on light. Literature describes an increased biomass yield on light for all available studies on the effect of a day:night cycle on biomass yield on light [25, 26]. It is unlikely that the baseline with continuous light conditions for the strains tested in literature were performed at the optimized growth conditions for maximum

biomass yield on light of the applied strains, as done in this study. A light intensity of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ was used in both literature studies that showed increased biomass yield on light by the implementation of a day:night cycle [25, 26].

Under higher light conditions of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ in experiments DN-HL-BT and DN-HL-HT, the biomass yield on light is significantly reduced compared to the baseline scenarios for both temperatures studied ($P < 0.05$). Daytime temperature shows a significant effect on the biomass yield on light with the experiment at 25°C (DN-HL-HT) showing a higher biomass yield on light ($0.70 \pm 0.04 \text{ g mol}^{-1}$) than the experiment at 21°C (DN-HL-BT - $0.64 \pm 0.04 \text{ g mol}^{-1}$) ($P < 0.05$). The significantly lower biomass yield on light for experiments with high light conditions ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$) compared to the baseline experiment correlates to the negative effect of increased light intensity on biomass yield on light as described in literature [47]. Compared to previous experiments under continuous conditions for light and temperature performed at similar light and temperature levels, the results of the experiments including a day:night cycle, at high light levels (DN-HL-BT and DN-HL-HT) do show an increased biomass yield on light. A biomass yield on light of $0.53 \pm 0.01 \text{ g mol}^{-1}$ and $0.57 \pm 0.01 \text{ g mol}^{-1}$ for continuous conditions with equal light conditions ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$) was reported at 20°C and 25°C , respectively [96]. In the current work we show a 20% increase of biomass yield on light for the DN-HL-BT experiment compared to previous experiments at continuous conditions of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 20°C . A 22% increase of the biomass yield on light for the DN-HL-HT experiment in the present study is achieved compared to continuous conditions of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 25°C in previous work [96]. These results show the biomass yield on light can be significantly improved with the implementation of a day:night cycle under suboptimal conditions such as high light intensities ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$) or suboptimal temperature (25°C). These results are in line with results in literature that showed 10-15% increase biomass yield on light for *Neochloris oleobundans* [25] and a 20% increase for *Acutodesmus obliquus* [26] when grown under day:night cycle conditions compared to continuous light using light intensities of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Under extreme temperature conditions for *Rhodomonas* (30°C in experiment DN-BL-XT), the positive effect of day:night cycles on *Rhodomonas sp.* growth under suboptimal conditions is further confirmed. Good reproducible growth was observed at a temperature of 30°C with oscillations to 15°C during the dark phase at a light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. The biomass yield on light under these conditions is not significantly different from the baseline experiment with $0.89 \pm 0.06 \text{ g mol}^{-1}$ ($P > 0.05$). Previous experiments showed that continuous light (light levels between 60 - $600 \mu\text{mol m}^{-2} \text{s}^{-1}$) and a continuous temperature of 30°C did not result in cell growth for *Rhodomonas sp.* [96]. Similar biomass yield on light of experiment DN-BL-XT compared to the baseline emphasizes the benefits of synchronized cellular processes for microalgae growth under day:night conditions. The

detrimental effects of temperature during the daytime are compensated during the dark phase with lower temperatures. The combination of high light ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the extreme temperature (30°C in experiment DN-HL-XT) shows that the regenerative capacity of the dark phase are limited. Unstable results were obtained and only one successful experiment with steady state was obtained with a low average biomass yield on light of $0.34 \pm 0.03 \text{ g mol}^{-1}$.

The increased biomass yield on light by implementation of a day:night cycle has been described in literature before. Here we show that a positive effect is only present under sub-optimal growth conditions. This indicates that under temperature and light conditions where no detrimental effect of either of these growth parameters is present, no additional gains are obtained by including a day:night cycle. The optimal conditions for biomass yield on light, typically represent conditions with relatively low biomass production rates and these conditions would therefore be uninteresting for large scale microalgae production. Conditions with higher light intensities and higher biomass production rates, benefit with the implementation of a day:night cycle.

4.3.2 Fatty acid composition

The fatty acid content and composition of the biomass was determined at the end of the steady state, and was measured at $t=16\text{h}$ after sunrise, the transition moment between light and dark phase. The results are depicted in Figure 21 where the fatty acid content is divided in four categories: Saturated fatty acids(SFA), Mono unsaturated fatty acids (MUFA), Poly unsaturated fatty acids excluding EPA and DHA (PUFA) and EPA+DHA, the total fatty acid content (TFA) is the sum of all four categories.

The high TFA content for DN-HL-XT does not fit with the other observed trends. This deviating TFA content is the most likely a stress response to the extreme conditions of light and temperature. As these growth conditions did not result in reproducible growth and only one reactor was sampled. Experiment DN-HL-XT is not considered in further fatty acid results. The TFA content of the full day:night cycle experiment (DN-BL-BT) is significantly lower ($P<0.05$) at $76 \pm 1 \text{ mg gDW}^{-1}$ than the baseline experiment at $85 \pm 1 \text{ mg gDW}^{-1}$. All other day:night cycle experiments (DN-HL-BT, DN-HL-HT and DN-BL-XT) appear to show a lower TFA content as well but these difference are not significant ($P>0.05$) compared to the baseline. The results are not significantly different as a result of the relatively large standard deviations and the low number of measurements for each individual experiment ($n=2$). Comparing the average TFA content of all day:night cycle experiments combined (DN-BL-BT, DN-HL-BT, DN-HL-HT and DN-BL-XT – $n=8$) to the baseline, a significant difference can be seen. A lower average TFA content of $75 \pm 5 \text{ mg gDW}^{-1}$ is found for the day:night cycle

experiments compared to a TFA content of $85 \pm 1 \text{ mg gDW}^{-1}$ of the baseline ($P < 0.05$). The results of EPA+DHA content show the same trend as found for the TFA. Individual experiments show no significant difference compared to the baseline. The average EPA+DHA content over all day:night experiments ($14 \pm 3 \text{ mg gDW}^{-1}$) is significantly lower ($P > 0.05$) than the baseline ($18 \pm 1 \text{ mg gDW}^{-1}$). This lower TFA and EPA+DHA content in the day:night cycle experiments indicates an effect of the fluctuating conditions. A fluctuation in temperature only (Base-Temp) results in a significant increase of the TFA and EPA+DHA content ($P < 0.05$). This is consistent with previous experiments with

continuous conditions where a lowered temperature showed increased TFA and EPA+DHA content [96]. The relatively large increase of TFA content as found here is higher than expected. The large deviation found for the BT experiment could be the results of daily fluctuations and the moment of sampling. The effect of daily fluctuations on the fatty acid is further discussed in section 4.3.3.2.

Figure 21: Fatty acid content of *Rhodomonas sp.* grown under different day:night cycle conditions for temperature and light. Saturated fatty acids (SFA), Mono unsaturated fatty acids (MUFA), Poly unsaturated fatty acids excluding EPA and DHA (PUFA) and EPA+DHA. Values are averages over two replicates, error bars depict standard error of the mean. Means with the same letter are not significantly different ($P > 0.05$).

4.3.3 Daily oscillations

The 24hour-profile of the DN-HL-HT experiment was measured by sampling every two hours for the duration of 14 hours in two reactor systems to represent the full 24 hours cycle.

4.3.3.1 Biomass concentration

Results of the biomass-concentration (C_x in g l^{-1}) and cell-concentration (cells ml^{-1}) are shown in Figure 22. Each datapoint represent a measurement of duplicate measurements from 2 separate days. The datapoints of $t=11\text{h}$ and $t=23\text{h}$ were taken from two reactors

and act as control for reproducibility between the reactors and represent data of duplicate measurement for two days of steady-state in two different reactors. The low standard deviation on these two measurements, relative to the other data points, shows that both reactors showed the exact same pattern. The biomass concentration shows a slight fluctuation over the day:night cycle. The highest biomass concentration ($1.55 \pm 0.01 \text{ g l}^{-1}$) is found at $t=15\text{h}$ followed by a 21% decrease in biomass concentration (to $1.23 \pm 0.05 \text{ g l}^{-1}$) during the dark phase.

Figure 22: Daily oscillation of *Rhodomonas* sp. in a 16:8 day:night cycle for biomass concentration (left -black circles - g l^{-1}) and average cell count (left - grey circles - million cells ml^{-1}) and average cell diameter (right - μm). Grey areas indicate the dark phase. Error bars show standard deviation (black circles $n=6$, blue circles and black diamond $n=4$ for $t=11\text{h}$ and $t=23\text{h}$ $n=\text{doubled}$)

The biomass loss during the night is the result of maintenance and energy used for cell-division. The fluctuating biomass concentration during the light period is the result of the turbidostat settings and changing absorption properties of the biomass (data not shown). During the daily fluctuations the average diameter of a cell increases from $7.03 \pm 0.07 \mu\text{m}$ at $t=0\text{h}$ to $8.38 \pm 0.20 \mu\text{m}$ at $t=15\text{h}$ after which the cell diameter decreases again during the dark phase (Figure 22). The increased average cell diameter during the first 15 lighthours results in a decrease of the cell concentration (cells ml^{-1} - Figure 22) and indicates the storage of energy-rich component in the cell in preparation for cell-division. Cell division occurs during the dark phase where cells decrease in size but increase in number. The oscillations from cell diameter and cell number are comparable to results described for *Acutodesmus obliquus* showing synchronized cell cycles under 16:8 day:night cycles [26].

The oscillation for biomass concentration as found for *Rhodomonas* sp. has not been described for other species under similar growth conditions. This oscillation of biomass

concentration over a 24hour period could have some implications for the results described in section 4.3.1. All experiments were measured at $t=16h$, the moment with the highest biomass concentration for *Rhodomonas sp.* under day:night cycles, followed by a loss of biomass during the night. The moment of sampling could result in a slightly overestimated biomass yield on light for day:night cycle experiments as the biomass concentration was assumed to be constant during steady-state.

4.3.3.2 Fatty acid content and composition

During the 24h cycle the fatty acid content of the biomass fluctuates (See Figure 23). The fatty acid composition data shows a clear daily pattern with a maximum TFA content of $94 \pm 2 \text{ mg gDW}^{-1}$ at $t=5h$ and minimum of $76 \pm 2 \text{ mg gDW}^{-1}$ at $t=13h$, $15h$ and $17h$ after sunrise. All four defined fatty acid categories follow the same trend as the TFA content. The fluctuation in TFA content is not the result of fluctuations in one specific category. The TFA content seems to be mostly correlated to the average cell size (Figure 22). This indicates that *Rhodomonas sp.* does not utilize fatty acids as energy storage but TFA content is most likely a function of the fraction of membranes of the total biomass. This result suggests that fatty acids of *Rhodomonas sp.* are mainly present in the cell membrane resulting in the correlation between cell size and fatty acid content. This trend is opposite to the trend described by Lacour et al for *Isochrysis sp.* which showed an increased fatty acid content for larger cells [99]. The EPA+DHA content shows small deviations from the other fatty acids with the EPA+DHA content decreasing from $16 \pm 1 \text{ mg gDW}^{-1}$ at $t=24h$ to $12 \pm 0 \text{ mg gDW}^{-1}$ at $t=13h$, $15h$ and $17h$. Previous experiments under continuous conditions showed a EPA+DHA content of 12 mg gDW^{-1} at 25°C conditions and 20 mg gDW^{-1} at 15°C [96]. These results correlate with those finding and show that EPA+DHA is mostly affected by temperature. During daytime temperatures (25°C) the EPA+DHA content is equal to that of continuous growth conditions at this temperature. During the dark phase at 15°C the EPA+DHA content reaches the highest level but not as high as the content of cell grown under continuous 15°C conditions.

