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Scale-up cultivation of Australian algae

New approaches to isolation, mid-scale cultivation and harvesting of Australian wild type algal strains

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Abstract

Algae biotechnologies offer one possible path to addressing the forecast challenges of human society which are the increased demands of food, fuel and water due to population growth and large scale lifestyle change. The development of renewable algae production technologies helps to establish a basis for sustainable development and provide options to decrease CO_2 emissions to address climate change.

Algae are increasingly recognised as a promising bioresource and the range of cultivated species and their products is expanding. Compared to terrestrial crops, microalgae are highly biodiverse and offer considerable versatility for a range of biotechnological applications including the production of animal feeds, fuels, high value products and waste-water treatment.

Despite their versatility and capacity for high biomass productivity microalgae represent a relatively unexplored bioresource both for native and engineered strains. Success in this area requires (1) appropriate methods to source and isolate microalgae strains, (2) efficient maintenance and preservation of parental stocks, (3) rapid strain characterisation and correct matching of strains to applications, (4) ensuring productive and stable cultivation at scale, and (5) ongoing strain development (breeding, adaptation and engineering).

In chapter 2 a streamlined process was developed for the isolation, identification and maintenance of over 150 local microalgae strains as a bioresource for ongoing strain development and biotechnological applications. 121 algal strains could be purified to the axenic level, whereas the rest were maintained as unialgal, non-axenic cultures. All algae used in the large scale trials were obtained as axenic cultures. A range of isolating techniques were explored. Fluorescence activated cell sorting (FACS) proved to be a useful method for high throughput isolation and purification of physically robust algal species, while micromanipulation was most beneficial to obtain a high species diversity. A separate survey by Wolf *et al* (2014) optimised the nutrient requirements and identified the highest biomass producing strains.

Eight algae isolates identified as high production strains based on lab experiments, as well as an isolated invasive algal species and a polyculture of five morphological different strains were tested outdoors to identify key variables limiting optimised production at pilot-scale. The results showed that based on the maximum specific growth rates achieved in sterile laboratory systems, exposed pond systems achieved ~30-50%, indicating limitations imposed by environmental and biological

factors. Highest daily growth rates in open pond trials single strain trials were obtained for *M. pusillum* (5_H4) (0.959 d^{-1}), *Chlorella* sp. (11_H5) (0.719 d^{-1}), *Chlorococcum* sp. (12_02) (0.755 d^{-1}), and the polyculture (0.743 d^{-1}). *M. pusillum* (5_H4), *Scenedesmus* sp. (Pinjarra001). The polyculture trials achieved the highest optical densities (up to OD_{750}=4). In addition to fast growth, other variables influencing performance included the ability to grow to high densities, autoflocculation, and resistance to predators proved important.

Polyculture trials achieved the highest maximum areal and volumetric productivity (24.3 g m^{-2} day and 266.7 g m^{-3} day). However the polyculture productivity values are only about 6-10 % higher than those of the highest single strain cultivations and may have resulted from the different cultivation timings. The polyculture growth during spring was favoured by higher mean solar energy, higher absolute temperatures and greater temperature flux than cultivations during autumn and winter. Nevertheless the polyculture trials are considered to be a potential alternative to single species cultivation due to their increased robustness against invasive predatory influences.

Contamination by invasive organisms is expected and observations revealed that their impact on the algal culture is dependent both on the cultivated species and the predatory organism. Grazing and competition for nutrients had negative impacts on algal growth and change of algal morphology (e.g. spines and flocculation). Positive impacts of certain grazers included reductions in bacterial and protozoa load, providing opportunities to use “predators” as a tool to sustain algal cultures and optimise the culture production. The flocculation of *C. sorokiniana* (8_C4) in the presence of a *Tetrahymena* like ciliate was one of the most promising leads for the optimisation of algae production systems in the form of a novel harvesting technology.

Chapter 4 focused on the characterisation and optimisation of a novel bioflocculation method using the native isolated ciliate *Tetrahymena*. *Tetrahymena* is a common invader of microalgae cultures, typically feeding on bacteria rather than on the microalgae themselves. *Tetrahymena thermophilia*, a freshwater ciliate, is well studied for its ability of undergoing dramatic metabolic changes during starvation, its secretory granule biogenesis and exocytosis. Here a locally isolated and identified *Tetrahymena* culture was used as a controllable bioflocculation agent for the microalgae *Chlorella sorokiniana* (8_C4). The process was triggered by adding chemical substances to the starved ciliate leading to exocytosis of extracellular polymeric substances (EPS) which functioned as a binding substance between the algae cells. The ratio of ciliate to algae could be reduced to as little as 1:400 cells to initiate rapid bioflocculation. Furthermore stimulators of ryanodine receptors (caffeine and *p*-chlorocresol) were identified as useful triggers for exocytosis. Future experiments at larger scale

can prove that the protocol is transferable to greater volumes which could have significant potential for industrial scale application. The work described in this chapter has been the basis for a patent application and has been included in a manuscript published in the journal *Algal Research* [1].

