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Preface

This PhD thesis comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from April 2012 to December 2015. Professor Irini Angelidaki and postdoctoral fellow Davide De Francisci were supervisor and co-supervisor respectively. Susan Løvstad Holdt initially served as an additional co-supervisor. This project was funded by EU project E4Water (EU grant agreement No. 280756) and by DTU.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-VI**.

I Van Wagenen, J, Holdt, SL, De Francisci, D, Valverde Perez, B, Plósz, BG & Angelidaki, I 2014, 'Microplate-based method for high-throughput screening of microalgae growth potential' Bioresource Technology, vol 169, pp. 566-572., 10.1016/j.biortech.2014.06.096

II Van Wagenen, J, De Francisci, D & Angelidaki, I 2015, 'Comparison of mixotrophic to cyclic autotrophic/heterotrophic growth strategies to optimize productivity of *Chlorella sorokiniana'* Journal of Applied Phycology, vol 27, no. 5, pp. 1775-1782., 10.1007/s10811-014-0485-1

III Van Wagenen, J, Pape, ML & Angelidaki, I 2015, 'Characterization of nutrient removal and microalgal biomass production on an industrial waste-stream by application of the deceleration-stat technique' Water Research, vol 75, pp. 301-311., 10.1016/j.watres.2015.02.022

IV Van Wagenen, J, Pape, ML Safafar, H, D'Este, M, DeFrancisci, D & Angeldaki, I Photobioreactor design and operation influences biochemical composition of waste-grown microalgae. Submitted to Algal Research

In this online version of the thesis, paper **I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

Safafar, H, Van Wagenen, J, Jacobsen, C, Møller, P Carotenoids, phenolic compounds and tocopherols contribute to the antioxidative properties of some microalgae species grown on industrial wastewater. Marine Drugs. Accepted December 2015.

Wágner DS, Valverde-Pérez, B, Sæbø, M, de la Sotilla, MB, Van Wagenen, J, Smets, BF & Plósz, BG. Towards a consensus green microalgal growth model (ASM-A) – uptake and storage of nutrients. Submitted to Water Research December 2015.

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To all the colleagues, especially Manfred, Yoshi, Fabio, Elham, Carson, Pauly, Pedram, Kos, Ana, Arnaud, Gizem and the whole group of PhD students who were around. It was always awesome to have such a fun, international group of people who were there and up for something. Thanks to the guys who let me play football with them, Evangelos especially for converting the finish on the best cross I ever had. It was a lot of fun until I broke my nose. Thanks to the Danish health care system for fixing my nose better than before. Thanks to DTUBasket, the Geckos for letting me play I think of you every day when my ankle hurts. Thanks to FCK for two below standard seasons and to Uli for enjoying them with me.

To my extended family who support me from across the sea. To my parents, for years of investment in my education and sacrifices I appreciate more every day.

To Linnea who gave me daily support throughout the process and to Ingvar who is both a glorious distraction from work and an inspiration to do it better.

Summary

Microalgae production for the purpose of clearing wastewater has been researched for at least half a century. Such systems have a dual benefit: first, they prevent nutrients from entering water bodies and causing eutrophication; second, they transform sunlight and carbon dioxide into a biomass that has many potential uses. Unfortunately, the current high costs of cultivation have limited the development and exploitation of such systems, resulting in only a few full-scale algae wastewater treatment installations and a small industry based mostly around food and pigments. This thesis contributes to a growing body of knowledge with the aim to make algae cultivation viable for the production of sustainable products. Specific contributions include: improvement in the methods of screening the growth potential of different microalgae species; identification of an industrial wastewater that allows good algae growth; knowledge about the mixotrophic utilization of chemical energy present in organic waste; demonstration of a method to optimize efficiency of culture growth and nutrient removal; and biochemical characterization of the produced biomass.

When designing algae cultivation, one challenge is that there are many potential combinations which must empirically screened. Tens of thousands of microalgae species have been identified so far and there are numerous wastestreams that potentially could be of interest. A screening system was developed using the microplate as cultivation vessel and measurement cuvette. Fluorescence was demonstrated to be an order of magnitude more sensitive than optical density for detecting biomass growth, which increased the length of time in which exponential growth was observable from hours to days. This enabled growth rate-light intensity (μ-I) curves to be measured in microplates which were found to be equivalent to those obtained in typical lab-scale photobioreactors. As μ-I curves are the key biological input to an already existing model, it was validated that low density microplate cultivations can be used to make predictions about industrially relevant autotrophic cultivation.

When algae are grown within a wastewater treatment plant, the use of the chemical energy stored in the organic carbon dissolved in the wastewater could also be a useful option. Conventional aerobic sewage treatment expends much energy in breaking down the biomass to CO_2. However, various anaerobic treatment methods would result in effluent containing dissolved organic molecules suitable for algae species that have the ability to grow as

mixo- or heterotrophs. *Chlorella sorokiniana* was cultivated in a lab scale photobioreactor under daily light dark cycles and various timing strategies were tested for adding acetate at concentrations that can be obtained in waste streams of 1 – 2 g L^{-1}. The results showed that the fastest growth occurred when adding the acetate at night (cyclic autotrophy/heterotrophy). However adding the acetate during the day (mixotrophty) also improved growth compared to autotrophic controls.

Industrial wastewater was used as cultivation medium of *Chlorella sorokiniana*. The culture was able to grow at high rates upto a density of 4 g L^{-1}. The deceleration-stat technique was used to create a series of pseudo-steady states to give information about the expected results of continuous cultivation of microalgae in the selected wastewater. At light intensities of 2100 and 200 µmol photon m^{-2} s^{-1} the algae grew at a rate of over 5 and 1.67 g $L^{-1}day^{-1}$, respectively. The corresponding removal rates of nitrogen were 238 and 93 mg $L^{-1}day^{-1}$ and 40 and 19 mg $L^{-1}day^{-1}$ for phosphorous. Ammonium removal varied from below 40% to 99%, while phosphate removal was always nearly total.

When the biomass was characterized, it was found that fertilizer value N and P content increased with growth rate. For animal feed, the amino acid content was about 40% of biomass. The content of the nutritionally important α-Linoleic fatty acid increased when light intensity and dilution rate were higher. Valuable pigments lutein, carotene and other carotenoids were higher in low-light conditions.

The results from this thesis demonstrate that industrial wastewater can be a suitable replacement for algae cultivation medium. The screening method developed will reduce the cost of identifying the best conditions to test at lab scale. The D-stat method offers a way to identify the best conditions for biomass production and nutrient removal. Various options for heterotrophic and mixotrophic utilization of waste organic carbon in effluents are identified. Further advances in microalgae cultivation and processing will be needed for the production of sustainable products from wastewater in the future.

Dansk sammenfatning

Mikroalgedyrkning som led i spildevandrensning har været studeret i mindst et halvt århundrede. Sådanne systemer har to gavnlige effekter: de forhindrer udledning af næringssalte, der ellers ville medføre eutrofiering, og derudover omformer mikroalgerne sollys og kuldioxid til biomasse, der har mange potentielle anvendelsesmuligheder. Desværre har de nuværende omkostninger ved mikroalgedyrkning begrænset udviklingen og udnyttelsen af sådanne systemer, hvilket har resulteret i kun få fuldskala alge-spildevandsrensningssystemer og en lille industri mest baseret på fødevarer og pigmenter.

Denne afhandling bidrager med en tiltagende viden med det formål at skabe en realistisk algedyrkning for at producere bæredygtige produkter. Helt specifikt inkluderer dette: optimering af metoder indenfor screening af vækstpotentiale af forskellige arter; identificering af et industrielt spildevand, der tillader god algevækst; viden om mixotrofisk udnyttelse af kemisk energi fra organisk affald; påvisning af en metode til at optimere effektiviteten i væksten af algedyrkning og fjernelse af næringssalte; og biokemisk karakterisering for at identificere kvaliteten af den producerede biomasse.

Når en algedyrkning designes, er den store udfordring at der er mange potentielle kombinationer, der skal screenes empirisk. Ti-tusinder af mikroalgearter er allerede identificeret og der er utallige spildevande, der kunne have potentiel interesse. Der er i denne afhandling blevet udviklet et screeningssystem baseret på mikroplader som dyknings- og målingsbeholder (cuvette). Fluorescens, sammenlignet med optisk densitet, viste sig at være overlegen i følsomhed ved påvisning af biomassevækst. Dette resulterede i et forøget tidsforløb fra timer til dage, hvor det var muligt at observere den eksponentielle vækst. Dette muliggjorde at væksthastighed-lysintensitets (μ-I) kurver kunne måles i mikropladerne, hvilket er tilsvarende de kurver fundet i typiske laboratorieskala fotobioreaktorer.

Da μ-I kurver er et biologisk nøgleinput i allerede eksisterende modeller, blev det valideret at mikroplade-dyrkninger med lave algedensiteter kan anvendes til at forudsige industrielle relevante autotrofiske dyrkninger.

Når alger dyrkes i spildevandsanlæg, så kan den kemiske energi, der er lagret i opløst organisk kulstof, også være et muligt dyrkningsmedie. Konventionel aerobisk spildevandsrensning anvender meget energi på at nedbryde biomasse til CO_2, men forskellige anaerobiske behandlingsmetoder vil resultere i spil-

devand indeholdende opløste organiske molekyler, der er egnede til mikroalger, der kan gro mixo- eller heterotrofisk. Mikroalgen *Chlorella sorokiniana* blev dyrket i laboratorieskala i en fotobioreaktor ved daglige lys/mørke cyklus og med forskellige strategier for tilsætning af acetat i koncentrationer, der forefindes i spildevand (1 – 2 g L^{-1}). Resultaterne viste at den hurtigste vækst forekom ved acetat tilsætning om natten (cyklisk autotrofi/heterotrofi). Dog øgedes væksten også ved acetat tilsætningen i dagscyklus (mixotrofi) sammenlignet med de autotrofiske kontrol forsøg.

Industrielt spildevand blev anvendt som dyrkningsmedie for *Chlorella sorokiniana*. Det var muligt at dyrke algekulturen op til en densitet på 4 g L^{-1}. Hastighedsnedsættelses (deceleration)-stat (D-stat) teknikken blev anvendt således at der dannedes en serie af pseudo ligevægtstilstande, hvilket gav information om de forventede resultater for kontinuerlig dyrkning af mikroalger i det valgte spildevand. Algerne voksede med en hastighed på over 5 og 1,67 g $L^{-1}dag^{-1}$ ved henholdsvis 2.100 and 200 µmol fotoner m^{-2} s^{-1}. De tilsvarende fjernelseshastigheder af kvælstof (N) var 238 og 93 mg $L^{-1}dag^{-1}$, samt 40 and 19 mg $L^{-1}dag^{-1}$ for fosfat (P). Ammonium-fjernelsen varierede fra under 40% til 99%, mens fosfatfjernelsen altid var stort set total.

Biomassekarakteriseringen viste at gødningsværdien, i form af N og P indholdet, øgedes med vækshastigheden. Algebiomassen indeholdt omkring 40% aminosyrer, hvilket kunne have potentiale for dyrefoder. Indholdet af den næringsmæssige α-linol fedtsyre øgedes når lysintensiteten og fortyndingshastigheden var højere. Indholdet af værdifulde pigmenter såsom lutein, karoten og andre karotenoider var højere i lavt-lys forholdene.