The daily oscillations show the lowest TFA content at $t=13$, 15 and 17 hours indicating that the decreased TFA content as found for the day:night experiments of section 4.3.1 are most likely the result of the moment of sampling. The average TFA content of all day:night experiments of $76 \pm 1 \text{ mgFA gDW}^{-1}$ correlates with the TFA content found at $t=13$, 15 and 17 hours for the 24h-cycle at $76 \pm 2 \text{ mgFA gDW}^{-1}$. The daily oscillation of TFA content from $91 \pm 4 \text{ mg gDW}^{-1}$ at $t=5h$ to $76 \pm 1 \text{ mg gDW}^{-1}$ at $t=13-17h$ (Figure 23) is larger than the total deviation found between the baseline experiment and the day:night cycle experiments of section 4.3.2 (Figure 21). This shows that the moment of sampling during a day:night cycle

is the most important parameter affecting the TFA content found in the biomass for all described scenarios in this study.

The average cell size of the baseline experiment was $7.68 \pm 0.20 \mu\text{m}$, corresponding to the cells at $t=5\text{h}$ and $t=7\text{h}$, the time point of highest TFA content ($91 \pm 4 \text{ mg gDW}^{-1}$) during the 24h-cycle. The TFA content of the baseline experiment is $86 \pm 1 \text{ mg gDW}^{-1}$. The combination of all results strongly indicate that for *Rhodomonas sp.* the TFA content is mostly affected by cell size. The EPA+DHA is mostly influenced by temperature, showing higher levels at lower temperatures, corresponding with previous experimental data under continuous temperatures [96]. The EPA+DHA concentration fluctuates significantly during a 24h-cycle, these oscillations are not expected for continuous temperature conditions.

When *Rhodomonas sp.* is produced under day:night cycles an optimized harvesting strategy could therefore be selected to maximize the nutritional value of the biomass. This harvesting optimization is not required under continuous production conditions with the trade-off of a slightly lower TFA content under equal production conditions for light and temperature.

Figure 23: Fatty acid content of *Rhodomonas* sp. over a full 16:8 day:night cycle. Saturated fatty acids (SFA), Mono unsaturated fatty acids (MUFA), Poly unsaturated fatty acids excluding EPA and DHA (PUFA) and EPA+DHA. Values are averages over 2 replicates, error bars depict standard deviation ($n=4$ for all time points except $t=11h$ and $t=23h$ with $n=8$).

4.4 Conclusion

Rhodomonas sp. was grown under different day:night cycle conditions for light and temperature and compared to a baseline with all growth parameters at continuous levels. The cells showed clear synchronized cell cycles with cell growth during the light hours and cell division in the dark phase. It can be concluded that when growth conditions are maintained close to the optimized settings for biomass yield on light, no effect of a day:night cycle on the biomass yield on light is observed. An improved biomass yield on light was found for growth with a day:night cycle under suboptimal conditions with high light intensity and high temperature, compared to the same conditions applied for 24h day⁻¹. High temperature (30 °C) that did not result in growth under continuous conditions did result in good growth when implementing a day:night cycle, but only at low light levels. The fatty acid content of *Rhodomonas sp.* seems to be correlated to cell size and growth temperature. Implementation of a day:night cycle for *Rhodomonas sp.* production at large scale for aqua feed applications could allow for specific fatty acid composition and EPA and DHA content based on the time of harvesting.

Chapter 5

Production of *Rhodomonas sp.* at pilot scale under sunlight conditions

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Production of *Rhodomonas sp.* at pilot scale under sunlight conditions

Abstract

Rhodomonas sp., is an important microalga for aquaculture feed applications and gained increased research interest over the past few years. While efforts to optimize cultivation of the strain have been studied in detail under laboratory conditions, *Rhodomonas sp.* has never been grown in photobioreactors at large scale under outdoor light conditions. To study the industrial potential of this strain, we cultivated *Rhodomonas sp.* in three identical tubular photobioreactors with 200L working volume each, located in a greenhouse using sunlight conditions only. Growth experiments were performed from February with winter light conditions ($<10 \text{ mol m}^{-2} \text{ d}^{-1}$) up to high light conditions of summer ($>50 \text{ mol m}^{-2} \text{ d}^{-1}$) in July, representing all sunlight conditions in the Netherlands. All nutrients were supplied in surplus and temperature and pH were maintained at optimum values for growth of *Rhodomonas sp.*, based on lab data. The total light per reactor was calculated using a ray-tracing analysis to allow calculations based on the light reaching each individual reactor. *Rhodomonas sp.* grew under all tested light conditions. Biomass yield on light decreased with increasing light conditions from $0.43 \pm 0.21 \text{ g mol}^{-1}$ to $0.18 \pm 0.04 \text{ g mol}^{-1}$ at $0\text{-}10 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$ to $30\text{-}40 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$. Biomass productivities increased with increasing light from $0.09 \pm 0.04 \text{ g l}^{-1} \text{ d}^{-1}$ to $0.19 \pm 0.06 \text{ g l}^{-1} \text{ d}^{-1}$, for $0\text{-}10$ and $30\text{-}40 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$. We obtained a 2-5 fold increase in biomass productivity compared to previous reports on *Rhodomonas sp.* cultivation using artificial light at large scale. Our results show that *Rhodomonas sp.* can be grown at pilot scale using sunlight conditions and further improvements can be reached in the future.

5.1 Introduction

In aquaculture, specifically for hatcheries or copepod production, the use of the microalga *Rhodomonas sp.* is of great importance [9, 32, 101, 102]. The production of *Rhodomonas sp.* however has been poorly described in literature and the strain has been known for its difficulty to grow in mass-culture [7, 32]. *Rhodomonas sp.* cultures are reported as unstable with sudden culture death [32]. Recent studies at lab scale showed large improvements in respect to growth rates and stability of *Rhodomonas sp.* [33, 59-61, 67, 96]. We recently published advancements in continuous cultivation of the strains in lab-scale photobioreactors [96]. As a next step we would like to demonstrate production at larger scale. The increased interest in large scale production of this important strain for aquaculture is evident from recent studies [59, 67, 68]. Vu et al showed continuous *Rhodomonas sp.* cultivation in a bubble-column type reactor at a size of 84 L working volume and Thaisen et al in a 200 L tubular reactor [67, 68]. In both aforementioned studies artificial light conditions were used for consistent growth conditions. In these two studies, the biomass productivity from one single reactor and was in the range 0.02-0.13 g l⁻¹ d⁻¹. The growth of *Rhodomonas sp.* using only sunlight conditions has never been shown before and the behavior of this strain in tubular reactors has not been described with enough detail to allow scale-up to industrial processes. Microalgae production outdoors is subjected to fluctuating light conditions throughout the year and day, therefore being not as stable and robust as indoor production under continuous and constant illumination. To study the industrial potential of outdoor cultivation of *Rhodomonas sp.* we cultivated this microalga in three tubular reactors. The reactors are located in a greenhouse under sunlight conditions. Multiple experimental runs were done over the course of half a year representing all sunlight conditions (from low light <10 mol m⁻² d⁻¹ up to summer conditions of >50 mol m⁻² d⁻¹) found during a full year of outdoor cultivation.

5.2 Materials and methods

5.2.1 Experimental setup and reactor operation

Three tubular photobioreactors (LGem MK1-200, LGem, The Netherlands) with a working volume of 200L, each, located in a greenhouse in Vlissingen, the Netherlands were used for *Rhodomonas sp.* cultivation (Figure 24). Sunlight was the only light source during all experiments. Recirculation of the culture in the reactor was provided by air only with an approximately 60L air volume in the tubular section of a reactor. The air was recirculated

Figure 24: Picture of three tubular photobioreactor operated with *Rhodomonas* sp. as seen from the North-East, Vlissingen, The Netherlands.

over the reactor with a fresh flow of approximately 4 l min^{-1} (filter sterilized at 2.0 and $0.2\mu\text{m}$). The pH was measured online and maintained at 7.5 ± 0.3 by on-demand CO_2 addition in the gas phase of the reactor. Temperature of the cultures was maintained at $21.0 \pm 2.0 \text{ }^\circ\text{C}$ by circulation of hot or cold water over a heat exchanger located in the reactor tank with the cold and hot water provided by a heat pump (Carrier, 30RQ 017 CHE). Offline daily measurements (OD_{750} , cell-count, V_{harvest}) and online measurements (temperature, pH, light intensity) were performed to monitor the culture in all experimental conditions. These measurements are used to calculate the biomass productivity and biomass yield on light. Online measurements (temperature, pH, light intensity) were logged in 10-minute intervals using the reactor control systems (Neptune Systems, APEX Fusion). The light intensity in the greenhouse was measured by a PAR-sensor located inside the greenhouse on top of the middle reactor.

Reactors were operated in chemostat mode, i.e. with a constant daily dilution rate during the full length of the experiment. A dilution rate of $6.5 \pm 1.0 \text{ l hr}^{-1}$ was applied during the initial 20-days in of the experiment (experiment day 10-30) and $5.5 \pm 1.0 \text{ l hr}^{-1}$ during all other days. A dilution time of $10 \text{ hours day}^{-1}$ was applied for the duration of the experiment with the exception of experimental days 59-66 when a dilution time of $12 \text{ hours day}^{-1}$ was used corresponding to dilution rates of $0.30 \pm 0.025 \text{ d}^{-1}$ for the full duration of the experiment. The cultivation medium consisted of saline groundwater from a 30 meter deep groundwater-well located at the reactor location. The groundwater was aerated to oxidize

soluble iron to insoluble precipitates and further removed by a sand filter. Peristaltic pumps were used for the mixing of nutrient stocks with the ground water. Addition of nutrient stocks from the L1-culture medium [70] were added in 10ml l⁻¹ or 20ml l⁻¹ to maintain a nutrient-rich condition during reactor operation. Water mixed with nutrients was filter sterilized (0.5µm and 0.2µm - SupaPleat) before use in the reactors.

The three reactors were operated over a 167-day period in multiple reactor runs from February till July. A selection of data points from all available data was made to create the final data set used for data analysis. This selection only contains data points of operational days without nutrient limitations or temperatures outside the selected range. A batch phase of a newly started reactor was excluded from the final dataset. A final dataset of 143 data points over a total of 67 operational days was assembled for final data analysis of *Rhodomonas sp.* cultivation. A detailed timeline of reactor operation and selected data points is found in section 5.5.

5.2.1.1 Daily measurements

The biomass concentration (C_x) and harvest volume (V_{harvest}) were determined daily during the morning. The biomass concentration was determined by optical density (OD_{750}) and cell count measurements in duplicate. OD_{750} was determined by UV-VIS Spectroscopy at 750nm, (Hach, DR5000). Cell-count was done using a Beckman Coulter MultiSizer 3 Particle counter equipped with a 100µm aperture tube and samples diluted 100x using Isotone II diluent. Total harvest volume was determined daily by measuring the water level (in cm) in the cylindrical harvest vessels of known diameter. Weekly measurements of nitrogen and phosphate content of the culture medium were performed to ensure non-limiting conditions of nutrients for the full duration of the experiment. Nitrogen and phosphate concentrations were determined by UV-VIS Spectrophotometer using Hach test kits for nitrate (NO_3^- - method number 10049), ammonia (NH_3 - Nessler Method) and orthophosphate (PO_4^{3-} - method number 8048). The nutrient concentration (10ml l⁻¹ or 20ml l⁻¹ of L1-culture medium stocks) was adjusted accordingly (data not shown). The biomass concentration (g l⁻¹) was calculated based on the cell-count (cells ml⁻¹) and an assumed average cell mass (120 pg cell⁻¹), based on literature and previous results under laboratory conditions [27, 69, 96, 103].

5.2.1.2 Available light and Ray-Tracing

The reactors were placed in a north-south facing orientation with a total distance of 132cm between two reactors and a total length of the tube section of 450cm. The control panels

of all reactors are located on the east side of the tube section. Buildings were present in the immediate facility of the reactors location. The total available light to all reactors (PFD_{total}) was determined by the 10-minute interval measurements by a PAR-sensor located in the greenhouse in the middle of the reactor tube section area. A ray-tracing analysis was performed to determine the total available light for each reactor in the used location as a function of the measured light from the PAR-sensor. Calculations were performed based on a CAD-drawing of the full scenery including building as used in the experiments. From the results of this ray-tracing model a monthly average of light received by each individual reactor ($PFD_{reactor}$) is determined as a function of the total available light in the greenhouse (PFD_{total}) as measured by the PAR-sensor inside the greenhouse. These light correction values are summarized in Table 13 in section 5.5. With this specific method, only the light reaching the culture is used in the calculations.