In conclusion, 150 native microalgae were successfully isolated, cryo-preservation applied (Bui *et al*., 2013) and their nutrient media optimised (Wolf *et al*., 2014). The performance of 9 strains was analysed in high rate ponds both in mono and polyculture and a novel *Tetrahymena* based harvesting system identified (paper published and patent filing submitted).

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Research Papers:

Gisela Jakob, Juliane Wolf, Tony VL Bui, Clemens Posten, Olaf Kruse, Evan Stephens, Ian L Ross and Ben Hankamer. Surveying a diverse pool of microalgae as a bioresource for future biotechnological applications. *Journal of Petroleum & Environmental Biotechnology*, 2013, 4(5): 153

Tony V L Bui, Ian L Ross, Gisela Jakob, Ben Hankamer. Impact of Procedural Steps and Cryopreservation Agents in the Cryopreservation of Chlorophyte Microalgae. *PLoS ONE*, 2013, 8(11): e78668

Khairul A Radzun, Juliane Wolf, Gisela Jakob, Eugene Zhang, Evan Stephens, Ian L Ross, Ben Hankamer. Automated nutrient screening system enables high-throughput optimisation of microalgae production conditions. *Biotechnology for Biofuels*, 2015, 8: p.65

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Incorporated as **Chapter 2**

Contributor	Statement of contribution
G. Jakob (Candidate)	Designed experiments (15%) Performed experiments (50%) Wrote and edited paper (30%)
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Contributions by others to the thesis

In chapter 2, Juliane Wolf contributed on algae strain purification techniques and identification approaches. With her developed nutrient screen assays she contributed to the identification of top production strains. Tony Bui contributed with his developed cryopreservation protocol and the application on the collected algae to maintain motherstocks. Contribution (writing, discussion, critical review) to the publication was provided by Prof. Ben Hankamer, Dr Ian Ross, Dr. Evan Stephens and Juliane Wolf.

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Chapter 1

1. Introduction

1.1 Microalgae as an energy solution for the future

The global economy is valued at ~$100 Tn pa [2] and is powered by the $6 Tn energy sector. Importantly approximately 80% of global energy is used as fuels and only ~20% as electricity [3]. By 2050, the expansion of the human population to over 9 billion people and political demand for continued global economic growth, will necessitate 50% more fuel [4], 70% more food [5] as well as 50% more fresh water [6]. This needs to be achieved while cutting CO_2 emissions by approximately 80% (IPCC) [7] to avoid temperature rises above 2°C and maintain political, social, fuel and climate security.

In recent years renewable electricity production systems such as photovoltaic and wind turbines have made significant gains in supplying the electricity sector. Some limited replacement of fuels with electricity is possible through new technology (e.g. small electric vehicles); however to meet the required CO_2 emissions reductions, renewable fuel solutions (largely biofuels) are desirable to supply the fuel sector (80% of global energy demand). Currently, "first-generation" biofuel production mainly relies on food crops (e.g. corn ethanol, soy) and environmentally problematic crops (palm oil) [8], however, to avoid a future fuel vs. food scenario, it is critical to fast-track the development of environmentally sustainable, 'commercial-ready' CO_2 neutral fuel systems that do not compete with food and water needs. The G7 nations have pledged to phase out fossil fuels to drive this process. In parallel the UK chief scientist Sir David King and prominent economist Lord Stern are leading an international call for a 'Global Apollo Program' to address this issue and deliver renewable energy systems as cheap as coal within 10 years [9]. This is critical to enable the global economy to uncouple from fossil fuels in a controlled manner and provide a solid basis for sustainable long-term global economic development with significant international benefits. In addition to providing benefits in terms of CO_2 emissions reductions, renewable fuel technologies have the potential to enhance international fuel security, as much of the world's oil is derived from politically volatile regions (e.g. from the Persian Gulf), which is essential to ensure stable economic development.

The production of clean fuels for the future at a globally significant level requires a renewable energy source that is sufficiently large to drive this process. Solar energy is by far the largest energy source available to us, with 5500 ZJ yr^{-1} (1 ZJ = 10^{15} J) arriving at the Earth's atmosphere (Fig.1.1 light yellow box). 1300 ZJ yr^{-1} of this is Photosynthetically Active Radiation (Fig.1.1 PAR green box), able to drive photosynthesis [6]. It is worth noting that this annual level of irradiance (1300 ZJ yr^{-1}) dwarfs the total of all reported oil, coal, gas and uranium reserves (82.2 ZJ - Fig.1.1 Dark grey box;) as well as annual global fuel demand (0.46 ZJ – Fig.1.1 red box) [10]. It can be calculated that at a 2% solar to biomass conversion efficiency, which is already achievable for microalgae, ~1.7% of the Earth's surface would be sufficient to supply the current global energy demand. Furthermore as the maximum efficiency of photosynthesis is ~8%, area requirements could potentially be reduced through advances in biology and engineering.