Resultaterne fra denne afhandling viser at industrielt spildevand kan være egnet til at erstatte algedyrkningsmedie. Den udviklede screeningsmetode vil reducere omkostningerne til at identificere de bedste forhold, der kan videretestes i lab-skala. D-stat teknikken bidrager med at kunne identificere de bedste forhold til biomasseproduktion og næringssaltfjernelse. Flere muligheder er identificeret til heterotrofisk og mixotrofisk udnyttelse af organisk affald i spildevand.

Yderligere fremskridt indenfor mikroalgedyrkning og forarbejdning er nødvendige for produktionen af bæredygtige produkter fra spildevand i fremtiden.

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Abbreviations

DHA	Docosahexaenoic acid
IC	Internal Circulation reactor.
μ-I	Exponential growth rate- Light Intensity relationship
VFA	Volatile fatty acids
$Y_{X,E}$	Yield on light energy (g mol^{-1})
$Y_{X,S}$	Yield on substrate (g g^{-1})

1 Introduction

1.1 Motivation: Global Challenges

The motivation for sustainable technology research can be described with the help of a simple equation:

$$I = PAT$$

According to the "IPAT" equation, the total environmental impact (I), as expressed in resource depletion and environmental pollution, depends on the *population* (P) and the *affluence* (A) GDP per capita. These factors are multiplied by *technology* (T), which is the resource use or pollution per unit of GDP (Huesemann and Huesemann 2011).

Population is increasing. In 1950 the global population was about 2.5 billion; it has currently reached about 7 billion and in 2050 it is expected to be around 10 billion (UNPD).
Affluence is increasing. Despite the growing global population, the number of people living below the accepted poverty threshold has fallen below 1.1 billion (Economist). The prolific consumers of the middle class are on track to increase from 1.8 to 3.2 billion in the period 2009- 2020 (Pezzini).
Technology, then is the remaining variable over which there is more control.
The environmental impacts (I) include anthropogenic climate change which has been well documented by the International Panel on Climate Change (IPCC). However, changing climate is not the only threat to our environment. A recent framework describes nine conceptual "planetary boundaries" safe operating conditions for humanity, beyond which the abrupt or irreversible environmental change could occur (Steffen et al 2015). The other planetary boundaries are: i) change in biosphere integrity (biodiversity loss and species extinction), ii) stratospheric ozone depletion, iii) ocean acidification, iv) biogeochemical flows (phosphorus and nitrogen cycles), v) land-system change (e. g. deforestation), vi) freshwater use, vii) atmospheric aerosol loading and viii) introduction of novel entities (e.g. radioactive materials, nanomaterials, and micro-plastics). All of these impacts are expected to increase with increasing population and affluence, unless specific technological improvements or changes are made to the current modus operandi. Some of the impacts have already, or may soon cross the limits described by Steffen et al. (Figure 1).

The motivation for sustainable technologies is the maintenance of the planet in conditions that benefit humanity. To reach this target, leaders are calling for a circular economy where waste emissions to landfill, waterbodies and atmosphere are reduced -- ideally eliminated (European Commisson 2015). A pivotal characteristic of circular economies is a paradigm shift where waste is viewed as a resource.

Microalgae play a role in the definition of one planetary boundary, namely the "biochemical flows." The flow of nutrients, especially phosphorous into water bodies stimulates algae growth and axoxia will occur when the algae are consumed by other microbes. This can disrupt food webs and eventually fisheries (e. g. the dead zone in the Gulf of Mexico). Therefore Steffen et al. point out that there must be a threshold of P emissions above which our oceans will be irreversibly damaged. Excess P, along with Nitrogen can lead to eutrophication of fresh water bodies as well. (Steffen et al).

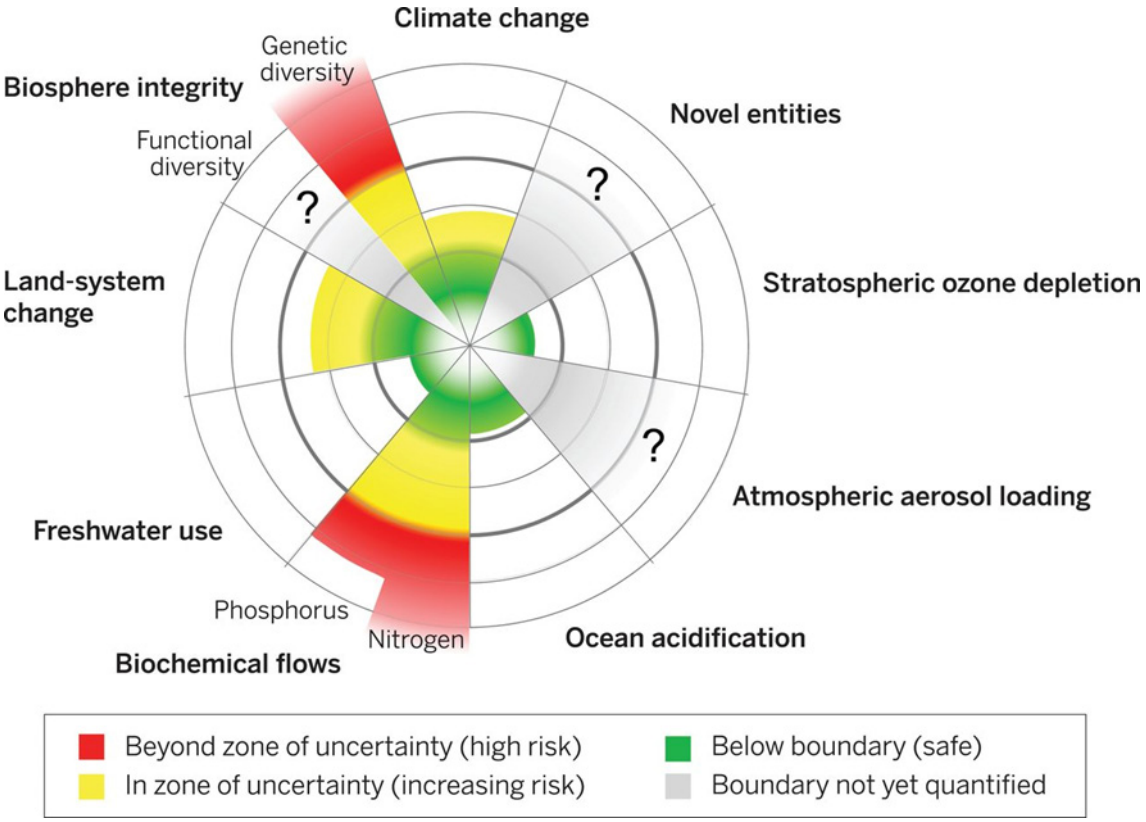

Figure 1. Current status of planetary boundaries Source: Steffen and others, 16 January 2015, Science. (Permission request granted 04/09/2015).

1.2 Sustainable microalgae technologies

This thesis will focus on micoralgae use in wastewater treatment, reducing biochemical flows and fresh water use.

Algae can utilize the reduced N in wastewater, which leads to several indirect benefits. The Haber-Bosch process that fixes atmospheric nitrogen for fertilizers consumes about 1% of yearly energy (Notman 2012). Conventional wastewater treatment returns N to its inert atmospheric state, but produces nitrous oxide (N_2O) as a byproduct. If wastewater treatment is not successfully implemented, N can also compromise water supply via nitrate toxicity. So overall reduction in the need to fix and treat nitrogen benefits the environment.

To the extent that algae are used to uptake emitted carbon dioxide and replace non-sustainable resources, they can also contribute to reducing climate change. Today algal biomass is a product that is used for many applications as diverse as fertilizers, feed, food, nutritional supplements, cosmetics and pharmaceuticals (Borowitzka 2013). There is hope that algae can play a role in displacing some of the bulk chemicals that are derived from petroleum resources, even perhaps fuels one day. This is limited by a cost of production that is currently too high (Wijffels and Barbosa 2010). In the near term, algae will be marketed for high value products, while a biorefinery, where multiple products are extracted from the algae biomass, is thought to be necessary to make bulk products economically feasible (Vanthoor-Koopmans et al 2013).

Despite the current economic limitations, research on the cultivation of algae in wastewater should continue. Consider the advantages:

- The inputs are air pollution, polluted water and sunlight.
- The outputs are oxygen, cleaner water and useful biomass.

1.3 Objectives and thesis structure

The main aim of this thesis is to make technical progress towards sustainably growing microalgae on wastewater. In order to do this, several objectives were laid out which are considered meaningful steps in the process of advancing the field towards lower-costing, higher-yielding algae production in wastewater.

The first of the specific objectives was to improve available platforms for screening of microalgae growth. It had been noted that there are many spe-

cies of microalgae which may be interesting for commercial exploitation, but that each species would need to be tested again each time that a new wastewater stream was considered. In order to bring down the cost and increase the ability of future workers to contemplate more strain-wastewater combinations, a microplate based platform was tested. The results were validated by comparing them to the measurements obtained from relevant larger scale cultures (Paper I).

A second objective was to utilize the abilities of the microalgae to heterotrophically or mixotrophically assimilate the types of organic carbon that are usually present to some extent in wastewaters. Specifically, it was examined if these substrates were more preferentially added in the day or night (Paper II).

A third objective was to demonstrate the maximum attainable biomass productivity of a given species of microalgae on at least one specific wastewater. To maximize productivity in continuous cultures, the culture dilution rate was shown to be a critical factor. Therefore, a method to study the productivities arising from a range of biomass densities was employed. (Paper III).

Additionally, generated biomass was characterized for the valuable products that can serve as the main motivation for microalgae to be produced in the future. In this specific scenario, a fourth objective was to describe the role of cultivation conditions on biorefinery potential, as defined by biochemical composition (papers II and IV).

Chapter 2 covers the biological diversity of microalgae and the diversity of their metabolism. This diversity is one of the motivations for attempting to increase our ability to screen algal isolates. Chapter 2 also discusses the history of research into microalgae cultivation as a source of products that leads to the field of microalgae bioprocess engineering. It also describes the state of research and deployment of microalgae wastewater systems. This contextualizes papers II, III and IV.

Chapter 3 describes various efforts on phototrophic, mixotrophic and heterotrophic growth of microalgae on wastewaters and waste carbon sources.

Chapter 4 discusses a screening methodology which enables estimation of microalgae growth at industrial scale from measurements made in small-scale cultures.

Chapter 5 is about the optimization of lab-scale photobioreactors on an industrial wastewater and the first application the deceleration-stat method for quickly characterizing algae wastewater treatment performance.

Chapter 6 discusses how cultivation conditions will change content of various valuable components of the biomass and other considerations when industrial wastewater is used as the algae cultivation medium. Conclusions and future perspectives follow.

2 Microalgae Biotechnology

2.1 Biological and physiological diversity

Microalgae are by definition microscopic and by definition have chloroplasts or chlorophyll. The group of organisms which meet this definition is polyphyletic and contains much diversity. Eukaryotic microalgae[1] evolved from endosymbiosis events. In primary endosymbiosis, a eukaryote engulfed a cyanobacterium and gained the ability to conduct photosynthesis in the structure called a plastid. In secondary (or tertiary) endosymbiosis events, eukaryotes engulfed other photosynthetic eukaryotes (Keeling 2004). These events apparently took place multiple times, which accounts for the situation where microalgae occur across 4 of the 6 eukaryotic super groups: mostly among the Plantae and Chromalveolata, but also excavate such as Euglenoids and Rhizaria such as Foraminifera (Figure 2) (Simpson and Roger 2004). A few thousand algae isolates are available for researchers from culture collections, tens of thousands of them are listed in taxonomic databases and the possible number of species in existence may be higher by an order of magnitude (Guiry 2012). This massive diversity is the basis for developing methods to accelerate screening of species (Paper I).