5.2.1.3 Biomass productivity

The biomass productivity is calculated using the biomass concentration at the time of sampling (C_x) and the total volume harvested ($V_{harvest}$) between samples. Actual biomass concentration in the harvest could not be determined due to fast precipitation, cell death and continued cell division in the harvest volume. It is therefore assumed that the biomass concentration in the harvest volume ($C_{x-harvest}$) is equal to the biomass concentration measured in the reactor ($C_{x-reactor} = C_{x-harvest}$). The biomass productivity is determined as both volumetric and areal biomass productivity as given in Equation 5.1 and 5.2.

Equation 5.1:

$$\text{Volumetric biomass productivity } R_{xVol}(g * l^{-1} * d^{-1}) = \frac{C_{x-harvest} * V_{harvest}}{(V_R / \Delta T)}$$

Equation 5.2:

$$\text{Areal biomass productivity } R_{xA}(g * m^{-2} * d^{-1}) = \frac{C_{x-harvest} * V_{harvest}}{(A / \Delta T)}$$

Where $V_{harvest}$ = the volume of harvest between two measurements (liter), V_r = Culture volume of a reactor (liter), A = ground area for a reactor (m^2) and ΔT the time between two measurements (days).

5.2.1.4 Biomass yield on light

The biomass yield on light ($Y_{x,ph}$) describes the efficiency of light use by the algae culture. The biomass yield on light is calculated using equation 5.3 where R_x is the biomass

productivity (in $\text{g reactor}^{-1} \text{ day}^{-1}$) and photon flux density is the total available light that reaches the reactor ($\text{PFD}_{\text{reactor}}$ in $\text{mol}_{\text{ph}} \text{ reactor}^{-1} \text{ day}^{-1}$).

Equation 5.3:

$$\text{Biomass yield on light (g * mol}^{-1}\text{)} = \frac{R_x}{\text{PFD}}$$

5.3 Results and Discussion

Cultivation of *Rhodomonas sp.* under sunlight conditions showed variable results based on the available light. During the experiments the three reactors behaved equally during days of operation, indicating good reproducibility. Continuous cultivation was maintained during multiple reactor runs under all available sunlight conditions. The overall trends showed increased biomass productivities at higher light levels but with decreased biomass yield on light. These trends were all similar to trends observed for outdoor production of other algae species [50]. No unexpected behavior, such as culture crashes as described in literature, were observed under controlled conditions. With cultivation parameters maintained within the boundaries set (temperature below 25°C, non-limiting nutrients and $\text{pH} 7.5 \pm 0.3$) no biofilm formation or sudden culture dead was observed. Deviating from the set points resulted in the formation of biofilm in the reactors and decreased biomass productivity. Data of reactor with biofilm formation was not included in the final dataset.

All data points were combined in average values per photon flux density (PFD) intervals of $10 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$ (0-10, 10-20, 20-30 and 30-40 $\text{mol m}^{-2} \text{ d}^{-1}$) with error bars representing the total standard deviation of all days represented within each PFD interval (Figure 25Figure 24). Visualization of all data points within the selected dataset can be found in section 5.5, Figure 27. The results of single data points fluctuate between biomass concentrations of 0.10 and 0.90 g l^{-1} , biomass productivities of 0.03 to 0.30 $\text{g l}^{-1} \text{ d}^{-1}$ and biomass yield on light between 0.07 and 0.88 g mol^{-1} . The light conditions on the reactor surface ($\text{PFD}_{\text{reactor}}$) fluctuate between 1.7 and 38.6 $\text{mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$.

5.3.1 Biomass concentration

For chemostat conditions (fixed dilution rate) the biomass concentration is a result of the total available light. Under fixed light conditions a steady-state could be achieved, with higher biomass concentrations at higher light intensities. Under outdoor cultivation the biomass concentration is constantly adapting to the available light. The biomass concentration is expected to increase with increased light conditions. The average biomass

concentration over the full length of the experiment increased with increasing light availability for $0.30 \pm 0.15 \text{ g l}^{-1}$ in February with an average a $\text{PFD}_{\text{reactor}}$ of $8.34 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$ up to $0.55 \pm 0.20 \text{ g l}^{-1}$ in June/July with an average $\text{PFD}_{\text{reactor}}$ of $20.9 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$. In literature it is described that higher biomass concentrations could be achieved for other algae species grown in very similar conditions [50]. *Nannochloropsis sp.* is described in vertical tubular reactors, operated in chemostat mode under comparable light conditions (The Netherlands, July and August) and dilution rates (0.27 d^{-1}) obtaining an average biomass concentrations of 2.1 g l^{-1} . *Rhodomonas sp.* showed biomass concentration of to $0.55 \pm 0.20 \text{ g l}^{-1}$ under similar light conditions and a dilution rate of 0.30 d^{-1} . Although the work with *Nannochloropsis sp.* is the most comparable work available in literature, many differences exist between literature and our work with *Rhodomonas sp.*. Equal outdoor light conditions could be assumed, but in our work with *Rhodomonas sp.* the reactors were located in a greenhouse. An average loss of light of 14.6% is calculated with the ray-tracing analysis for the summer months (June-August) by placing the photobioreactors in a greenhouse. The tube diameter in this work was 6.0cm compared to the 4.6cm of the reactors described for *Nannochloropsis sp.*. Besides these differences the difference in strain could also result in very different biomass yield on light and biomass concentrations. The lower biomass concentrations found for *Rhodomonas sp.* does seem to indicate lower biomass yield on light for this strain under chemostat conditions utilizing sunlight, compared to *Nannochloropsis sp.*.

Rhodomonas sp. did show higher biomass concentrations of $1.2 - 1.5 \text{ g l}^{-1}$ in lab experiments using the same nutrient, temperature and pH settings with a dilution rate up to 1.02 d^{-1} [96]. These lab experiments were performed with the light applied at a constant level ($600 \mu\text{mol m}^{-2} \text{ s}^{-1}$) for 24hours per day resulting in a total PFD of $52 \text{ mol m}^{-2} \text{ d}^{-1}$. From these lab results it can be concluded that *Rhodomonas sp.* is able to reach higher biomass concentrations under favorable light conditions. We hypothesize that sunlight conditions with high peak light intensities ($>1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$) had a negative effect on the growth rate of *Rhodomonas sp.* during moments of high incident light intensities. The high incident light intensity combined with the relatively low biomass concentrations ($<0.5 \text{ g l}^{-1}$) during peak light hours of the day may have resulted in photo-inhibition. Photo-inhibition could have halted or decreased growth during hours of high light intensity, keeping the biomass concentrations relatively low under the applied dilution conditions.

5.3.2 Biomass productivity

From the biomass concentration and the dilution rate of the reactors the daily biomass productivity was calculated. The biomass productivity is represented in Figure 2A (areal) and Figure 2B (volumetric). An average areal biomass productivity of 3.11-6.25 $\text{g m}^{-2} \text{d}^{-1}$ is found. The overall trend of increased biomass productivity with increasing total photon flux density is equal to that described for other algae strains [47, 50]. Values observed in literature for a vertical tubular reactor under Dutch summer outdoor conditions described 3-4x higher areal biomass productivities of (10.6 - 24.4 $\text{g m}^{-2} \text{d}^{-1}$) with *Nannochloropsis sp.* [50, 104, 105]. The lower areal biomass productivity for *Rhodomonas sp.* is also represented in relatively low volumetric biomass productivities. The volumetric biomass productivity of *Rhodomonas sp.* (0.10-0.19 $\text{g l}^{-1} \text{d}^{-1}$) is lower than values in literature for *Nannochloropsis sp.* at 0.31-0.71 $\text{g l}^{-1} \text{d}^{-1}$ [50, 104, 105]. These lower volumetric and areal biomass productivities for *Rhodomonas sp.* are the result of the low biomass concentration obtained in the reactors. The significantly lower biomass productivity suggests lower biomass yield on lights obtained for *Rhodomonas sp.*. Nevertheless, the productivity data we obtained for *Rhodomonas sp.* at larger scale are higher than described in literature. The average volumetric biomass productivity over the full length of the experiment of $0.12 \pm 0.05 \text{ g l}^{-1} \text{d}^{-1}$ is very similar to that described by Vu et al growing *Rhodomonas salina* in a bubble column reactor under artificial light conditions ($981 \pm 133 \mu\text{mol m}^{-2} \text{s}^{-1}$) at a dilution rate of 0.46 d^{-1} and biomass concentration of approximately 0.29 g l^{-1} resulting in a biomass productivity of approximately $0.13 \text{ g l}^{-1} \text{d}^{-1}$ [68]. Thoisen et al obtained a volumetric biomass productivity of approximately $0.02\text{-}0.08 \text{ g l}^{-1} \text{d}^{-1}$ for *Rhodomonas sp.* grown in a tubular photobioreactor under artificial light conditions ($225 \pm 35 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a dilution rate

Figure 25: Areal biomass production rate (Figure 2A), Volumetric production rate (Figure 2B) and biomass yield on light (Figure 2C) averages over the total available light on the tube surface area per unit ground area ($\text{PFD}_{\text{reactor}}$). Data shows average per interval of 10 for $\text{PFD}_{\text{reactor}}$ (0-10, 10-20, 20-30 and 30-40 with $n=39,60,33,6$). Data of three independent reactors was combined to create the full dataset.

of approximately 0.3 d^{-1} [67]. Both literature studies are performed under constant artificial light conditions and 24 hours per day of light. It has to be noted that the biomass productivities are presented in $\text{g l}^{-1} \text{ d}^{-1}$ but growth only occurs during the light hours of the day. For the outdoor production of *Rhodomonas sp.* this is limited to a maximum of 15-16 hours of light per day, whereas artificial light experiments described in literature used constant light intensities 24 hours per day. The biomass productivity corrected for hours of light received ($\text{g l}^{-1} \text{ h}_{\text{light}}^{-1}$) shows a more realistic comparison between the different values from literature and the growth under sunlight conditions. The maximum biomass productivity in our study is found for the highest light conditions with an average of $0.19 \pm 0.06 \text{ g l}^{-1} \text{ d}^{-1}$ from 6 data points at a PFD of $30\text{-}40 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$. A maximum average hourly biomass productivity of $12 \pm 3 \text{ mg l}^{-1} \text{ h}_{\text{light}}^{-1}$ was achieved. Literature described $5.4 \text{ mg l}^{-1} \text{ h}_{\text{light}}^{-1}$ for Vu et al and $0.8 - 3.3 \text{ mg l}^{-1} \text{ h}_{\text{light}}^{-1}$ for Thoisen et al. [59, 68]. The volumetric biomass productivity expressed per hour of light shows that the actual biomass productivity obtained under sunlight conditions in our results is 2-5x higher than those reported in literature. This shows the potential of *Rhodomonas sp.* production under sunlight conditions. Biomass productivities (up to $1.4 \text{ g l}^{-1} \text{ d}^{-1}$ or $60 \text{ mg l}^{-1} \text{ h}_{\text{light}}^{-1}$) at lab scale have recently been reported [96]. It is therefore likely that with *Rhodomonas sp.* a much higher biomass productivity at large scale production could be achieved than presented here.