Given the wide biological diversity, it is unsurprising that various physiologies have evolved. These give several possibilities for biotechnological exploitation. A photo-autotrophic metabolism with light as the energy source and carbon dioxide as the carbon source is possible for many, but not all microalgae. For example, *Crypthecodinium cohnii*, despite having a chlorophyll, is an obligate heterotroph that can grow on glucose or acetate (De Swaaf et al 2003). The species is used commercially for the production of the Omega-3 fatty acid docosahexaenoic acid (DHA). The ability of many photosynthetic

[1] Note that cyanobacteria can be cultivated with similar or identical techniques to those of eukaryotic microalgae. Throughout this thesis and much of the literature "microalgae" can be considered a shorthand term for eukaryotic microalgae and cyanobacteria. An exception to this is paper I, where the fluorescence techniques described would need to be modified to work with cyanobacteria (Schubert et al 1989).

algae strains to also grow on glucose or acetate in the dark was demonstrated by Pringsheim in the 1920s (Preisig and Andersen 2005). By the early 1960s, there were detailed characterizations of 41 strains of the genus *Chlorella,* with various stimulatory and inhibitory responses to various carbon sources in light or darkness (Pore 1972). These properties, called mixotrophy, have also been exploited for the commercial production of *Chlorella* sp. in outdoor ponds with acetic acid (Richmond 2004). Exploitation of autotrophic metabolism has been more widely researched and is discussed below.

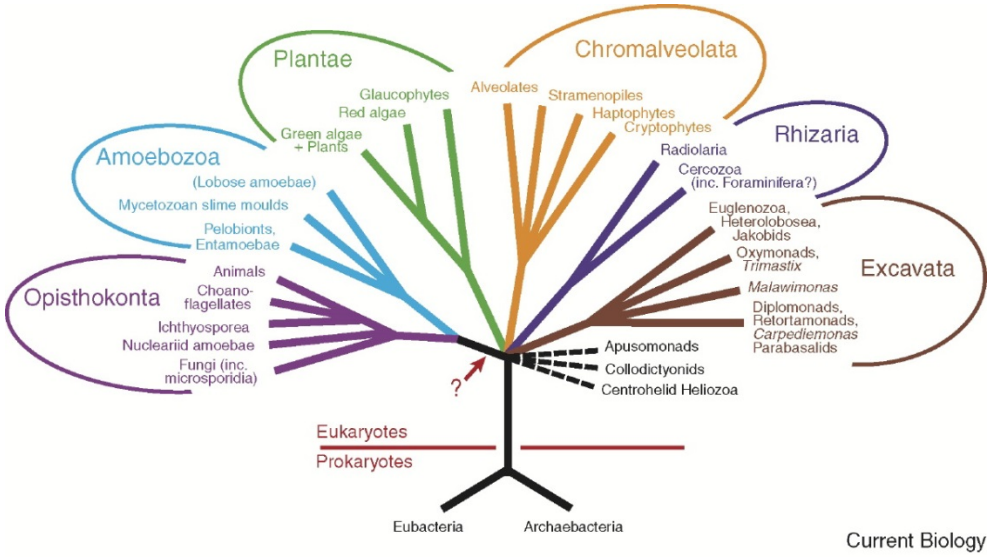

Figure 2. Eukaryotic diversity. Reproduction from (Simpson and Roger 2004) request granted 8282015.

2.2 Development of algal mass culture

Human collection of microalgae was documented already when the Spanish first contacted the Aztecs and noticed the collection of *Arthrospira* biomass from lake Texcoco for processing to cakes known as "tecuitlatl" (Gantar and Svirčev 2008). Such traditional collection has also been noted in Africa (for example Lake Chad) and has been refined by current practitioners in four volcanic lakes in central Myanmar, where production was estimated at 30 tons yearly (Richmond 2004). Other species of filamentous microalgae that readily agglomerate are eaten as vegetables in South America and Asia (Gantar and Svirčev 2008).

The current interest in microalgae as a sustainable technology can be traced to the period following the Second World War (Burlew 1953). At that time,

algae were considered an interesting source of food, given their protein content; the prospect of burning the produced algae for biofuels was also mentioned. At this point most of the fundamental challenges of large algae culture were described: light limitation, solubility of gasses in the liquid medium, contamination problems and so on. *Chlorella,* a "weed" alga was chosen as one of the best to perform initial characterization experiments due to its robust nature.

William Oswald was completing his dissertation research around the time of Burlew et al and he began a long career describing the possibilities to clean wastewater with microalgae (Oswald and Golueke 1960). Oswald would go on to design a system where algae in paddle-wheel driven ponds provide oxygen so that bacteria are able to consume wastewater. His physical legacy can be seen among a series of ponds with treatment capacity from 1 – 200 million liters per day. The ponds are concentrated in California, where Oswald worked, but are also found globally (Gutierrezl 2011). The algae turf-scrubber (ATS) is another simple and robust system for nutrient removal that was developed in the 1970s and is built on larger scales lately (Calahan et al 2015).

Motivated by the oil crisis in the 1970s, the US Department of Energy (DOE) Office of Fuels Development began a program to develop fuels from microalgae. The ASP ran between 1978 and 1996, when it was defunded during a period of low fuel prices (Sheehan 1998). The program isolated over 3000 strains from the across the country, which were characterized and screened. Cultures were eventually scaled up and tested in raceway ponds in the New Mexico desert. Japan also invested in a "Biological CO2 fixation and utilization program" throughout the 90s.

Algae research would bloom again when the gas prices rose in the later part of first decade of the new millennium. Spurred on by NAABB, a $ 60 million DOE research program, there were large sums devoted to research by oil companies, philanthropists and many start-ups (Mascarelli 2009). This rush to biofuels was called "The summer of algae" by industry observers, who joked "a breakthrough in algae fuel is always five years away" (Lemos Stein 2009). Many of the biofuel companies have begun to refocus on profitability through valuable products, as fuels remain too far away. Examples can be found on the company websites of Algenol, Sapphire, Synthetic Genomics, Aurora, Cellana and Solix. The interest in these products is not merely from small companies or biofuel start-ups looking to change course into short-term

profitability. After an investment in research in 2010, Unilever has launched soaps that feature heterotrophically produced algae oils (Sonne 2010). Active industrial-academic research collaboration is on-going about using waste-grown algae for pigment production.

2.3 Light limited growth, potential and benchmarks

Enough solar energy to fuel the planet for a year hits the earth every hour. Therefore, efficient conversion of solar to usable electrical or chemical energy offers a technical solution that can greatly reduce environmental impact (Tsao et al). However, photosynthesis by algae or plants faces several limiting factors.

By the time of (Burlew 1953) many of the fundamental issues of microalgae cultivation had been described. Chief among these is the utilization of light (Figure 3). A growth rate- light intensity (μ-I) curve demonstrates that initially algae use increasing light in a linear way, but well before full sunlight is reached, efficiency stops increasing. Light does not penetrate deeply into algae cultures due to self-shading (Huesemann et al 2012).

Two apparent solutions exist to maximize production given these limitations. The first is option is to increase the efficiency of algal culture by genetically modifying the strains so to produce fewer pigments and therefore allow light to penetrate more deeply. Despite success in genetic modification, implementation of the modified microbes has not yet achieved the potential (de Mooij et al 2014). Secondly, to increase the surface area to volume ratio of the algae culture, so that the more of the reactor volume is illuminated (Zijffers et al 2008)(Cuaresma et al 2011). Many reactor types have been designed up to today, and it is useful to have a simple way to compare their efficiency.

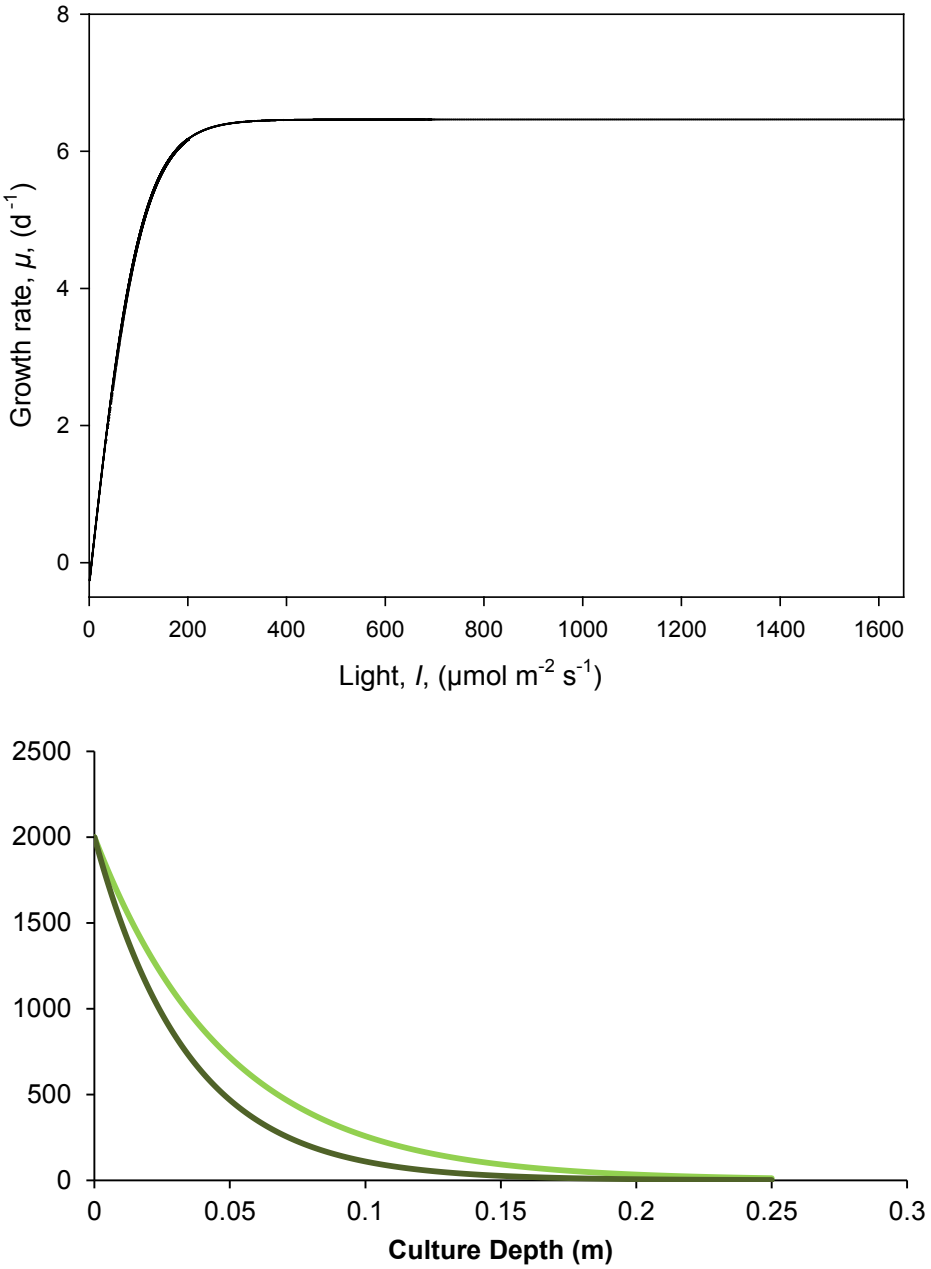

Figure 3. a. Typical growth rate light intensity curve for C*hlorella sorokiniana*. **b.** example light absorption curve when initial light intensity is 2000. and k_a is 0.12 or 0.17 indicating acclimation to high or low light respectively. Data from Paper I.