5.3.3 Biomass yield on light

The biomass yield on light (Figure 2C) clearly shows the negative effect of increased total daily photon flux density on the efficiency of light use. This trend correlates to similar trends described in literature for other algae strains at pilot scale production [47, 50, 105]. The results for *Rhodomonas sp.* represent inefficient light conversion to biomass by the algae strain under the applied experimental conditions with an average biomass yield on light of $0.29 \pm 0.16 \text{ g mol}^{-1}$ over the full length of the experiment. Studies on *Nannochloropsis sp.* showed a similar trend. A decrease in biomass yield on light from approximately 0.93 g mol^{-1} at low light conditions of $0\text{-}15 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$ to about 0.44 g mol^{-1} at high light conditions of $45\text{-}60 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$ was described [47, 50]. *Rhodomonas sp.* in our study shows an average of $0.43 \pm 0.21 \text{ g mol}^{-1}$ under low light conditions of $0\text{-}10 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$ to $0.18 \pm 0.04 \text{ g mol}^{-1}$ at high light conditions of $30\text{-}40 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$. Our results for *Rhodomonas sp.* only use the light reaching the culture, as calculated using the ray-tracing method. The total light in the studies on *Nannochloropsis sp.* describe all light reaching the ground surface of the reactor including light not reaching the culture. Values of the work with *Nannochloropsis sp.* are expected to be slightly higher if a similar ray-trace approach would have been used for the determination of light reaching the culture, further increasing the gap between our results with *Rhodomonas sp.* and data on *Nannochloropsis sp.* [50]. Although the same

trend is observed with decreasing biomass yield on light with an increased PFD, the average values are significantly lower for *Rhodomonas sp.* in our results. It was not possible to calculate a biomass yield on light for *Rhodomonas sp.* in other studies in literature due to limited data on total available light. Improving the biomass yield on light is key to the large scale success of *Rhodomonas sp.* as a production species for aquaculture. It is likely that the low biomass concentrations achieved during the study resulted in inefficient light use during moments of high incident light intensity. Light not reaching microalgae cells leaves the reactor and is not used for photosynthesis, lowering the biomass yield on light. This loss of light should be avoided to obtain more efficient biomass production.

5.3.4 Improved productivity under sunlight conditions

The results of the biomass concentration, biomass productivity and biomass yield on light of *Rhodomonas sp.* in our study all represent the same overall trend. A lower average biomass concentration resulting in a lower biomass productivity and lower biomass yield on light compared to data in literature for *Nannochloropsis sp.* were found. In lab-scale experiments *Rhodomonas sp.* has shown higher biomass concentrations (over 1.4 g l^{-1}), biomass productivities (up to $60 \text{ mg l}^{-1} \text{ h}^{-1}$) and biomass yields on light (up to 0.91 g mol^{-1}) than the values found at pilot-scale [96]. Laboratory experiments with *Rhodomonas sp.* showed higher growth rates ($> 1.0 \text{ d}^{-1}$) than those obtained at pilot scale utilizing sunlight. For industrial implementation of *Rhodomonas sp.* the gap between what has been achieved in lab studies and what our study shows at large scale should be closed. To achieve this the biomass productivity should be increased under the applied sunlight conditions. In chemostat the biomass productivity is a function of the dilution rate and the biomass concentration. A higher biomass concentration should be maintained under chemostat production to obtain higher biomass production rates. Increasing the dilution rate would reduce the biomass concentration further, not resulting in more efficient biomass production. As discussed in section 5.3.1 the biomass concentration is assumed to have remained low as a result of the high incident light intensity combined with a low biomass concentration. It is hypothesized that the high amount of light per cell could have resulted in photo inhibition. Strategies that reduce the total light per cell are therefore expected to result in higher biomass concentration and biomass production rates.

One strategy to decrease the amount of light per cell is an increased biomass concentration. A higher biomass concentration could be achieved by changing the production strategy or the reactor setup. A changed production strategy could include a longer batch phase, increasing the biomass concentration before starting a chemostat or a chemostat operation with a lower dilution rate. If the increased biomass concentration indeed results in higher

growth rates with high light conditions it is hypothesized to maintain this higher biomass concentration in the reactor during chemostat operation. Lab scale experiments of *Rhodomonas sp.* under high light conditions with high and low biomass concentration could show the effect of photo inhibition on the cells. Lab scale experiment could facilitate determination of cultivation strategies that could effectively increase the biomass production rate under sunlight conditions. Turbidostat experiments with a low biomass concentration under high incident light intensities could show the photo inhibiting effect. *Rhodomonas sp.* has not been produced at lab scales with light intensities over $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ [96]. Increasing the range of light conditions tested at lab scale could provide valuable information on the biomass yield on light and suitable biomass concentration for these light conditions.

5.4 Conclusion

Rhodomonas sp. was successfully cultivated at pilot-scale utilizing the sunlight conditions of the Dutch climate from February till July. This is the first reported cultivation of *Rhodomonas sp.* at pilot scale utilizing natural sunlight conditions. Biomass productivities on sunlight conditions are higher than previously published results of *Rhodomonas sp.* at pilot-scale utilizing stable artificial light conditions. Based on literature of *Rhodomonas sp.* production at lab scale and comparison to cultivation of other algae species at pilot-scale outdoors, a large potential for future improvement is still possible. Lab scale studies of *Rhodomonas sp.* with high light levels should determine the further possible improvements of *Rhodomonas sp.* as an industrial production strain for aquaculture using sunlight conditions.

5.5 Supplementary Files

Table 13: Correction factors for total light hitting the reactor tube surface area as determined using the ray-tracing analysis displaying monthly averages of light received per reactor relative to the measurement of the PAR-sensor located inside the greenhouse.

	Reactor #1	Reactor #2	Reactor #3
Jan	99%	74%	87%
Feb	90%	76%	76%
Mar	88%	76%	76%
Apr	82%	75%	79%
May	78%	75%	78%
Jun	73%	74%	74%
Jul	76%	75%	76%
Aug	82%	77%	80%
Sep	86%	76%	78%
Oct	86%	74%	74%
Nov	100%	78%	89%
Dec	87%	72%	72%

Figure 26: Overview of operational days per reactor over the full duration of the experiment (top figure) and the successful days of operation selected in the dataset and represented in the results (bottom). First day of operation: 4-2-2019. Last day of operation: 20-7-2019

Figure 27: Data plots of all data points in the final dataset as used in the results section of the experimental runs for each of the three reactors. Top: Volumetric biomass production rate ($g l^{-1} d^{-1}$) Bottom: Biomass yield on light ($g mol^{-1}$). X-axis: Total photon flux density reaching the reactor surface as calculated by ray-tracing analysis ($PFD_{reactor}$ in $mol_{ph} m_{ground}^{-2} d^{-1}$).

Chapter 6

General discussion: Opportunities for industrial microalgae production in aquaculture hatcheries

This chapter is prepared for publication as:

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Opportunities for industrial microalgae production in aquaculture hatcheries

Abstract

A recent techno-economic model has revealed the cost price of microalgae production for aquaculture hatcheries. Although multiple cost reduction strategies were proposed, the feasibility for implementation of these strategies remained untested. With recent advances made in the production of *Rhodomonas sp.* at lab scale, extensive data is now available on the effect of growth conditions on biomass yield on light, fatty acid content and composition of this strain. Coupling the laboratory and pilot-scale data of *Rhodomonas sp.* to the techno-economic model enabled us to evaluate the efficiency of the proposed cost reduction strategies for a real production strain relevant to aquaculture. Furthermore, the effect of cost reduction strategies on the produced biomass quality was assessed. The most cost efficient production does not occur for growth conditions with the highest biomass yield on light nor for those with maximal biomass production. *Rhodomonas sp.* is most efficiently grown at a temperature range of 23-25 °C and light levels between 400-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The implementation of a day:night cycle does not result in lower biomass production cost at the scale of biomass production required for aquaculture hatcheries. The calculation confirms that the production of microalgae in state of the art scalable photobioreactors is most cost efficient and production at a larger scale can greatly reduce the production cost. Cost efficient and reliable production can be achieved under controlled growth conditions using artificial light and state of the art scalable photobioreactors. The techno-economic model coupled to the experimental data is a powerful tool for optimization of microalgae production towards cost efficiency. This strategy can be used for any microalgae species if the effect of growth conditions on the biomass yield on light is known.

6.1 Cost price of microalgae in aquaculture hatcheries

The production of microalgae is important in aquaculture. Up to 60% of the total hatchery costs are allocated to microalgae production [106]. Cost efficient microalgae production for aquaculture industry is therefore very relevant to the sector. The techno-economic model developed in Chapter 2 describes the cost price of microalgae production in aquaculture hatcheries for two different production systems (bubble-columns and tubular photobioreactors), each under two light conditions (artificial light and sunlight). The results of Chapter 2 give an in depth analysis of the cost price in the different production methods. Bubble-columns represent labor intensive, traditionally applied production systems still widely used in aquaculture today [7, 17-19]. The scale up of these systems is based on increasing the number of individual reactor units. Tubular photobioreactors represent modern, more complex production systems with high levels of automation and control. Production of microalgae at larger scale using state of the art photobioreactors is done by increasing the volume of a reactor unit rather than the number of reactors, making the production technology better scalable. The two production systems are further referred to as modular production systems for the bubble-columns and scalable production system for the tubular photobioreactors.

In chapter 2 a techno-economic model is described and multiple strategies to reduce the cost price of microalgae in aquaculture hatcheries are given. We discuss the feasibility of the most significant cost reduction strategies: optimization of growth (section 6.2) and production at large scale (section 6.3). We combine laboratory data obtained from the experimental chapters 3 and 4 with the techno-economic model. *Rhodomonas sp.* was used in all experimental work as an example species due to the high relevance of industrial implementation of this strain in aquaculture and the limited literature available on this strain. The effect of the cost reduction strategies on the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content of the biomass is discussed in more detail in section 6.4 of this chapter.

In section 6.3, the effects of scaling up microalgae production in aquaculture hatcheries on the production cost and the biomass yield on light is assessed. Comparison of experimental lab-scale results (chapters 3 and 4) and pilot (chapter 5) results is done to discuss the future potential of the strain for industrial applications. Finally, an outlook to future potential for industrialization of microalgae production in aquaculture hatcheries is given.

6.2 Growth optimization

The techno economic model showed that high biomass yields can significantly reduce the cost price of microalgae (chapter 2). Increased biomass yields on light of *Rhodomonas sp.* were obtained at laboratory scale using two strategies; 1) optimization of growth parameters (light and temperature – chapter 3) and 2) implementation of a day:night cycle (chapter 4). The impact of these strategies on the production costs is evaluated using the techno-economic model to assess feasibility for their implementation at industrial scale.

6.2.1 Light and temperature

With the techno-economic model it was shown that scenarios with a higher light input but with equal biomass yield on light compared to the reference scenarios resulted in a significant decrease of the cost price for biomass production. An increased biomass yield on light with equal light levels also showed large cost reductions. From literature it is known that the biomass yield on light is influenced by temperature and light intensity [47, 50, 51, 105, 107, 108]. A detailed understanding of the influence of growth parameters (light and temperature) on the biomass yield on light is very relevant for reliable cost price calculations.

Figure 28: (Left) Cost price of microalgae production (€ kgDW^{-1}) in a 100m^2 scenario using scalable production systems based on (right) biomass yield on light data of *Rhodomonas sp.* (chapter 3) combined with the techno-economic model (chapter 2).

Biomass yield on light has a negative correlation with the applied light intensity [47, 50, 105, 107, 108]. The negative correlation between the applied total photon flux density (PFD) and incident light intensity (I_{ph}) on the biomass yield on light was shown for *Rhodomonas sp.* in the experimental results of chapter 3, 4 and 5. Literature shows that also temperature can have a large effect on growth rate and biomass yield on light [51]. Microalgae typically show a higher growth rate with increasing temperatures until the optimum temperature. Above the optimum temperature a quick decrease in growth rate is observed due to damage of proteins required for photosynthesis [109, 110]. With the techno-economic model it was shown that temperature control does not significantly influence the cost price of algae production in aquaculture hatcheries. Therefore, optimizing culture temperature for optimized biomass production is more efficient than saving costs on temperature control. Coupling data on the effect of light and temperature on the biomass yield on light from chapter 3 with the techno-economic model allows for optimization of the growth conditions for the lowest biomass production costs. The biomass yield on light results resulting from stable light and temperature conditions (chapter 3) are applicable to scenarios of scalable production systems under artificial light conditions as described in chapter 2. An example of the combination of laboratory data with the techno-economic model for a scenario of scalable production systems at a scale of 100m² utilizing artificial light is given in Figure 28.