Table 1. Microlagae growth benchmarks

Parameter	Benchmark	Condition	Source
Exponential growth, μ	6.5 day^{-1}	Lab PBR cont. illumination 2100 μmol m^{-2} s^{-1}	(Cuaresma et al 2009)
Volumetric productivity P_V	0.4 g L^{-1} h^{-1}	Above	(Cuaresma et al 2009)
	0.9 g L^{-1} h^{-1}	2000* μmol m^{-2} s^{-1}	(Qiang et al 1998)
	1.2 g L^{-1} h^{-1}	8000* μmol m^{-2} s^{-1} *on both sides, lab PBR	(Cuaresma et al 2011)
	4 g L^{-1} day^{-1}	Simulated day day/night cycle PBR	
Areal productivity P_A	7.7 g m^{-2} h^{-1}	Above	(Cuaresma et al 2009)
	40 g m^{-2} d^{-1}	Small Pond (year av.)	(Norsker et al 2011a)
	10 g m^{-2} d^{-1}	Industrial pond (year av.)	
Yield on photons $Y_{X,E}$	0.85 g mol^{-1}	High light intensity PBR	(Cuaresma et al 2011)
	1.3 g mol^{-1}	Low light intensity PBR	(Cuaresma et al 2009)
	1.8 g mol^{-1}	Theoretical max	
Photosynthetic efficiency, PE%	1.5 %	Ponds (est. year av.)	(Norsker et al 2011a)
	3 %	Tubular reactors (est. year av.)	
		Flat panels (est. year av.)	
	5%	Theoretical "Max. Photosynthetic Efficiency"	(Tredici 2010)
	12.4%		

To discuss culture productivity, it is useful to compare to the highest reported literature values as a benchmark (table) (when excluding light intensities that exceed solar maximum (Qiang et al 1998). As we want to compare production efficiency, a rate is a logical starting point for comparison. For cultivation of microbes, the exponential growth rate μ ($hour^{-1}$ or day^{-1}) is a useful characteristic to describe how fast the organism can grow and how fast a chemostat could be diluted. As we are interested in the production of biomass, we must multiply μ by culture density, $C_{x,}$ in order to get the volumetric productivity (g L^{-1} day^{-1}) of the reactor. Concentration and growth rate are inversely related in a typical, light limited culture. For comparing algae cultivation systems (eg shallow ponds to tubular photobioreactors) to one another, volume per surface area ratio allows calculation on the basis of areal productivity (g m^{-2} day^{-1}). Neither areal nor volumetric productivity normalizes for input light, therefore biomass yield on photons, $Y_{X,E}$ (g mol^{-1}) allows practical comparison of all systems to see how close they are to achieving their theoretical maximum photosynthetic efficiency. While often sufficient for the studies conducted herein $Y_{X,E}$ provides less information than photosynthetic

efficiency: the fixed chemical energy divided by energy of incoming light (%) (Tredici 2010). However, as photosynthetic efficiency requires knowing the energy content of the biomass, its calculation can be more laborious, as it requires either calorimetry or relatively complete biochemical characterization (ie protein, carbohydrate, lipids per gram of biomass). Figure 4 below demonstrates how the energy content of the biomass and the $Y_{X,E}$ found in estimates of photosynthetic efficiency.

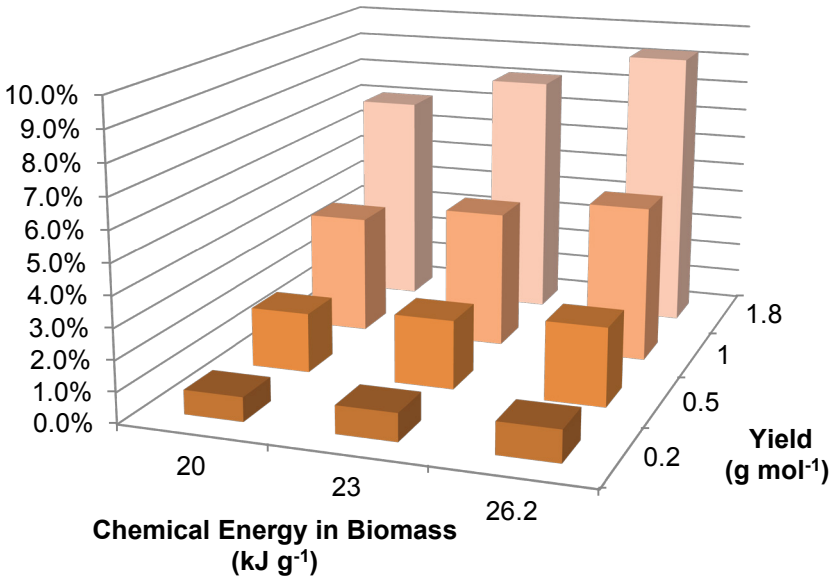

Figure 4. Photosynthetic efficiency is dependent on photosynthetic yield and biomass energy content. Chemical energy values from literature: (Tredici 2010; Norsker et al 2011a; Blanken et al 2013).

Burlew (1953) predicted that at maximum productivity, algae could generate biomass at upto 70 $gm^{-2}d^{-1}$, which was about five times more than they had observed in outdoor experiments at Stanford University. Six decades later, a thorough review of published data found that although short term productivity of 30 $gm^{-2}d^{-1}$ had been observed, on a yearly average, 20 $gm^{-2}d^{-1}$ was very rarely exceeded (Richmond 2003) (Norsker et al 2011a). Note that the correlation factor is 1 $gm^{-2}d^{-1}$ is 3.65 ton ha^{-1} $year^{-1}$, so this corresponds to 73 tons ha^{-1} $year^{-1}$. This value is very similar to the 2012 average production of sugar cane in Brazil: 69.4 (da Silva and Chandel 2014), while in the hot, humid northern regions of Brazil, 95 tons ha^{-1} $year^{-1}$ were yielded. Therefore, it is important to note that the maximum productivity potential of algae biomass alone is not significantly higher than all land plants. However, the potential

benefits would include higher oil content, lack of non-useful parts of the biomass (roots and leaves) longer growing season and implementation on otherwise non-arable land (Chisti 2007; Tredici 2010).

To understand algae potentials, based on empirical results and available light- see figure 5, which converts yield on photons and light received per year to yearly biomass. Furthermore, this chart is useful when performing literature review, one is also tasked with toggling between different units.

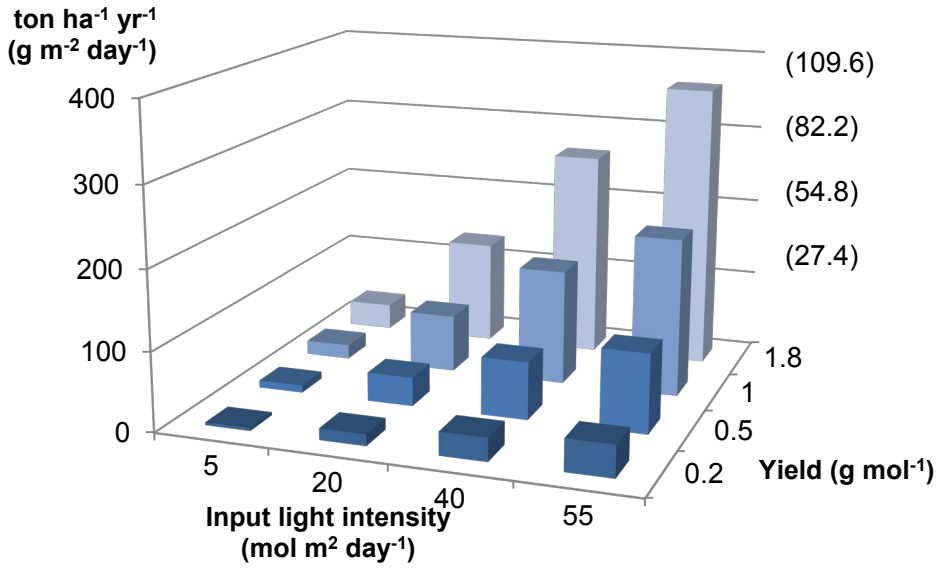

Figure 5. Productivity as a function of incoming light and $Y_{X,E}$. Light intensity of 40 mol m^{-2} day^{-1} is a Brazilian year round average (da Silva and Chandel 2014) or a Northern European summer (Olofsson 2015). Southern European summer and winter are represented by 55 and 20 mol m^{-2} day^{-1} respectively, while at best 5 mol m^{-2} day^{-1} will be available in northern European winters (Olofsson 2015).

The various metrics presented above set the limit for what algae may achieve under the best circumstances. One fundamental question addressed, especially in Paper III, is the extent to which algae grown in wastewater can meet the same productivity of algae grown in optimized mineral medium. Furthermore, Paper II investigated the extent to which a wastewater with appropriate amounts of organic carbon nutrients might increase production above that possible in purely autotrophic cultures. Given that these studies were performed in continuous, lab scale photobioreactors, $Y_{X,E}$ was chosen as the most relevant measure of comparison to the benchmarks. One key finding was that the studied wastewater in Paper III did allow $Y_{X,E}$ comparable to

what might be achieved on a mineral medium when low light intensities relatable to those of vertically oriented photobioreactors were used.

Paper II also demonstrated that, indeed, the yield achieved when heterotrophic growth took place at night exceeded that which is possible during phototrophic growth. Heterotrophic yields and wastewater performance metrics will be discussed in chapter 3. Finally, it should be noted that the metrics discussed above are generalized to biomass production, but in many cases (Klok et al 2014) the correct unit would be expressed in the yield or production rate of a biomass component of interest.

3 Wastewater and Waste Carbon Utilization

The advantages of algae for waste-treatment have long been acknowledged seen. In this chapter we examine various scenarios in which algae have been cultivated in waste-streams and the potential advantages and disadvantages of each scenario are compared and discussed. The lines between a phototrophic, mixotrophic or heterotropic culture may be blurred in scenarios in which small amounts of organic nutrients are present and there is always the probability that an outdoor culture which is mixotrophic in the daytime will be somewhat heterotrophic at night. Still the categories are useful in dividing the various efforts which have been recorded in the literature.

3.1 Photoautotrophy

3.1.1 Waste Carbon dioxide

All photoautotrophic cultures will fix CO_2 and therefore remove it. Thus, microalgae carbon sequestration has been touted as beneficial (Rosenberg et al 2011). One kg of algae removes about 1.8 kg of CO_2 from the atmosphere. The known production costs are in the range of 5000 euro per ton (Spruijt et al 2015a), but the cost of carbon dioxide credits are less than 100 euro per ton (Jotzo 2012). The cost of food grade CO_2 is also on the range of less than 100 euro per ton (Doty). Until the price of carbon is valued higher, algae carbon sequestration is not likely to provide an economic motive for algae cultures.