Results of chapter 3 showed that maximum biomass yield on light for *Rhodomonas sp.* is achieved at a temperature of 21 °C and light intensity of 100-150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 28). The light intensity for maximal biomass yield on light is relatively low resulting in a low total biomass output of the reactors. The low biomass production leads to a high biomass production cost (€200-300 kgDW⁻¹ - Figure 28), meaning that optimized conditions for biomass yield on light do not lead to minimized biomass production cost. Growth conditions for maximal biomass productivity ($T = 21\text{-}24\text{ °C}$ $I_{ph} > 600\text{ }\mu\text{mol m}^{-2} \text{s}^{-1}$) as described for *Rhodomonas sp.* in chapter 3 also do not correspond with the growth conditions with the lowest biomass production cost. Optimized growth conditions for most cost efficient biomass production for the scenario shown in Figure 28 are $\pm$ €100 kgDW⁻¹. The lowest production cost is obtained at a temperature of 23-25 °C and light levels between 400-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The growth conditions optimized for cost efficient production of *Rhodomonas sp.* remain equal when changing to smaller or large scales (data not shown).

Literature available on *Rhodomonas sp.* lacks studies with growth conditions similar to the ones leading to the lowest biomass production costs ($T = 23\text{-}25\text{ °C}$ and $I_{ph} = 400\text{-}500\text{ }\mu\text{mol m}^{-2} \text{s}^{-1}$).

Figure 29 A: The cost of artificial light (€ kgDW⁻¹) as a function of the biomass yield on light, based on fluorescent tubes and an electricity price of €0.08 kWh⁻¹. Figure 29 B: Effect of implementation of a 16:8 day:night cycle (D:N – open symbols) on the production cost in modular (squares) and scalable (circles) production systems, in comparison to the reference scenario with continuous light conditions (closed symbols). Scenarios of 25-100-250-500-750-1500m² are shown.

Literature mostly reports temperature ranges of 18-21 °C combined with light levels not exceeding 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for experimental studies [33, 57, 58, 60, 61, 64, 65]. Literature describing *Rhodomonas sp.* cultivated in industrial settings for copepod production shows even lower temperatures (10-17 °C) as summarized by Drillet et al. [92]. The cost prices of *Rhodomonas sp.* production at growth conditions reported in literature would result in values of €200-300 kgDW⁻¹ as calculated using the model for the scenario illustrated in Figure 28. Combining the experimental laboratory data of chapter 3 with the techno-economic analysis shows a potential cost reduction of up to 65% for *Rhodomonas sp.* compared to the commonly used growth conditions in studies or industrial applications as presented in the base case scenarios of chapter 2. This cost reduction is achieved by minor changes in the operation of the production system (temperature and light settings). The combination of laboratory data with the techno-economic analysis proves to be a powerful tool for strategies for cost reduction for industrial applications of microalgae production as shown in the example of *Rhodomonas sp.* presented in Figure 28. This strategy could also be applied to other reactor systems or production strains if the combined effect of light and temperature on the biomass yield on light is known.

6.2.2 Day:night cycles

The combined effect of light and temperature on the cost price of microalgae production shows how the production of a specific algal species can be optimized when utilizing continuous light and temperature conditions. From literature it is known that daily oscillations in light and temperature could positively impact the biomass yield on light [25, 26, 36, 77, 97]. For *Rhodomonas sp.* a positive effect on the biomass yield on light is found when implementing a day:night cycle, specifically at higher light levels (chapter 4). Reduced hours of light in a day:night cycle will reduce the cost of electricity while decreasing the total daily biomass production. The implementation of a day:night cycle will only result in a lower cost price for biomass production if the reduction in costs for energy and CAPEX of the artificial lights can fully compensate the relative increase of the cost of other categories per kgDW (e.g. labor and major equipment cost) resulting from the lower total biomass output from the same amount of reactors operated. The cost of artificial light per kgDW is a function of the biomass yield on light, the type of light source and the cost of electricity (Figure 29A) [49]. The potential cost reduction strategy by implementation of a day:night cycle can be studied with the techno-economic model of chapter 2, specifically for artificial light conditions.

Reference scenarios for microalgae production in aquaculture industry (chapter 2) using artificial light (AL) under continuous conditions are compared to the same scenario using a 16:8 day:night cycle (D:N) with identical light intensity ($580 \mu\text{mol m}^{-2} \text{s}^{-1}$) during day time. A 20% increase in biomass yield on light is assumed (0.22 and 0.264 g mol^{-1} for AL-BC and AL-BC (D:N) and 0.45 and 0.54 g mol^{-1} for AL-TPBR and AL-TPBR (D:N)) based on the results of chapter 4. The results in Figure 29B show an increase in cost price with the implementation of a day:night cycle in the modular production systems (represented by the bubble-column systems). The scalable production systems (represented by the tubular photobioreactors) at a scale $> 250\text{m}^2$ show a cost reduction when implementing a day:night cycle. The energy component of the cost price is reduced by $\text{€}16.09 \text{ kgDW}^{-1}$ and $\text{€}12.91 \text{ kgDW}^{-1}$ under 16:8 day:night cycles for the modular and scalable production systems, respectively. For the modular production systems this cost reduction for energy does not result in an overall reduced cost price. The increased cost price for day:night scenarios is the result of the relative increase in labor and major equipment costs as a result of lower total biomass output from the same number of reactors. For the scalable systems the implementation of a day:night cycle at the largest tested scale (1500m^2) results in a total cost reduction of 10% ($\text{€}86.62 \text{ kgDW}^{-1}$) compared to the same scenario with continuous light ($\text{€}96.48 \text{ kgDW}^{-1}$). A lower total biomass output of $7436 \text{ kgDW year}^{-1}$ is obtained under day:night cycles compared to continuous light ($9295 \text{ kgDW year}^{-1}$). The results show that at the small scale applied in most aquaculture hatcheries ($20\text{-}50\text{m}^2$ or $125 \text{ kgDW year}^{-1}$),

the implementation of a 16:8 day:night cycle with the coupled 20% increase in biomass yield on light will not result in a lower cost price for biomass production.

6.3 Large scale production

Centralizing biomass production into one large facility has been described as an efficient cost reduction method [2], but centralized large scale production processes have not yet been implemented in the aquaculture industry. The cost price of microalgae produced with modular systems under artificial light is about €613 kgDW⁻¹ at small scale (25m²) and decreases to €428 kgDW⁻¹ at large scale (1500m² - Chapter 2). The scalable systems result in a cost price of €286 kgDW⁻¹ at small scales (25m²) with a decrease to €96 kgDW⁻¹ at 1500m² scale, using state of the art technology under artificial light conditions. Despite the higher cost price of microalgae cultivated in the modular production systems at small scales, these systems are still the preferred method of algae production in the aquaculture industry. The preference for the modular systems is evident from their widespread use in aquaculture studies in literature that require live microalgae [111-115]. This could be due to the preference for a mixed diet in most aquaculture hatcheries [7, 17-19]. The total required biomass capacity at aquaculture hatcheries could also impact the decision. In Dutch aquaculture hatcheries the required microalgae capacity for one hatchery was determined at approximately 125 kg year⁻¹ corresponding to a production facility of about 30-50 m² (chapter 2). A centralized facility combining algae production for approximately ten hatcheries, producing 1268 kgDW year⁻¹ with scalable systems using artificial light could result in algae cost prices of about €123 kgDW⁻¹ assuming implementation of state of the art technology. The cost price for a centralized facility results in a cost reduction of 80% compared to current practice with modular production systems at individual hatcheries. Combining a large scale microalgae production process in scalable reactors with detailed knowledge on the biomass yield on light of the produced strains as described in section 6.2.1 could reduce the cost price even further from the example scenario described above. The added cost of transportation and storage of the produced biomass were not included in the techno-economic analysis. New and existing aquaculture hatcheries relying on live microalgae for feed applications should implement the new scalable photobioreactor and benefit from the increased knowledge in microalgae biotechnology as soon as possible. Investing in scalable photobioreactors and collaborations in centralized facilities for microalgae production will result in a large advantage over competitors by significantly reducing the cost for feed production.

6.3.1 Effect of scale up on biomass yield on light

Results for biomass yield on light of *Rhodomonas sp.* at pilot-scale in sunlight conditions ($0.29 \pm 0.16 \text{ g mol}^{-1}$ – chapter 5) show that the biomass yield on light obtained at laboratory scale (up to 0.90 g mol^{-1} – chapter 3 and 4) could not be achieved in the pilot-scale experiments. Laboratory experiments showed higher biomass concentrations and growth rates than those obtained at pilot scale. Results obtained at pilot scale however had very different light conditions. Understanding the differences found between laboratory and pilot scale results may indicate the future potential of *Rhodomonas sp.* as a production strain.

The biomass yield on light of laboratory and pilot-scale experiments compared on a daily photon flux density basis (PFD – $\text{mol m}^{-2} \text{ d}^{-1}$) shows a large difference between the experiments. Biomass yield on light at laboratory scale can be more than five times higher than those obtained at pilot-scale, especially at higher photon flux densities ($>30 \text{ mol}_{\text{ph}} \text{ m}^{-2} \text{ d}^{-1}$). However, the photon flux density does not contain information on how the light was applied to the culture. The incident light at laboratory scale was applied at a constant level and in addition, an optimized biomass concentration for the applied light conditions was maintained constant in each experiment (chapter 3).

Under sunlight conditions the light intensity can vary every minute due to cloud movement. This large fluctuation in light levels under sunlight conditions results in non-optimized biomass concentrations for the light levels during most part of the day. The non-optimized biomass concentration may have led to the low biomass yield on light obtained under sunlight conditions, due to two reasons: 1) light leaving the reactors and 2) photo inhibition due to high light conditions.

The low biomass concentration in the pilot scale reactors ($< 0.5 \text{ g l}^{-1}$) during moments of high light intensity allowed part of the light to pass through the reactor. Light leaving the reactor is not measured at pilot-scale, leading to an underestimation in the calculation of the biomass yield on light. From literature it is known that high incident light intensities during peak light hours ($> 1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$) of the day on a low biomass concentrations can decrease photosynthetic efficiency, as described in detail for *Chlorella sp.* [116]. It is hypothesized that the high light conditions with low biomass concentrations resulted in photo inhibition, and a consequent low biomass yield on light. The low biomass yield on light combined with the sustained dilution rate of the chemostat settings resulted in a maintained low biomass concentration in the reactors during the experiments.

In laboratory scale conditions the biomass yield on light for *Rhodomonas sp.* as a function of the light intensity and temperature was described in detail (chapter 3). The biomass yield on light showed a decreasing trend for increasing light intensities. This trend was only confirmed for incident light intensities up to $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Under sunlight conditions the

incident light intensity reached $>1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ regularly with peaks above $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ during summer months (chapter 5). The response of *Rhodomonas sp.* on these light intensities is unknown and should be tested at laboratory conditions. The biomass yield on light under high light conditions ($> 1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) should be tested in laboratory experiments under continuous conditions with the same approach as experiments described in chapter 3. Such experiments could indicate the effect of high incident light intensities on a low biomass concentration for *Rhodomonas sp.*. It is recommended to collect this additional information from laboratory data before performing new outdoor pilot-scale experiments with *Rhodomonas sp.*.

The cost price of biomass production in scalable production systems at a relevant scale for aquaculture hatcheries is lower under artificial light ($\text{€}290 \text{ kgDW}^{-1}$) than under sunlight conditions ($\text{€}329 \text{ kgDW}^{-1}$) as described in chapter 2. In these calculations an equal biomass yield on light for artificial light and sunlight was assumed. Based on the data of *Rhodomonas sp.* production at laboratory and pilot scale it is concluded that production under sunlight conditions results in lower biomass yield on light than production with controlled artificial light (chapter 3, 4 and 5). The most cost efficient production of microalgae at aquaculture hatcheries will be obtained under artificial light conditions. Production of *Rhodomonas sp.* specifically should focus on production with artificial light using the growth conditions described under section 6.2. Further improvements on outdoor *Rhodomonas sp.* should be made under laboratory and pilot-scale conditions before more realistic calculations for outdoor production can be made.

6.4 Effect of cost reduction strategies on fatty acid content and composition

Temperature is the growth factor most influencing the fatty acid content and composition of *Rhodomonas sp.* under non-limiting nutrient conditions in continuous production systems (chapter 3 and 4). Lower temperatures increase the eicosapentaenoic acid and docosahexaenoic acid (EPA + DHA) fraction of the fatty acids (TFA). The TFA content in our experimental work is relatively low (7-10 %DW) compared to some values found for *Rhodomonas sp.* in literature (up to 31%) [117]. Most studies in literature describe batch cultivation or study the effect of nutrient limitations on the fatty acid composition [54, 79]. Literature describes an increased TFA content for *Rhodomonas sp.* under nutrient limiting conditions with a TFA content above 25 %DW [30, 54, 79, 117, 118]. Nutrient limiting conditions result in an increased TFA content but never an increased EPA+DHA content (mg kgDW^{-1}) [31]. Although the TFA content as described in our experiments is relatively low,

the EPA+DHA content of the biomass (mg gDW^{-1} - Figure 30) shows some of the highest values described in literature (up to 22 mg gDW^{-1} -).

6.4.1 Light and temperature

Cost efficient *Rhodomonas sp.* production using the growth conditions as suggested in section 6.2 ($21\text{-}24^\circ\text{C}$ and $400\text{-}500 \mu\text{mol m}^{-2} \text{s}^{-1}$) to attain the lowest biomass production cost, will result in biomass with a relatively low EPA+DHA content ($\pm 13\text{-}15 \text{ mg gDW}^{-1}$). Higher EPA+DHA contents of $19\text{-}20 \text{ mg gDW}^{-1}$ are found for growth conditions (16°C and 100 or $500\text{-}600 \mu\text{mol m}^{-2} \text{s}^{-1}$) that show relatively high production cost ($\pm \text{€}100\text{-}150 \text{ kgDW}^{-1}$ - Figure 28). This imbalance between increased EPA+DHA content and cost efficient biomass production could result in trade-offs when selection a production strategy at large scale, balancing the quality of the biomass with the cost price of production.

Figure 30: EPA + DHA content (mg gDW^{-1}) of *Rhodomonas sp.* produced as a function of the temperature (x-axis) and light intensity (y-axis)

6.4.2 Day:night cycles

Daily oscillations of the EPA+DHA content of *Rhodomonas sp.* biomass described in chapter 4 suggest that the EPA+DHA content can change within a timeframe of a few hours. The fluctuations showed an EPA+DHA content of 12.2 mgEPA+DHA gDW⁻¹ during the day (at 25 °C) and 16.1 mgEPA+DHA gDW⁻¹ at night (at 15 °C). Although the EPA+DHA content is 30% higher during the night than at daytime, the high levels reported in chapter 3 (> 20 mgEPA+DHA gDW⁻¹) are not obtained during daily fluctuations. The daily oscillation with a 30% increased EPA+DHA content shows a potential strategy towards EPA+DHA enriched biomass production for feed applications by selecting a specific moment to harvest if daily temperature oscillations are applied in the microalgae production process. Daily temperature oscillations at large scale production are only feasible in sunlight conditions, as a results of the weather. Production of *Rhodomonas sp.* under sunlight conditions is cost intensive and should not be applied at small scales. For controlled EPA+DHA production using artificial light scenarios an increased EPA+DHA content could be obtained using a two-step *Rhodomonas sp.* production strategy. Such an approach could contain an initial phase for cost efficient biomass production, using the growth parameters as described in section 6.2 followed by a second phase where the biomass is cooled to 15 °C or lower, increasing EPA+DHA content of the biomass. The effect of this enrichment phase on the biomass production cost should be evaluated separately to indicate if this would be a cost efficient process or if the higher production cost resulting from production at lower temperature in a continuous process (Figure 28 and Figure 30) would be a more cost efficient approach.

Table 14: Overview of the total fatty acid content (TFA in %DW), the EPA+DHA content (%TFA) and EPA+DHA content (mg gDW⁻¹) as found in literature studies working with *Rhodomonas sp.*. Single values indicate only one fatty acid composition was available in this source.

Strain	TFA (%DW)		EPA+DHA (%TFA)		EPA+DHA (mg gDW ⁻¹)		Source
	Min	Max	Min	Max	Min	Max	
<i>Rhodomonas</i> (NT15)	8.00	12.70	7.30	11.50	5.84	14.35	[31] Renaud - 2002
<i>Rhodomonas baltica</i>	15.70	30.70	4.50	7.50	11.8	13.8	[117] Fosse
<i>Rhodomonas baltica</i>	-	-	± 15	-	-	-	[120] Kreibich - 2008
<i>Rhodomonas baltica</i>	-	-	19.80	-	-	-	[121] Kreibich - 2011
<i>Rhodomonas baltica</i> NIVA-5/91	5.50	-	8.40	-	4.40	-	[69] Patil - 2007
<i>Rhodomonas baltica</i>	12.10	-	16.40	-	19.84	-	[81] v Houcke - 2017
<i>Rhodomonas baltica</i> NIVA-5/91	12.00	31.00	6.40	10.40	-	-	[118] Wang - 2019
<i>Rhodomonas lens</i>	± 10	-	15.30	-	15.3	-	[27] Seixas - 2009
<i>Rhodomonas marina</i>	18.90	20.50	-	-	-	-	[80] Fernandes - 2016
<i>Rhodomonas marina</i>	7.10	15.00	8.40	23.90	12.58	24.51	[79] Fernandes - 2016
<i>Rhodomonas marina</i> K-0435	-	-	17.90	-	-	-	[59] Thoisen - 2018
<i>Rhodomonas salina</i>	10.00	29.30	2.60	14.80	5.95	15.02	[30] Aussant - 2018
<i>Rhodomonas salina</i>	-	-	3.80	23.40	-	-	[33] Minh Thi Thuy Vu -
<i>Rhodomonas salina</i>	-	-	8.40	-	-	-	[122] Tremblay - 2007
<i>Rhodomonas salina</i> CCMP/NCMA 57	-	-	18.20	-	-	-	[123] Peltomaa - 2018
<i>Rhodomonas salina</i> CS-174	-	25.40	5.10	16.70	-	-	[54] Guevara - 2016
<i>Rhodomonas salina</i> CS-24	-	18.50	6.70	16.00	-	-	[54] Guevara - 2016
<i>Rhodomonas salina</i> K-0294	-	-	17.20	-	-	-	[59] Thoisen - 2018
<i>Rhodomonas salina</i> K-1487	-	-	16.90	18.70	-	-	[59] Thoisen - 2018
<i>Rhodomonas salina</i> K-1487	-	-	20.10	22.90	-	-	[68] Vu 2019
<i>Rhodomonas salina</i> LB 2763	-	-	22.50	-	-	-	[59] Thoisen - 2018
<i>Rhodomonas sp.</i> - this study	7.80	9.60	13.90	24.90	10.92	21.42	Chapter 3
<i>Rhodomonas sp.</i> - this study	7.20	8.60	13.50	21.50	8.46	21.74	Chapter 4
<i>Rhodomonas sp.</i> (NOAIF040)	9.50	20.50	6.90	17.30	6.90	17.3	[82] Huerlimann - 2010
<i>Rhodomonas sp.</i> CCAP 995/5	-	-	24.20	-	-	-	[59] Thoisen - 2018
<i>Rhodomonas sp.</i> Hf-1	-	-	11.90	18.60	-	-	[61] Yamamoto - 2019

6.4.3 Improved EPA+DHA content from *Rhodomonas sp.*

Other methods for improved EPA+DHA content of *Rhodomonas sp.* could include a more detailed screening of different *Rhodomonas* species available in literature. All strains tested in literature were grown under different growth conditions for temperature and light or under nutrient limiting conditions, not allowing direct comparison with the strain studied in this thesis. Some literature is available comparing different strains under equal growth conditions. The variation in the TFA between *Rhodomonas* strains is illustrated by a study showing a total fatty acid content of 18.5 and 25.4 %DW between two strains [54]. Although both strains experienced equal growth conditions, these were nutrient limiting conditions and not optimized for continuous production. Another study comparing a total of 5 strains showed significant differences in the EPA and DHA content of the five strains tested [59]. Differences in the TFA content could be the result of the tested conditions: low temperatures (17 °C) and very low light ($13 \mu\text{mol m}^{-2} \text{s}^{-1}$) [59]. The differences between the tested strains in literature suggest that improvements to the TFA and EPA+DHA content of *Rhodomonas sp.* for feed applications could be made when a more suitable strain would be found. With *Rhodomonas sp.* as used in chapter 3 and 4 regarded as the standard for current potential of *Rhodomonas sp.* production, new strains could be compared when grown under comparable continuous temperature and light intensity. Only strains with an equal or higher growth rate and equal or higher TFA and EPA+DHA content than the strain presented in this thesis would require further investigation. A comparison using the growth conditions with most cost-efficient biomass production as described in section 6.2 would allow for direct comparison of the large scale potential of the tested strains.

It has to be noted that although multiple studies describing *Rhodomonas sp.* for feed applications can be found in literature and multiple studies link the EPA+DHA profile of *Rhodomonas sp.* to their successful use in aquaculture applications, a desired and specified biomass composition of *Rhodomonas sp.* has never been described [7, 17, 119]. Most aquaculture applications of microalgae do not know the exact feed composition required for optimized production of the end product. With the detailed information on the EPA+DHA as a result of the growth parameters for *Rhodomonas sp.* now available (Figure 30) it is possible to design a production process with a known biomass composition output. An aquaculture production process that requires *Rhodomonas sp.* as feed can now use this information to produce algae biomass with different compositions and determine the optimal biomass composition for the specific feeding application. Combined with the data from the techno-economic model an algae production progress balancing cost of production and biomass quality can be developed.

6.5 Main conclusion and outlook

From this thesis, using *Rhodomonas sp.* as an example for microalgae production, clear strategies for cost efficient industrial microalgae production for aquaculture hatcheries can be developed. Hatcheries could be operated much more efficiently if state of the art, commercially available, scalable production systems with advanced control systems and a high level of automation would be used. The use of labor-intensive production systems with a low level of control of growth parameters is expensive. The use of artificial light is cost efficient and guarantees reliable production of biomass. Aquaculture hatcheries could collaborate in microalgae production for life feed by operation of a central large-scale microalgae production unit. A centralized industrial microalgae production facility focusing solely on the production of microalgae and achieving highly cost-efficient production processes could have a great impact on the aquaculture productions relying on algae biomass.

It has been shown that *Rhodomonas sp.* can be efficiently grown at industrial scale. There are a lot of possibilities to improve the process further. For that, studies on the behavior of this strain under high incident light intensities, including day:night cycles is required.

The techno-economic model which was developed in this thesis, is a very powerful tool to analyze the economic feasibility of the production of microalgae for aquaculture. This model was developed for *Rhodomonas sp.* but could be used for other algae as well. Cost price calculations can be done for other strains if the combined effect of temperature and light intensity on the biomass yield of light of that strain is known. Combining detailed information of any production strain with the techno-economic model allows to determine the most cost efficient growth conditions for large scale production of selected strains and production scenarios.

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Summary

Summary

In this thesis the cost price of microalgae production in aquaculture hatcheries and the effect of cost reduction strategies on the biomass quality is studied. The cost price of microalgae production in aquaculture hatcheries and cost reduction strategies is described with a techno-economic model in. Using *Rhodomonas sp.* as an example species for microalgae production in aquaculture, the effect of growth conditions on the biomass yield on light and the biomass quality is studied on both laboratory scale and pilot scale.