3.1.2 Mixed cultures

As mentioned in section 2.2, Oswald designed algae ponds that oxygenate wastewater and remove mineral nutrients. The provision of Biological Oxygen Demand (BOD) from photosynthetically produced oxygen prevents the need for blowers in the typical municipal sewage systems. This equals savings of energy and capital. The apparent drawback is that the lower cell densities supported mean that the pond systems are mainly suited to areas with low population and much available land (Gutierrezl 2011).

Such simple systems avoid the difficulties of providing sterile mono-cultures. Instead, they cultivate whatever robust algae predominate. Sometimes the reseeding of well-flocculating algae is practiced as a strategy to manage the species present in an otherwise uncontrolled system (Craggs et al 2011). Still, such strategies do not give the operator the ability to choose to produce

the most valuable organism, and flocculation and downstream processing may pose additional challenges.

The algae turf scrubber (ATS) also relies on mixed cultures, essentially an "artificial ecosystem" (Craggs et al 1996). These cultures are also mostly phototrophic, but instead of being suspended in water, they only receive water sporadically and remain attached to a surface until they are harvested. Despite their different appearance, they have reported productivities in the same range as conventional algae ponds 50 ton ha^{-1} yearly (Calahan et al 2015) or 25 g m^{-2} daily over peak periods (Craggs et al 1996). The quality of the biomass may not be the same as pond-grown algae, and Calahan et al did not recommend the biomass for high-value products. Still there would undoubtedly be pigments which could be extracted.

In mixed cultures bacteria releasing CO_2 could also help reduce the CO_2 demand and recycle algal extracellular products (Bai et al 2014). Note that the study only deals with very low productivity (below 20 mg L^{-1} day^{-1}) so it is doubtful the bacterial CO_2 production would be meaningful in industrially relevant conditions without the supplementation of additional organic carbon sources.

3.1.3 Monocultures

Monocultures are also well-researched in phototrophic wastewater cultures. (Cai et al 2013) used various concentrations of anaerobic digester effluent as the nutrient source for *Nannochloropsis* or *Synechocystis*. In outdoor cultivation, the AD effluent was equivalent to commercial nutrients in the maintenance of algae growth in ponds (Sheets et al 2014). Photobioreactors have been tested in various configurations for wastewater remediation. (Al-hadabi et al 2012). The obvious drawback is the need to design strategies to prevent contamination of unwanted species. Still, if a proper selective pressure is applied, the monoculture will result in a higher content of desired products (Darzins et al 2010). Such monocultures were apparently maintained even for culture of *Scenedesmus* in outdoor cultures using swine digestate centrate with negligible contamination (Morales-Amaral et al 2015a).

3.2 Heterotrophy

Heterotrophic cultivation of microalgae is a proven industrial technology. It currently provides for example most of the DHA in baby formula. However, when considering purely heterotrophic processes, it is important to note that whether the carbon source comes from a waste-stream or from pure sugar

cane the algae production system is now competing with a full range of well-characterized and genetically pliable heterotrophic organisms that have a proven industrial track record. Given that the main costs for heterotrophic production are the costs of reactor volume and substrate, the competition is on the terms of yield, productivity and final titer (Villadsen et al 2011). So the main advantage microalgae provide over other heterotrophs is the genetic diversity that can make them a reliable source of phytochemicals not found in other organisms. However, in the long term, if phytochemicals of interest are observed in microalgae that prove difficult to cultivate heterotrophically, it is likely that genetic engineering of the pathways producing the products of interest will quickly be tried. In the case of lower value chemicals and biofuels, where feedstock costs play a key role in economics, it is important to have benchmarks, to be sure whether a heterotrophic algae process is competitive. Fortunately, there is no limitation in cells yielding at least Y_{XS} 0.5 g cells per g glucose (Shi et al) (0.4 C-mol per C-mol) as one also finds in yeast (Villadsen et al 2011).

More characterized organisms like *E. coli* have achieved higher cell density (190 g L^{-1}) at higher growth rates up to 0.5 h^{-1} (Bauer and Shiloach 1974; Shiloach and Fass 2005) often reaching the point where the limit is not biological growth rate, but the ability to supply the organisms with oxygen. However, *E. coli* still requires significant engineering to produce fatty acids as well as microalgae do (Lu et al 2008). The oleaginous yeast *Yarrowika* offers an example of an organism that may produce lipids similar to those of microalgae, (although usually lacking the omega 3 fatty acids). It was found to have Y_{XS} of about 0.5 g g^{-1} on glucose, glycerol or acetic acid, while also producing on propionate or butyrate at slightly lower yields (Fontanille et al 2012). VFA utilization was also mentioned in the patent literature (Stephanopoulos 2011). A modified strain achieved lipid content over 60%, lipid titer of 55 g L^{-1}, lipid yield of 0.234 g g^{-1} and lipid productivity of 17 g^{-1} L^{-1} day^{-1} (Qiao et al 2015). (In over 100 algae fermentations reviewed by (Perez-Garcia, O., & Bashan 2015), the best recorded lipid productivity was only 11 g^{-1} L^{-1} day^{-1}). Based on projections from these values, Stephanopolis claimed that $ 250 per ton glucose and lipid yield of 0.3 g g^{-1} could put biological replacement of fossil fuels within reach. (DTU lecture 2015). Therefore, it is important to note that algae are directly competing with other, perhaps more easily genetically modified organisms for the utilization of waste sugars and even volatile fatty acids.

A review (Eriksen 2008) found the highest biomass concentrations of heterotrophic algae were around 80 g^{-1} L^{-1} of biomass. Biomass production by *Galdieria sulphuraria* up to 50 g^{-1} L^{-1} day^{-1} while achieving over 0.5 g g^{-1} Y_{XS} and good titres of the product, valuable pigment phycocyananin 16 mg g^{-1} (Graverholt and Eriksen 2007). More recently, *Chlorella vulgaris* produced over 87 g L^{-1} day^{-1} and concentrations over 100 g L^{-1} and Y_{XS} over 0.6 gg^{-1} (Doucha and Lívanský 2011). Good production of *Crypthecodinium cohnii* for DHA is documented on either glucose, acetic acid, ethanol or Carob pulp syrup (Mendes et al 2008). On acetic acid, biomass yields were 0.13 g g^{-1}. One g DHA L^{-1} day^{-1} was produced, with biomass titres reaching upto 100 g L^{-1}, with a lipid content over 50% (De Swaaf et al 2003). With this brief review, we see that algae can heterotrophically produce at rates approaching other heterotrophic organisms. The main competitive advantage will be specific products with the other organisms lack.

Heterotrophic production on waste-resources has been tried. One common problem with such approaches is lack of sufficient substrate concentration. Even when a cheese whey waste-stream had 182 g L^{-1} of lactate, its hydrolysate had around 30 g L^{-1} of monomeric sugars. At these concentrations, it is not surprising that the final biomass could not reach high enough concentrations (Espinosa-Gonzalez et al 2014). The agro-industrial wastes, silage and (a product of maize ethanol production) soy whey have sugars to sustain heterotrophic growth, but a concentration more suitable for mixotrophic growth (Mitra et al 2012). The added cost of harvesting or developing perfusion cultures would limit process economics on such substrates. However, more concentrated waste streams like pure glycerol have been used to achieve culture density over 50 g L^{-1} (Cerón-García et al 2013).

Food waste hydrolysate also shows promise, but faces a similar challenge of getting high enough density (Pleissner et al 2013; Lau et al 2014). A second potential issue is the composition of the waste streams. It may be advantageous to mix a Nitrogen-containing stream into the Carbon source, such as adding brewer's yeast to crude glycerol (Feng et al 2014). The drawback is that it may be difficult to co-locate facilities so that they have access to the waste of a brewery and a biodiesel production facility. Alternatively, chemical nitrogen will be a lower proportion of the cost in heterotrophic processes.

The identification of valuable products in heterotrophic algae, and conversion of photosynthetic algae to heterotrophy will continue to be commercially viable. However, it should be noted that there is a drawback from switching

from the mode of solar energy collection to conversion of already fixed carbons. Such processes lose "the primary thermodynamic advantage … that derives from the algal cell's ability to harness light energy to drive CO_2 fixation" (Darzins et al 2010). Still, if waste substrate concentration is high enough, it may be possible to design a profitable algae fermentation strategy on the waste steam. The challenge is to identify a waste substrate with a composition that is defined-enough not to limit the value of the algae. For the production of bulk lipids, algae are not necessarily the most productive of the heterotrophic organisms. Finally, the heterotrophic yields mentioned above give us good benchmark to determine the efficiency of mixotrophic production.

3.3 Mixotrophy

While heterotrophic algal cultures are unlikely to outpace other heterotrophic organisms, the use of mixotrophic metabolism may offer a route to harness the heterotrophic metabolism, while performing cultivation in photobioreactors. Since wastewater treatment plants are also tasked with removing organic carbon, it is logical to consider exploiting the potential offered by using the carbon source to boost algae production in photobioreactors. A key issue is how the presence of bacteria can be accounted for. In many lab scale studies the wastewater is simply autoclaved, which is obviously not feasible at industrial scale (Min et al 2011). An open question remains of how these lab-scale studies can be up-scaled. Are the existing methods to reduce bacterial content cost-effective? Can the algae be given a significant advantage to uptake the carbon sources before bacteria? To address such questions, studies of uptake rate become critical (Turon et al 2014; Turon et al 2015). It is also possible that by allowing the algae to multiply during the day, and harvesting the culture while adding organic carbon at night (as in paper II) we may offer a competitive advantage to algae.

Mixotrophic production requires a choice about what waste-stream to use. One option is to use existing wastewater reactor effluents that typically contain relatively low organic carbon amounts. Alternatively, the waste treatment process can be chosen specifically to maximize the production of carbon sources. Examples include dark fermentation, acidogenic fermentation or hydrolysis of nutrient rich wastes (see Table 2). An advantage of mixotrophy is that the culture densities useful for phototrophic growth are more in line with the substrate concentrations achievable in most wastewaters, while heterotrophic growth often requires substrate concentrations upto 100 g L^{-1}.

Table 2. Carbon sources investigated for mixotrophic or heterotrophic algae (Paper II)

Waste type and carbon sources	Organic concentration (g L^{-1})	Reference
Industrial dairy waste		(Abreu et al 2012)
Lactose	10	
Glucose	5	
Galactose	5	
Soybean Processing Wastewater		(Hongyang et al 2011)
VFA	2.5	
Synthetic Wastewater		(Lopez et al 2010)
Glucose	10	
Acetate	2.5	
Acidogenically digested manure		(Hu et al 2012)
VFA	3 – 7	
Synthetic biodiesel plant effluent		(Cabanelas et al 2013)
Glycerol	0.56 - 4.6	
Food waste hydrolysate		(Pleissner et al 2013)
Glucose	2.6 – 18.5	
Synthetic wastewater		(Lim et al 2013)
Acetate	< 0.255	

Mixtrophy has a proven industrial track record. Acetate has been used as the main carbon source in some industrial mixotrophic cultivations of *Chlorella* (Richmond 2003).