The cost price of microalgae in aquaculture

A techno-economic model to describe the cost price for microalgae cultivation in aquaculture hatcheries was developed (**chapter 2**). The model is based on inputs from hatcheries in the Dutch aquaculture industry using commercially available microalgae production systems. The techno-economic model is used to calculate the cost price of microalgae production in modular production systems (bubble-columns), and scalable production systems (tubular photobioreactors). On a production scale presently applied in Dutch aquaculture hatcheries (125 kg year^{-1}) the commonly used modular reactor systems result in a biomass cost price of $\text{€}587 \text{ kgDW}^{-1}$ for production under artificial light and $\text{€}573 \text{ kgDW}^{-1}$ using sunlight. The scalable production systems show lower production costs with $\text{€}290 \text{ kgDW}^{-1}$ with artificial light and $\text{€}329 \text{ kgDW}^{-1}$ under sunlight conditions. Three cost reduction strategies with a high impact on the cost price were identified: 1) increasing biomass yield on light, 2) applying more artificial light and 3) reducing labor requirements. The cost price can be reduced to $\text{€}96 \text{ kgDW}^{-1}$ by implementation of cost reduction strategies at the same scale (20 m^2) using scalable production under artificial light conditions. Production of biomass at a larger scale (1500 m^2) using scalable production systems combined with cost reduction strategies can result in a cost price of $\text{€}23,47 \text{ kgDW}^{-1}$ at a production scale of $6892 \text{ kg year}^{-1}$. Implementation of scalable production systems in aquaculture is important for efficient cost production of microalgae. Modular systems always result in a high costs.

Light and temperature on *Rhodomonas sp.*

In **chapter 3** the optimization of *Rhodomonas sp.* at lab-scale, under continuous cultivation conditions is described. This is the first study describing continuous cultivation of *Rhodomonas sp.*. The effect of both light and temperature on the growth rate, biomass production rate, biomass yield on light and the fatty acid content and composition of

Rhodomonas sp. was studied. A wide range of light intensities ($60\text{--}600\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) combined with a wide range of temperatures ($15\text{--}30\ ^\circ\text{C}$) was studied. The results describe growth rates of $> 1.0\ \text{d}^{-1}$, with biomass production rates up to $1.5\ \text{g l}^{-1}\ \text{d}^{-1}$. The highest biomass yield on light ($0.87\ \text{g mol}^{-1}$) is found at a temperature of $22\text{--}24\ ^\circ\text{C}$ and light intensity of $110\text{--}220\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$. The total fatty acid content fluctuates between 8–10% of the dry-weight with an EPA+DHA concentration of 14–25% of the total fatty acids. The total fatty acid content and EPA and DHA content of the cells was only influenced by the cultivation temperature with higher EPA and DHA content at lower temperatures.

Fatty-acid content of *Rhodomonas sp.* under day:night cycles of light and temperature

In **chapter 4** the effect of daily oscillations of temperature and light on the biomass yield on light and on the biomass fatty acid content and profile was studied. Synchronized cultures of *Rhodomonas sp.* were found under day:night oscillations. Under the optimized growth conditions for biomass yield on light as described in chapter 3 the oscillations of both light and temperature in a 16:8 day:night cycle did not result in an increase of the biomass yield on light. At higher light conditions ($600\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) a 22% increase of the biomass yield on light was found with a day:night cycle compared to continuous light conditions. In a day:night cycle with daily oscillations for light and temperature the fatty acid content and compositions of the cells varied greatly with the moment of the day. Highest total fatty acid concentrations ($91 \pm 4\ \text{mg gDW}^{-1}$) were found in the first hours after sunrise whereas the highest EPA+DHA content ($16 \pm 1\ \text{mg gDW}^{-1}$) is found at the end of the dark period with a lower temperature.

Outdoor cultivation of *Rhodomonas sp.*

In **chapter 5** *Rhodomonas sp.* was produced using pilot-scale tubular photobioreactors to study the effect of production at large scale under sunlight conditions on the biomass yield on light. Successful cultivation of *Rhodomonas sp.* at pilot-scale using sunlight conditions is described, for the first time in literature. Using three tubular photobioreactors with a working volume of 200L each, *Rhodomonas sp.* was produced over a period of 6 months, from February till July representing all sunlight conditions available in the Dutch climate. An average biomass yield on light of $0.29 \pm 0.16\ \text{g mol}^{-1}$ was obtained, which is lower than the yields obtained in the laboratory experiments (up to $0.89\ \text{g mol}^{-1}$). The results show the potential of *Rhodomonas sp.* as a production species for aquaculture industry. The biomass production rates obtained ($< 0.25\ \text{g l}^{-1}\ \text{d}^{-1}$) were lower than those obtained in the lab

experiments ($<1.5 \text{ g l}^{-1} \text{ d}^{-1}$). Further optimization of *Rhodomonas sp.* production at pilot scale seems to be possible. For that, the effect of high light intensities on the growth of *Rhodomonas sp.* should be studied at lab scale. New lab experiments with high light intensities could reveal the potential for and higher biomass production rates at large scale under sunlight conditions.

General discussion

In **chapter 6** the integration of cost reduction strategies as proposed in **chapter 2** with experimental lab data from **chapters 3, 4 and 5** is discussed. With lab data on the effect of cost reduction strategies on the biomass yield on light and the biomass fatty acid content and composition a more realistic view on cost reduction potential is described. The combination of data of the combined effect of light and temperature on the biomass yield on light (**chapter 3**) and the techno-economic model (**chapter 2**) shows that optimization of the growth parameters towards cost efficient production of a strain can result in large cost reductions. However, the most cost-efficient production is not obtained at growth conditions for maximal biomass yield on light nor at the conditions where maximal biomass productivity is maximal. The most cost efficient growth conditions for *Rhodomonas sp.* production using scalable production systems and artificial light at a scale of 100m^2 is at a temperature of $23\text{--}25 \text{ }^{\circ}\text{C}$ and light levels between $400\text{--}500 \mu\text{mol m}^{-2} \text{ s}^{-1}$. **Chapter 3** is the first study in which *Rhodomonas sp.* production is described under these growth conditions. In addition, the increased biomass yield on light resulting from the implementation of a day:night cycle (**chapter 4**) does not result in a cost reduction if applied at scales typically applied at aquaculture hatcheries, or with the modular production systems. A cost reduction (up to 10%) can be achieved at a scale $>250\text{m}^2$ when using scalable production systems. Considering all experimental data and inputs on the techno-economic model it is concluded that the production of microalgae for aquaculture hatcheries should be performed under controlled growth conditions using artificial light and scalable production systems. The implementation of a centralized microalgae production facility will result in a great cost price reduction. A cost reduction of 80% can be achieved if algae production of ten hatcheries is combined in a production facility utilizing scalable production systems, compared to individual hatcheries maintaining a modular microalgae production facility.

The combination of the techno-economic model with laboratory data is proven as a powerful method for optimization of cost efficient microalgae production in aquaculture. This method can be applied to any microalgae species of which the effect of growth conditions on the biomass yield on light are known and for any production scenario based on scalable or modular production systems.

Samenvatting

Samenvatting

In dit proefschrift wordt het onderzoek beschreven naar de kostprijs van micro-algen productie voor de aquacultuur industrie en het effect van kosten verlagende maatregelen op de kwaliteit van de geproduceerde biomassa. De kostprijs van micro-algen productie in aquacultuur broedhuizen en strategieën om deze kosten te verlagen zijn onderzocht aan de hand van een techno-economisch model. De alg *Rhodomonas sp.* is gebruikt als model alg voor de productie van algen in de aquacultuur in experimenteel werk op laboratorium schaal en piloot-schaal. In deze experimenten is specifiek gekeken naar de efficiëntie van lichtgebruik door de alg; de biomassa-opbrengst op licht. De kwaliteit van de geproduceerde algen is bestudeerd door de vetzuurconcentratie en de vetzuursamenstelling van de biomassa te bepalen.

De kostprijs van algenproductie in de aquacultuur

De kostprijs van algen productie voor toepassingen in aquacultuur broedhuizen wordt berekend met een techno-economisch model dat is ontwikkeld als onderdeel van dit proefschrift en staat beschreven in **hoofdstuk 2**. Het techno-economische model is ontworpen in samenwerking met Nederlandse aquacultuur broedhuizen waar algenproductie op commerciële schaal wordt uitgevoerd. Aannames en input waardes voor het model, waaronder de biomassa-opbrengst op licht, zijn gebaseerd op algen productie faciliteiten in de Nederlandse aquacultuur. In het techno-economische model wordt de kostprijs van algen productie in twee verschillende typen productie systemen beschreven. Modulaire productie systemen beschrijven algen productie systemen van lage complexiteit waarbij op grotere schaal meer reactor van hetzelfde volume worden toegepast. Dit type systeem is gebaseerd op de veelgebruikte bellen-kolommen uit de aquaculture. Buizenreactoren zijn gebruikt als basis voor de berekeningen voor schaalbare productie systemen. Op grotere schaal worden bij dit soort productie systemen een grotere reactor gebruikt in plaats van meerdere kleine reactoren.

In de Nederlandse broedhuizen wordt over het algemeen gebruikt gemaakt van algen productie systemen met een algen productie capaciteit van ongeveer 125 kg droge stof algen (kgDW) per jaar. Op deze schaal is de kostprijs van de modulaire systemen het hoogst. De algen geproduceerd in de modulaire systemen hebben een kostprijs van €587 kgDW⁻¹ indien gebruik gemaakt wordt van kunstlicht. De kostprijs van algen geproduceerd in modulaire systemen geplaatst in een kas waarbij er gebruikt gemaakt wordt van zonlicht

is €573 kgDW⁻¹. De algen geproduceerd in schaalbare reactoren hebben een kostprijs van €290 kgDW⁻¹ bij gebruik van kunstlicht en €329 kgDW⁻¹ wanneer zonlicht wordt gebruikt.

Er zijn drie belangrijke strategieën voor het verminderen van de productiekosten: 1) verhogen van de biomassa-opbrengst op licht, 2) toepassen van meer kunstlicht en 3) verminderen van de benodigde arbeid. De kostprijs kan gereduceerd worden tot €96 kgDW⁻¹ als deze kostenbesparende strategieën gecombineerd worden op dezelfde productieschaal van 20m². Dit is de kostprijs voor het gebruik van schaalbare productie systemen die gebruik maken van kunstlicht. De productie van biomassa in een grotere productiefaciliteit met een oppervlak van 1500 m² kan de kostprijs laten dalen naar €23,47 kgDW⁻¹ indien er gebruik gemaakt wordt van schaalbare reactorsystemen en de kostenbesparende strategieën worden toegepast. De biomassa productie capaciteit van dit scenario is 6892 kgDW jaar⁻¹. Het toepassen van schaalbare reactor systemen is een belangrijke stap om tot kosten-efficiënte micro-algen productie te komen in de aquacultuur. Het gebruik van de modulaire systemen zal in alle scenario's leiden tot een hogere kostprijs voor de geproduceerde biomassa.

Effect van licht en temperatuur op *Rhodomonas sp.*

In **hoofdstuk 3** van deze thesis wordt de continue productie van *Rhodomonas sp.* op laboratorium schaal beschreven. Dit is de eerste studie beschikbaar in de literatuur waarin continue productie van *Rhodomonas sp.* beschreven wordt. De invloed van zowel de licht intensiteit als de water temperatuur op *Rhodomonas sp.* is onderzocht. De groeisnelheid, de biomassa-opbrengst op licht en de vetzuurconcentratie en vetzuursamenstelling in de biomassa zijn onderzocht. Een ruime reeks aan temperatuur condities (15-30 °C) en licht intensiteiten (60-600 μmol m⁻² s⁻¹) is getest onder verschillende combinaties. De resultaten laten zien dat *Rhodomonas sp.* groeit met groeisnelheden tot boven de 1.0 d⁻¹. Er zijn biomassa-productie-waardes hoger dan 1.5 g l⁻¹ d⁻¹ zijn gemeten. De hoogst behaalde biomassa-opbrengst op licht was 0.87 g mol⁻¹ en werd behaald bij een temperatuur van 22-24 °C en licht intensiteit van 110-220 μmol m⁻² s⁻¹. De vetzuurconcentraties in de biomassa fluctueren tussen de 8-10% van het drooggewicht. De EPA+DHA concentraties wisselen tussen de 14-25% van de totale vetzuurconcentratie. Zowel de vetzuurconcentratie en de EPA+DHA concentratie in de biomassa werden beïnvloed door de temperatuur, maar niet door de licht intensiteit. De hoogste EPA+DHA concentraties ontstaan in biomassa gegroeid bij lagere temperaturen.

Vetzuurconcentraties in *Rhodomonas sp.* onder dag:nacht ritmes voor temperatuur en licht

In **hoofdstuk 4** worden experimenten beschreven waarin het effect van dagelijks fluctuerende licht en temperatuur condities op de biomassa-opbrengst op licht en de vetzuurconcentratie en de vetzuursamenstelling wordt bestudeerd. Gesynchroniseerde cellen van *Rhodomonas sp.* werden bestudeerd onder dag:nacht fluctuaties voor temperatuur en licht. Indien een dag:nacht ritme werd toegepast bij groeicondities waarbij een maximale biomassa-opbrengst op licht bereikt werd, zoals beschreven in **hoofdstuk 3** werd geen verhoging van deze biomassa-opbrengst op licht gevonden ten opzichte van continu licht. Bij hogere licht intensiteit ($600 \mu\text{mol m}^{-2} \text{s}^{-1}$) werd de biomassa-opbrengst op licht verhoogd met 22% onder dag:nacht ritmes ten opzichte van continue licht. De vetzuurconcentratie en de vetzuursamenstelling fluctueerde dagelijks met een dag:nacht ritme. De vetzuurconcentratie in de biomassa is het hoogste in de eerste uren na zonsopkomst ($91 \pm 4 \text{ mg gDW}^{-1}$). De hoogste EPA+DHA concentratie ($16 \pm 1 \text{ mg gDW}^{-1}$) is aanwezig aan einde van donker periode waarbij de temperatuur laag was.

Productie van *Rhodomonas sp.* met zonlicht

In **hoofdstuk 5** worden experimenten beschreven waarin *Rhodomonas sp.* op piloot-schaal geproduceerd is in drie buizenreactoren. Deze reactoren zijn in een kas geplaatst en maken gebruik van zonlicht. In dit experiment wordt het effect van grootschalige productie op zonlicht op de opbrengst van biomassa op licht bestudeerd. Dit is de eerste studie in literatuur die succesvolle productie van *Rhodomonas sp.* op zonlicht beschrijft. *Rhodomonas sp.* is geproduceerd in drie buizenreactoren met een volume van 200 liter per stuk gedurende een periode van 6 maanden. De groei-experimenten vonden plaats van februari tot en met juli en vertegenwoordigen daarmee bijna alle mogelijk zonlicht-condities die voorkomen in het Nederlandse klimaat. Uit de resultaten blijkt de potentie van *Rhodomonas sp.* als productie alg voor de aquacultuur industrie. De behaalde maximum biomassa-productiviteit was $0.25 \text{ g l}^{-1} \text{d}^{-1}$. De gemiddelde biomassa-opbrengst op licht was $0.29 \pm 0.16 \text{ g mol}^{-1}$. Deze waarden zijn lager dan de waarden die in laboratoriumexperimenten zijn gevonden. In laboratoriumexperiment was de maximale biomassa productiviteit $1.5 \text{ g l}^{-1} \text{d}^{-1}$ en biomassa-opbrengsten op licht 0.89 g mol^{-1} . De laboratorium-resultaten laten zien dat verdere optimalisatie van *Rhodomonas sp.* op piloot-schaal mogelijk is. Nieuwe laboratorium experimenten onder hoge lichtintensiteit kunnen de potentiële hogere biomassa productiviteit onder zonlicht-condities aantonen.

Algemene discussie

In de algemene discussie, zoals beschreven in **hoofdstuk 6**, wordt de combinatie van het techno-economisch model met de experimentele data uit **hoofdstukken 3, 4 en 5** beschreven. De combinatie van het techno-economische model (**hoofdstuk 2**) met experimentele data (**hoofdstuk 3**) maakt het mogelijk om een realistisch toekomstbeeld te geven over de kosten verlaging voor algenproductie in de aquacultuur. De combinatie van deze gegevens laat zien dat het optimaliseren van de groei-condities tot een grote kosten verlaging kan leiden. De kosten voor algen productie zijn niet het laagste wanneer de groei-condities voor een maximale biomassa-opbrengst op licht worden gebruikt. Ook groei-condities die leiden tot een maximale biomassa productiviteit leiden niet tot de laagste kostprijs voor algen productie. Kosten-efficiënte biomassa productie voor *Rhodomonas sp.* in schaalbare reactoren met kunstlicht op een schaal van 100m² wordt bereikt bij een temperatuur van 23-25 °C en een licht intensiteit van 400-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. **hoofdstuk 3** is de eerste studie in de literatuur die *Rhodomonas sp.* productie onder deze groei-condities beschrijft. Het gebruik van een dag:nacht ritme met kunstlicht zorgt niet voor een kosten verlaging wanneer dit wordt toegepast op de schaal gangbaar voor algen productie in broedhuizen in de aquacultuur. Bij een productie schaal boven de 250m² met schaalbare reactoren, kan een kostenbesparing van ongeveer 10%. Worden bereikt met een dag:nacht ritme op kunstlicht. In modulaire systemen resulteert een dag:nacht ritme nooit in een kostenverlaging van de algen productie.

De combinatie van alle experimentele data met het techno-economische model laat zien dat productie van algen voor broedhuizen in de aquacultuur gebruik moet maken van gecontroleerde groeiomstandigheden. De laagste productie kosten worden bereikt bij gebruik schaalbare reactoren met kunstlicht. Bij gecentraliseerde productie van algen voor de aquacultuur kan de kostprijs van algen biomassa sterk verlaagd worden ten opzichte van individuele productie waarbij gebruik gemaakt wordt van modulaire systemen. Een totale kostenbesparing van 80% kan behaald worden als de benodigde biomassa hoeveelheid voor ongeveer tien broedhuizen geproduceerd zou worden in een gespecialiseerde algen productie faciliteit.

De combinatie van het techno-economisch model met de experimentele data is een krachtige methode voor de optimalisatie van algen productie gericht op kosten-efficiënte productie. Deze methode kan gebruikt worden voor alle algen soorten waarvan gedetailleerde gegevens bekend zijn van het effect van de groei-condities op de biomassa-opbrengst op licht en voor ieder productie scenario in de aquacultuur waarin modulaire of schaalbare algen productie systemen gebruikt worden.

Nomenclature

Nomenclature

AL	Artificial light
BC	Bubble-column photobioreactor
CAPEX	Capital expenditure
C_x	Biomass concentration (g l^{-1})
$C_{x \text{ harvest}}$	Biomass concentration in the harvest volume (g l^{-1})
$C_{x \text{ reactor}}$	Biomass concentration in the reactor (g l^{-1})
D	Daily dilution rate (d^{-1})
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GH	Greenhouse facility utilizing sunlight conditions
I_{ph}	Incident light intensity ($\mu\text{mol}_{\text{ph}} \text{m}^{-2} \text{s}^{-1}$)
MUFA	Mono unsaturated fatty acid
OPEX	Operational expenditure
PFD	Photon flux density ($\text{mol m}^{-2} \text{day}^{-1}$)
$\text{PFD}_{\text{total}}$	Total photon flux density ($\text{mol}_{\text{photons}} \text{m}_{\text{ground}}^{-2} \text{d}^{-1}$)
$\text{PFD}_{\text{reactor}}$	Photon flux density reaching reactor ($\text{mol}_{\text{ph}} \text{m}_{\text{floor}}^{-2} \text{d}^{-1}$)
PUFA	Poly unsaturated fatty acid
r_{ph}	Volumetric photon supply rate ($\text{mol}_{\text{ph}} \text{l}^{-1} \text{d}^{-1}$)
R_{xA}	Areal biomass productivity ($\text{g m}_{\text{floor}}^{-2} \text{d}^{-1}$)
R_{xVol} / r_x	Volumetric biomass productivity ($\text{g l}^{-1} \text{d}^{-1}$)
SFA	Saturated fatty acids
TFA	Total fatty acid content of the biomass
TPBR	Tubular photobioreactor
V_{dilution}	Daily dilution volume (l)
V_{harvest}	Harvested volume (l d^{-1})
V_{reactor}	Total reactor volume (l)
$Y_{\text{x,ph}}$	Biomass yield on light (g mol^{-1})

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About the author

About the author

Pieter Cente Oostlander was born on June 28th, 1990 in Utrecht, the Netherlands. He attended high school at the 'Revis Lyceum, Doorn, The Netherlands' where he obtained his VWO diploma in 2008. Pieter started his bachelor in Biotechnology at the Wageningen University and Research in Wageningen, the Netherlands in 2008. He obtained his Bachelor's degree in Biotechnology with a specialisation on Cellular and Molecular Biotechnology in the 2012.

Pieter started his Master in Biotechnology at Wageningen University and Research in Wageningen, the Netherlands in 2012. During his MSc thesis Pieter worked on microalgae production as part of his internship. In his internship he worked on a project at the Universidad Autónoma de Querétaro, Querétaro, Mexico in collaboration with Agropark Querétaro. The project worked on the production of microalgae on wastewater from the greenhouse industry. Pieter's work in the project focussed on the effect of geographical orientation on the efficiency of microalgae production systems in outdoor conditions.

After obtaining his MSc degree in 2015, Pieter started his doctorate (PhD) Research within a collaborative project between HZ University of Applied Sciences, Vlissingen, the Netherlands and the Bioprocess Engineering group at Wageningen University and Research, Wageningen, the Netherlands. His PhD project was carried out as part of the RAAQUA-project where he studied the cost price of industrial microalgae production for aquaculture hatcheries and the effect of temperature and light on the biomass yield on light for *Rhodomonas sp.* at different scales. The impact of the effects of these growth parameters on the cost price was also analysed. The results of his PhD research are described in this thesis.

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List of publications

Oostlander, P. C., van Houcke, J., Barbosa, M. J., & Wijffels, R. H. (2020). Microalgae production cost in aquaculture hatcheries. *Aquaculture*, 735310.

Oostlander, P. C., van Houcke, J., Wijffels, R. H., & Barbosa, M. J. (2020). Optimization of *Rhodomonas sp.* under continuous cultivation for industrial applications in aquaculture. *Algal Research*, 47, 101889.

Oostlander, P.C., van Houcke, J., Barbosa, M.J. & Wijffels R.H. Growth and fatty acid content of *Rhodomonas sp.* under day:night cycles of light and temperature, submitted for publication

Oostlander, P. C., Latsos, C., van Houcke, J., Wijffels, R. H., & Barbosa, M. J. (2020). Production of *Rhodomonas sp.* at pilot scale under sunlight conditions. *Algal Research*, 48, 101934.

Schipper, K., Fortunati, F., **Oostlander, P. C.**, Al Muraikhi, M., Al Jabri, H. M. S., Wijffels, R. H., & Barbosa, M. J. (2020). Production of phycocyanin by *Leptolyngbya sp.* in desert environments. *Algal Research*, 47, 101875.

Overview of completed training activities

Discipline specific activities

Conferences

Dutch Shellfish conference (Neeltje Jans, the Netherlands) ¹	2017
Young Algaeneers Symposium (Oban, Scotland) ²	2018
Algae Europe (Amsterdam, the Netherlands) ¹	2018
Algae Europe (Paris, France) ²	2019
Algae Biomass Summit (Orlando, Florida, USA) ²	2019
Symposium RAAQUA project (Vlissingen, the Netherlands) ²	2019

Courses

VLAG Microalgae process design	2016
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General courses

VLAG PhD Week	2016
WGS Project & Time management	2016
WGS Scientific writing	2017
WGS Career perspectives	2019

Optional courses

Preparation of the research proposal	2015
International PhD study trip (San Diego, California, USA) ^{1,2}	2018
BPE symposium ²	2016-2019
RAAQUA project meetings ²	2015-2019

¹ Poster presentation

² Oral presentation

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