Still there are several other potential approaches to obtain mixotrophy that blurs the border with heterotrophy. For example, if the organic carbon, is made available when the sun sets, the culture will shift from phototrophic to heterotrophic. Taken over a 24-hour period, the culture will be using both organic carbon and sunlight for energy, so it could also be considered a form of mixotrophy. By adding the organic carbon at night, one can increase the period of time of culture activity (paper II). Acetate values of 1-2 g L^{-1} were chosen in the low range that may be representative of what is available in various anaerobic, dark fermentation or acidogenic digester effluents. Here, we found that it was possible to achieve equivalent heterotrophic yields that match the benchmarks set in the heterotrophic section. The apparent heterotophic yield, Y_{Ac} *app.* (Y_{XS} above) exceeded 1 g g^{-1}, confirming the expectation that mixotrophic growth produces more than is possible with heterotrophic growth alone (Table 3). Furthermore, when we corrected this yield by

subtracting the growth expected in autotrophic mode, the yields Y_{Ac} *corr.* exceeded 0.3 g g^{-1}, indicating that the night-time heterotrophy was more efficient than that of *Crypthecodinium cohnii* (De Swaaf et al 2003) and less efficient than *Yarrowika* (Fontanille et al 2012).

Table 3. Kinetic parameters of continuous autotrophic, mixotrophic and cyclic autotrophic/heterotrophic growth of C. sorokiniana in 16:8 light:dark cycle with ca. 200 µmol photons m^{-2} s^{-1} T = 37° C (Paper II)

	no.	D_l (h^{-1})	D_d (h^{-1})	S_{Ac} (g L^{-1})	X_{Av} (g L^{-1})	q_{Ac} (g day^{-1})	P_V (g^{-1} L^{-1} d^{-1})	Y_{Ac} app. (g g^{-1})	Y_{Ac} corr. (g g^{-1})	Y_{PFD} app. (g mol^{-1})
Auto.	1a	0.016	-	-	0.98	-	0.50	-	-	0.64
	1b	0.016	-	-	1.08	-	0.53	-	-	0.64
	1c	0.031	-	-	1.39	-	0.76	-	-	0.97
	1d	0.031	-	-	1.49	-	0.69	-	-	0.84
	1e	0.031	-	-	1.40	-	0.99	-	-	1.27
	1f	0.047	-	-	0.84	-	0.81	-	-	1.04
Mixotrophic	2a	0.034	-	1	1.54	0.220	0.72	1.31	-0.16	0.93
	2b	0.063	-	1	1.53	0.405	1.04	1.03	0.23	1.34
Cyclic-constant dilution rate	3a	0.031	0.031	2	1.50	0.200	1.18	2.36	0.74	1.44
	3b	0.047	0.047	2	1.01	0.300	1.08	1.45	0.36	1.32
Cyclic-enhanced night hetero.	4a	-	0.094	2	1.67	0.602	1.33	0.88	0.34	1.61
	4b	-	0.140	2	1.47	0.899	1.57	0.70	0.34	1.91

Legend: Dilution rate (D), light period (l), dark period (d), acetate substrate concentration (S_{Ac}), average biomass concentration X_{Av}, rate of acetate addition q^{Ac} , Volumetric productivity (P_V), Yield on acetate (Y_{Ac}), apparent (app.) or corrected (corr.) by subtracting autotrophic growth, apparent photosynthetic yield (Y_{PFD} app).

There are many possible ways to combine mixotrophic metabolism with the diurnal light dark cycle. One alternative is to use substrate for inoculum preparation and then increase the content of desired lipids or pigments by exposure to light in a photobioreactor (Ganuza 2015). The reverse process was also considered (Lane et al 2012). A strategy they called "heteroboost" includes producing low cost biomass using sunlight and then transitioning over to a heterotrophic bioreactor. Here, the algae will be "fattened up" where the cells will accumulate lipids, perhaps in the absence of nitrogen so that cell division is not possible.

However the process is designed, some basic conditions should be met. If high enough concentrations of organic carbon substrate are available to produce algae biomass heterotrophically, then the production of many other heterotrophic organisms will also be possible at similar or lower production

costs. Therefore, the main reason to use concentrated organic carbon waste streams is likely the higher value algae-specific products. If lower concentration organic carbon is available, then mixotrophic processes will likely be favored to heterotrophic. Strategies and processes will have to be developed to prevent or manage the growth of other heterotrophs.

4 Screening

A result of the rich diversity of algae mentioned in section 2.1 is the desirability of systematic protocols to understand physiology of the algae and how they may be exploited, or conversely to determine how much algae may grow on a given wastewater. An early example by one of the leading American phycologists (Shihira and Krauss 1965) demonstrated the differences in substrate usage and physiology of 41 isolates of *Chlorella*. Oswald (Oswald and Gaonkar 1969) defined algae growth potential (AGP) as a screening characteristic, which was essential the amount of algae that accumulated in a flask after a significant waiting period. The interest in finding algae capable of growth on a given wastewater continues (Zhou et al 2011). Sheehan (Sheehan 1998) documents how many isolates were laboriously screened in bubbled columns and other methods throughout the Aquatic Species Program in order to find the fastest growing of a library. Algae are often screened to find the ones most productive of a substance of interest (Breuer et al 2012). It should also be noted that algae are often the "victims" of toxicological screens, so some screening technologies have also been developed for slightly different purposes (Eisentraeger et al 2003; Pavlic et al 2006).

Screening efforts are clearly useful and sometimes essential. However, despite this massive effort, there can be difficulty in knowing that the results obtained at the screening scale will apply at the industrially relevant scale. Put in another way, you get what you screen for, *exactly* what you screen for. For example, (Slocombe et al 2015) recently screened an entire culture collection for productivity and never found a value greater than 0.1 g L^{-1} day^{-1}. The results are published in Nature, and still considered of great value, but offer little guarantee that those judged to be faster and more productive at these slow rates will also be the fastest when we want to get 5-10 g L^{-1} day^{-1} in a photobioreactor. Such screens do give accurate information on the content of some components (Slocombe et al 2013), but not necessarily those which are only present during stress responses, and are not predictive of growth kinetics at scale.

To develop a screening protocol that could predict industrially relevant performance metrics, (Huesemann et al 2012) created a method that could translate two relatively easily measurable strain characteristics into predictions of volumetric productivity under given cultivation conditions. The two characteristics are the μ-I curve and light absorption coefficient (k_a) displayed in section 2.3. These characteristics are possible to measure at the screening

scale and the model predicts performance at industrial scales. The model was validated in the simple case, where light intensity is fixed. Work is ongoing to correct the model for fluctuating light and temperature along with loss to respiration in the dark (Van Wagenen et al 2012; Edmundson and Huesemann 2015) (Huesemann recently accepted ALGAL-D-15-00471R1). Given the existing model and framework for improvement, Paper I demonstrates a way to significantly increase the throughput of the screening method.

To begin with, this paper proved that the μ –I curve found in well mixed, very low density photobioreactors could be obtained in microplates for two species from different kingdoms of the eukaryotic tree (figures 6 and 7). This is the key, difficult to measure parameter in the Huesemann model. The other parameter, k_a, can easily be measured with one mL of photo-acclimated culture.

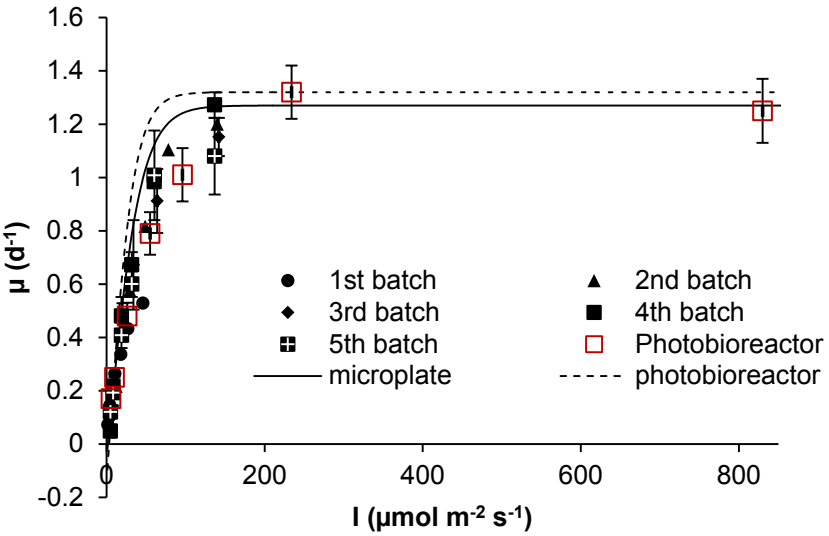

Figure 6. Growth rate (μ)–light intensity(I) curve for *N. salina* grown in f/2 medium. Black points from cultivation in microplates illuminated with fluorescent bulbs, open squares are from Hueseman et al (2013). Error bars represent one standard deviation, for microplates n =4. Lines represent the hyperbolic tangent function (Jassby-Platt) with the model parameters listed in Table 3 Paper I.

The demonstration that μ-I curves can be measured in microplates enables screening in such system. To this point, the screening has been done by human operators performing all dilutions and manually transferring the plates from the cultivation setup to the microplate reader. This is already a labor savings, as it is far easier to control and manipulate one or two microplates

than six to eight litter bottles with gas manifolds. Furthermore, massive screening is now possible with robots already available for pipetting and plate movement. Computer algorithms could decide when to dilute the culture again and how many times the culture needs to be repeated until a user-defined acclimation threshold had been reached. Furthermore, data transfer from raw format to μ-I curves could also be readily done. Higher frequency monitoring could overcome the challenge of some reported species which require a daily light dark cycle.

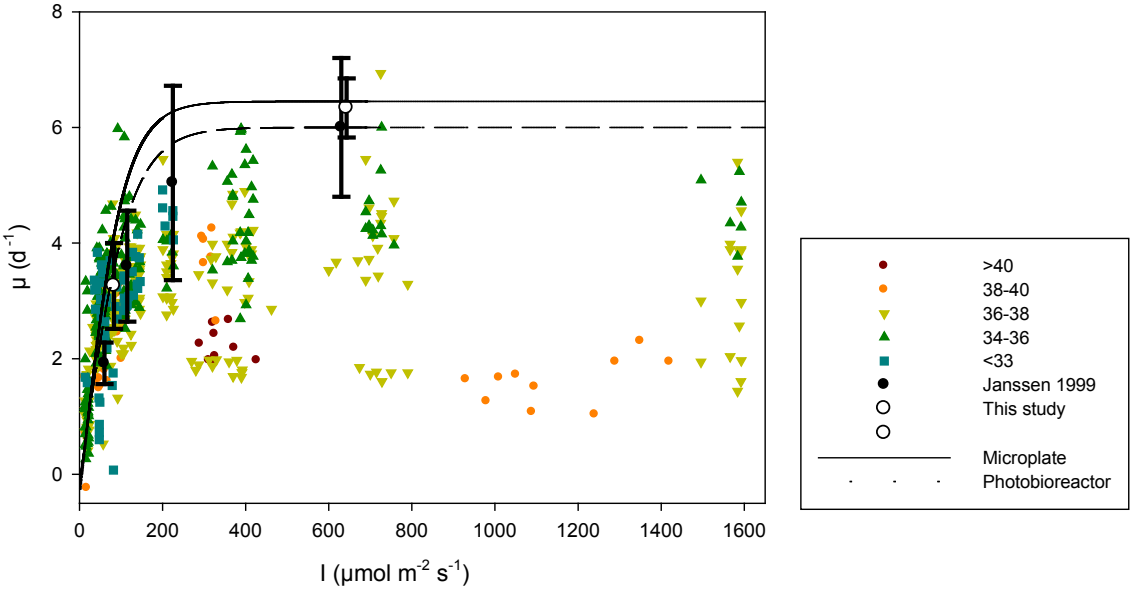

Figure 7. Specific Growth rate (μ)- light intensity (I) plot for *C. sorokinana*. White circles from this bioreactors in this study, filled circles modified from Janssen et al. 1999, 95% confidence intervals shown in error bars. Lines represent the hyperbolic tangent function (Jassby-Platt) with the model parameters listed in Table 3 Paper I. Colored points are individual data from microplates illuminated with LED with color indicating temperature (°C) measured: red >40, orange 40-38, yellow 36-38, green 34-36, blue <34.

Whether human or machines are operating, microplate screening of microalgae has a number of potential applications. It can determine if there is optimum dilution of a concentrated wastewater. For example, it was determined that after 2 growth cycles, *Chlorella sorokiniana* grew equally well in all concentrations of an industrial waste-stream (paper III, supplementary material). It has been used in our research group to profile growth rate of a number of species on a given wastewater (unpublished). Conversely, one could screen many wastewaters against a reference species to determine if they are non-inhibitory or optimal for growth as in (Morales-Amaral et al 2015b). It

could also be applied in mixotrophic scenarios (Mitra et al 2012). This methodology could also be used on genetically modified clones.

5 Cultivation on wastewaters at lab-scale

Screening using the framework above is only an early step in the process of industrial microalgal cultivation. Scaling up the best results to larger scales for verification is the normal progression. The Huesemann model allows us to make predictions about volumetric productivity when we have determined μ-I curves and values of k_a. However, the model assumes perfectly transparent medium and no nutrient limitation or chemical inhibition. While these assumptions are logical in the context of production on defined medium, they mean that waste-water cultivations will always require more verification in upscaling than cultivation on defined medium. Furthermore, the model does not account for the dynamic changing of culture pigmentation that occurs in photo-acclimation, which means that the exact k_a developed by the culture is not directly identifiable from microplate experiments because they vary depending on both light intensity and culture density (see paper III figure 7). In this chapter, the example of Paper III is used to show how a suit of methods can be used to quickly and effectively verify a screening result and determine the characteristics of culture that are possible on a given waste-water.

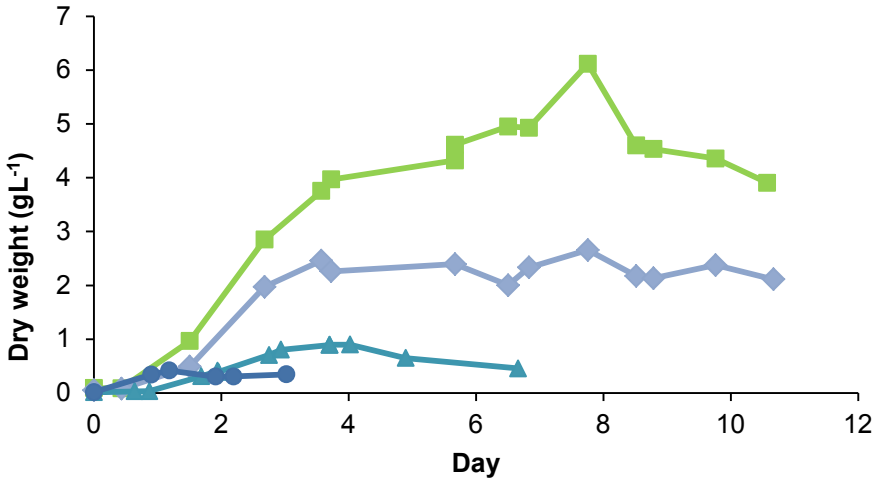

Figure 8. Biomass concentration in the flat panel reactors with 6% (●), 25 % (▲), 50 % (◆) and 100 % (■) wastewater, light intensity approx. 160 μmol m^{-2} s^{-1}

As mentioned in Chapter 4, microplate screening showed that *Chlorella sorokiniana* could readily be adapted to a wastewater we obtained from an internal circulation (IC) reactor being used to produce biogas and treat the effluent from the insulin and enzyme production facilities of Novo Nordisk and Novozymes at Kalundborg, Denmark. To verify this result, we up-scaled to 400

mL photobioreactors and ran batch cultivations at moderate light intensity (figure 8). Here we could see that growth rate was essentially the same, but the cultures ended growth due to nutrient limitation at different densities depending on the amount of nutrients available at the various dilutions. From the data in Table 3 of Paper III, it was unclear if N or P was the limiting nutrient. Most importantly, the un-diluted wastewater was an adequate substrate for production of at least 4 g L^{-1} of algal biomass.

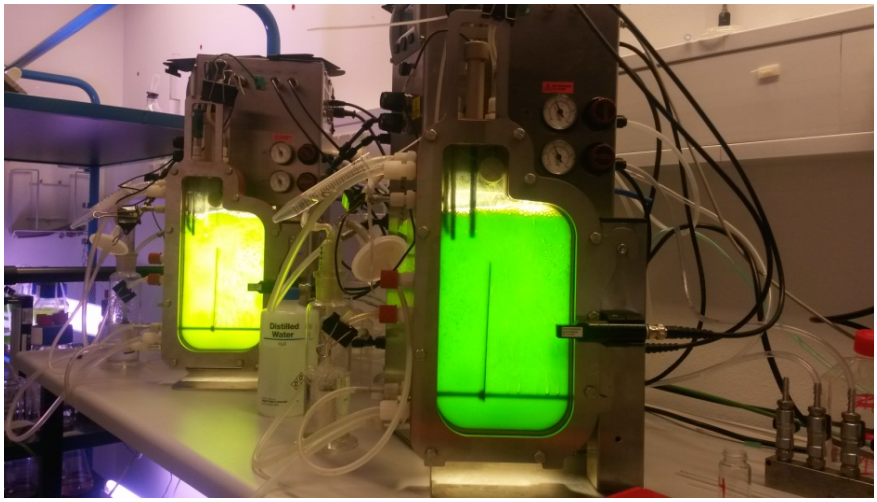

Figure 9. Experimental setup, high light 2100 µmol photon m^{-2} s^{-1} left and low light 200 µmol photon m^{-2} s^{-1}, right.

In order to make recommendations for the operation of the bioreactor at industrial scale, it was important to understand how the operating conditions would impact the productivity and the nutrient usage. Culture density and reactor configuration were judged to be the two parameters for nutrient removal and biomass productivity. We endeavored to determine the optimal culture density for production at two different incident light intensities representative of vertical or horizontal photo-bioreactors[2] (figure 9). Culture density is best controlled by dilution rate in continuous cultures. However, the establishment of many steady-states in continuous algae photobioreactors can be a time-consuming process. Therefore, the deceleration-stat (D-stat) technique was applied (Hoekema et al 2014).

[2] We also examined pond-like conditions, but due to the lower cell concentrations, we excluded these from further analysis, as the nutrient removal would be lower.

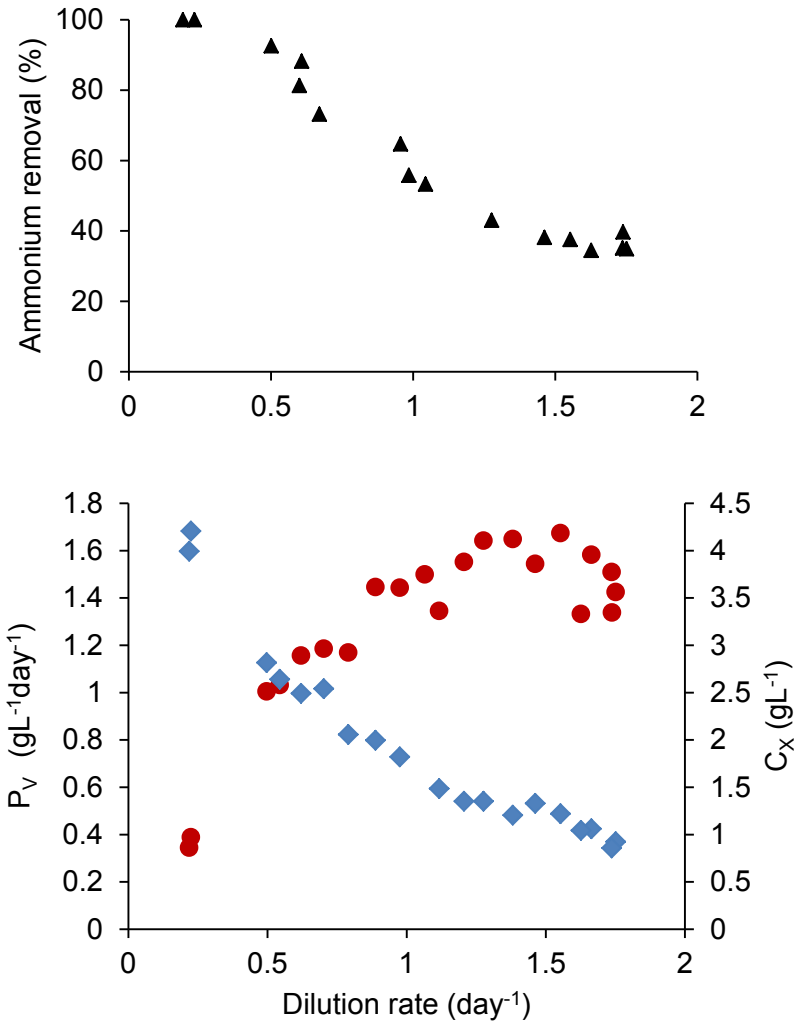

Figure 10. Impact of dilution rate in low-light culture on a. Ammonium removal (% of influent removed b. Volumetric productivity (P_V) during D-stat (●), smoothed D-stat P_V (—) and biomass (dry weight) concentration (C_X) (◆). Modified from Paper III.

This is apparently the first time that the D-stat technique was applied to a wastewater algae culture. The method can be used to find the optimal volumetric productivity and characterize nutrient removal possible (figure 10). Furthermore, the yield on photon $Y_{X,E}$ equaled the best observed for this species according to literature and was upto 70% of the theoretical maximum, when low light was used. This indicates that the wastewater used is equivalent to a mineral medium for the purpose of biomass production. At high light, the performance was significantly less than that seen in Cuaresma et al (2009). This, combined with the fact that the measurable phosphate remaining in the effluent was close to zero in nearly all conditions, is indicative that phosphate limitation of growth probably occurred in the high light cultures. As a result, it seems clear that the implementation of vertically oriented

arrays of photobioreactors would maximize biomass production. Unfortunately, the conditions that optimize the biomass productivity do not correspond to those which produce the effluent with the lowest ammonium content. Therefore, in the industrial context, there would need to be decisions about which is the most important parameter.

6 Considerations when operating wastewater-based algae cultures

The previous chapter showed that industrial wastewater can replace a defined culture medium without any loss of biomass yield on photons. However, there are further considerations as to whether this will be an effective business strategy. The economic benefits of replacing nutrients with waste stream can be estimated at about 0.72 € per kg of algae biomass (Table 4), which is consistent with the estimate in (Morales-Amaral et al 2015a). This value is about 14% of the current estimated production price of 5 € per kg (Norsker et al 2011b). This benefit must be weighed against the drawbacks. There may be variation between the optimal conditions for nutrient removal and production of valuable components. Furthermore, there may be conflicting motivations between operating the anaerobic waste reactor and the algae photobioreactor. Finally, the association with wastewater may constrain the biomasses acceptability as a source of higher value products.

Table 4. Cost of key medium components replaceable by wastewater

Component	Fertilizer cost[a]	Emission cost[b]	Requirement approx. per kg algae	Savings per kg algae
N	1.08 € kg^{-1}	4 € kg^{-1}	0.1 kg	0.5 €
P	2.22 € kg^{-1}	22 € kg^{-1}	0.01 kg	0.22 €
Water	0.878 € m^{-3}	-	1 L	0.001 €

a.(Spruijt et al 2015b) b. Paper III

The effects of the various dilution rates and light intensities on biomass composition of *Chlorella sorokiniana* was described in Paper IV. Fatty acid composition in the various dilution rates is shown in figure 11. If one would like to produce the omega-3 fatty acid alpha-Linolenic acid (ALA C18:3) at the rate, then high light and high dilution rate are required. However, in these conditions less than 40% of the ammonium will be removed by the algae culture (Paper III, fig. 8b) and the reactor effluent will have over 100 mg L^{-1} of NH4-N, requiring further treatment before release. Such trade-offs are considered in table 5.

Table 5. Optimal operation and design choices for various purposes.

	High light	Low light
High dilution	Fertilizer value Volumetric productivity N and P removal rate ALA (C18:3) Carotenoids	Yield on Photons LA (C18:2) Carotenoids Chlorophyll
Low dilution	Effluent quality (lowest remaining N) Biomass concentration (reduced harvesting costs)	Effluent quality (lowest remaining N) LA (C18:2) Carotenoids Chlorophyll

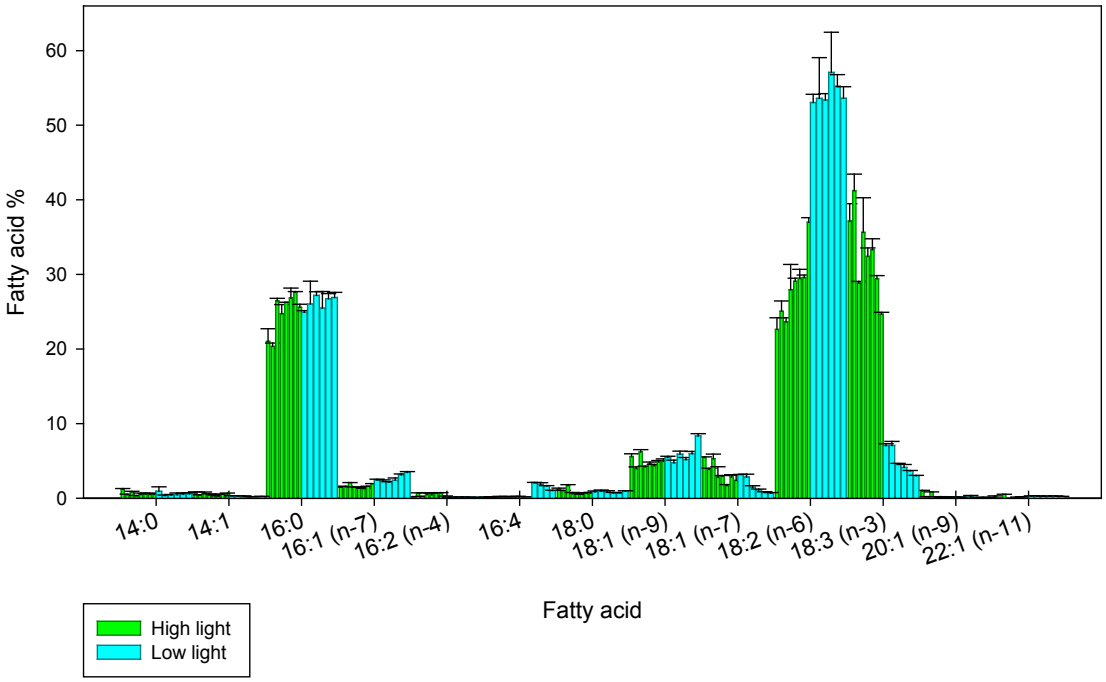

Figure 11. *Chlorella sorokiniana* fatty acid composition as a function of light intensity and dilution rate. Dilution rate is presented in decreasing order. Paper IV.

Many of the most valuable algae products are consumed by directly humans or fed to animals in the human food chain. There are a few potential industrial biomolecules that may be interest, namely enzymes and antioxidants (Paper IV), but these are mainly potential rather than current products. When cultivating with municipal wastewaters, this may lead to concerns of accumulation of pharmaceutical residues in the biomass (Tuantet 2013). This concern can be addressed by using industrial wastes with known compositions. Unwanted bacteria or viruses may also be present, even in anaerobic reactors. Therefore, in order to prove that industrial wastes are relevant for producing high added value compounds from microalgae, it must be shown that the ex-

tracts of interest are not contaminated. Perhaps in some cases the extraction process would also serve to remove contaminants, but this will need to be examined on a case-by-case basis. Proof of safety would serve as the basis for a new regulatory framework for legal acceptance of waste-grown products.

While domestic wastewaters are expected to have roughly similar daily flows, industrial wastewaters may be more periodic. For example, in agricultural processing stations there will be seasonal changes in waste quality and availability. In biotechnological or pharmaceutical production there are several expected causes of irregularity. For example, many companies will produce multiple products per year in individual batches. Also if a batch becomes spoiled and is discarded, the waste-treatment reactors will face a much higher load. This can lead to shocks to the microbial community populating the anaerobic digester and the resulting effluent will therefore be richer in nutrients (McHugh et al 2003). Thus, to fully integrate an algae reactor into a facility with an anaerobic digester, there are two options. Either, the operators of the production plant and wastewater treatment plant must take extra care to avoid shocking the anaerobic digester. Or the algae reactor will need a large buffer tank, capable of storing wastewater that makes good medium during the periods where the anaerobic digester is not operating properly. One context where the needs of the algae plant would always be considered are during algae medium recycling (Davis et al 2015). Therefore, the results shown here could be especially promising in regards to future efforts to recycle algae nutrients from processed biomass.

7 Conclusions

Microalgae production is a promising way to treat and utilize wastewaters. Despite the promise, there is still a need for scientific knowledge that can help reduce the costs of algae cultivation and enable profitable enterprises to be built. The results presented in this thesis demonstrate ways that algae cultivation on wastewaters can be performed more efficiently. More specifically, the main conclusions of this work are:

- Microalgae specific growth rate can be determined in low density cultures using microplates. This enables determination of growth rate – light intensity (μ-I) curves, always in microplates, which replicate the curves which are found in well-mixed, aerated, low-density photobioreactors. The μ-I curves are the key input for the Huesemann model, which predicts volumetric productivity given input conditions.
- The reduction of cultivate scale to microplate level offers the potential to reduce the cost and increase the throughput of screening. This is applicable in many contexts including matching algae species to wastewater or finding the optimal dilution of wastewater.
- Synthetic wastewater, with 1-2 g L^{-1} sodium acetate representative of products of anaerobic waste-treatment, was used to increase the productivity of photobioreactors operated in a day/night cycle. Both mixotrophy and cyclic heterotrophy/autotrophy could increase productivity.
- A cyclic autotrophic/heterotrophic approach, in which acetate-containing medium was added at the highest rate (0.14 h^{-1}) during the dark cycle only was found to be the most productive with 1.57 g L^{-1} day. Yield on photons was over 1.9 g mol^{-1}, which exceeds the theoretical maximum for purely autotrophic growth (1.8 g mol^{-1}).
- A second cyclic autotrophic/heterotrophic approach was also effective. In this approach dilution occurred continuously, but with acetate only present during the dark period. This strategy achieved productivity higher than autotrophic or mixotrophic cultivation, because of a better yield on acetate.
- The wastewater from the Internal Circulation (IC) reactor at an industrial biotechnology facility was able to support *Chlorella sorokiniana* growth at photosynthetic yields as high as any reported medium.
- The Deceleration stat method shows promise as a way to quickly characterize algal productivity and nutrient removal from wastewater.

- On IC effluent growth occurred at a rate of up to 1.33 $gL^{-1}day^{-1}$, with a high yield of up to 1.4 g mol^{-1} up to a density of at least 4 g L^{-1} in batch cultivation.
- In continuous cultivation, at high light intensity (2100 µmol photon m^{-2} s^{-1}) volumetric productivity reached up to about 5 g L^{-1} day^{-1}, but with a lower yield on photons about 0.4 g mol^{-1}. At low light intensity, the yield was over 1.2 g mol^{-1} and volumetric productivity was about 1.5 g L^{-1} day^{-1}.
- In continuous systems the effluent contained fewest nutrients at low dilution rates. However, the rate of nutrient removal was associated with higher dilution rates.
- Cultivation conditions change biomass composition. Lower light intensity increased Linoleic acid, chlorophyll and carotenoid contents. High light and higher dilution rate increased α-Linolenic acid content. Mixotrophy decreased chlorophyll content, but in some cases carotenoid remained unchanged.
- In *Chlorella sorokiniana*, the highest lutein content observed was over 10 mg g^{-1} and with total content of β carotene and other carotenoids of up to 4 and 2 mg g^{-1}, respectively.

8 Future perspectives

This research shows that cultivation on industrial wastewaters is possible, and that autotrophic growth can be just as high in a wastewater as in algae growth medium as measured by yield on photons or areal productivity. Cheaply available organic carbon sources could be used to boost this productivity. Screening of strains and conditions can be done in high-throughput ways at lower cost. These are promising results. Still, there are many research and practical questions:

- Production of multiple and higher value substances from cheap materials (biorefinery) developed more readily when one product was already quite profitable and available at very large scales. Such development is evident when we look at all the products derived from maize. It is much more complicated to simultaneously develop two or more components of a system. To optimize both production systems and bioextraction process for multiple products is difficult. Therefore, academics should collaborate with the algae industry as it currently exists to fully understand what bi-products are available from the algae production. Researchers should go to places where algae is already used in wastewater ponds or turf scrubbers and see if they can identify a potential high value product there.
- For academic studies of higher value products researchers need strong leadership from the existing algae industry, especially in identifying target products. What are the substances which are produced by robust algae or algal consortia that are of commercial interest? Are these substances compatible with wastewater? What kinds of the contamination found in wastewater do the currently known refining methods already remove? Identification of one high-value product that makes commercial sense when grown on wastewater could be the catalyst to new legislation to change the regulatory framework.
- A vision of the product and all downstream processes will help remove confusion about what contaminants pose a significant threat to microalgae production. Are the downstream processing efforts such that they purify the product from any contaminants? Or on the other hand will purification increase the content of certain unwanted contaminants? Is the downstream process very sensitive to contaminants which may increase the price? Specificity is the soul of narrative.

- The algae research field is hindered when journals accept papers using units that do not enable comparison.
- Mixotrophy based on waste sources is promising, but concentrated, pure carbon sources have other potential uses.
- Awareness of the commercial potential for higher value products is important so large companies that can invest in research and development. Development is needed not just in making products, but in marketing them.
- The algae industry needs to demonstrate profitability and growth. Improvements to bring down the costs of screening, equipment for mass culture, harvesting and refining are important.
- If other technical advances bring algae biofuels or cheap bulk products close to commercialization, then switching to wastewater as a nutrient source, along with and recycling of algae medium nutrients would make the difference.

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In this online version of the thesis, paper **I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
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