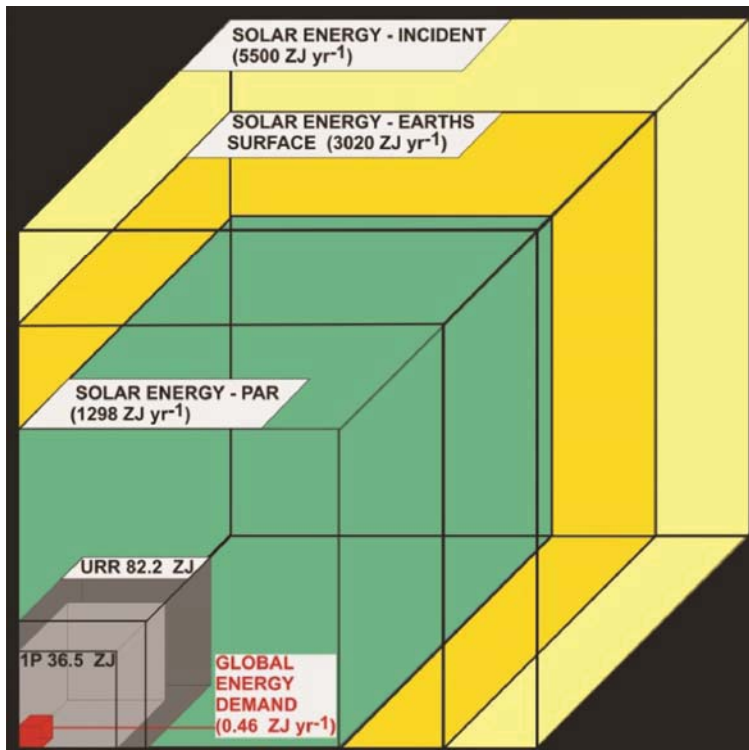

Figure 1.1: Schematic representation of the annual solar energy availability in comparison to the global energy reserves and its annual demand. *Courtesy of Ben Hankamer.*

The idea of replacing fossil fuels by solar energy stored in plants was described as early as 1912. At this time it was already proposed that fast growing plants of any kind could be found, and their growth rates enhanced with carbon dioxide and fertilisers, and that the sun dried harvest could be converted into gaseous fuel to run engines [11]. Algae systems have the advantage for solar driven fuel production that they can be established on non-arable land, and conserve fresh water and arable land for food production [10]. They therefore overcome many of the concerns related to first generation biofuels. Best-practice algae production systems currently produce crude oil at ~$250/barrel, in weeks, and with significantly improved efficiencies and greenhouse gas emissions, compared to $50-140/barrel and millions of years for fossil-derived sources. A sensible average target cost is therefore about $100/barrel. For commercial deployment, algae systems must therefore be a factor of 2-3x cheaper. Furthermore to support the energy needs of a civilized society the

'Energy Return on Energy Invested' (EROEI- a measure of process efficiency), must be increased from ~1:1 towards 10:1. To achieve this, photon conversion efficiency (PCE) must be increased both through microalgae strain [12] [13] and systems development and energy requirements reduced (e.g. through novel harvesting technologies). Increasing photon conversion efficiency (3x) and energy requirements (3x) could therefore allow algae technologies to be developed with an EREOI close to 10:1.
In addition to the major advantage of algae as solar fuels is that their light-capturing solar interfaces can be coupled to a wide range of downstream solar-powered biochemistries. This allows the production of high valuable products (e.g. therapeutic proteins, metabolites, vaccines and nutraceutical production in algae, ~$1,000-10,000 kg^{-1}; [13] [12] [14]) and mid-value intermediate products (e.g. bio-plastics and animal feeds, ~$500-1,000 kg^{-1}). This opens up significant market opportunities for a range of algae-based bio-technologies which will support the development of economically viable microalgae systems on the path to delivering urgently needed and cost competitive CO_2 neutral fuel systems.

The overall aim of this project is to advance knowledge to achieve this goal. This PhD project describes in Chapter 2 the isolation of ~150 Australian microalgae strains using a range of purification strategies. These were cryo-preserved (Bui) [15] to provide a firm basis for further selection and breeding. In parallel studies [14] [16], 100 of these strains were subjected to over 23,000 robotic high-throughput nutrient optimisation experiments to define the top 10 performers and production conditions in terms of specific growth rate. In Chapter 3 nine of these strains were subjected to pilot scale high-rate pond trials at the Solar Biofuels Research Centre, both as part of single strain and poly-culture trials to define the best strains and delineate the specific challenges involved in algaculture in subtropical conditions. Finally, at the conclusion of culture growth, improved methods of harvesting were sought to improve the energy balance of the overall process. In Chapter 4 a novel harvesting method was developed which has potential for wide scale application in this field. The following sections of Chapter 1 provide background to the research conducted.

1.2 Diversity of algae

The phylogenetic biodiversity of algae is very broad. Genetically, the variability between the different groups is much larger than for example among the terrestrial plants, owing to the fact that

microalgae represent a much broader and more ancient phylogenetic group than the land plants which originate from a single branch of the algal lineage [17]. In addition, algae are adapted to a very wide range of environmental conditions (e.g. salt and fresh water conditions, pH, temperature, light, nutrients, pathogens) and possess a short life cycle which is expected to enhance the rate of evolution.

Algae have been divided into three main groups based on their size: macroalgae (thalli up to 30m), microalgae (1-200µm) and picoalgae (0.2-2µm) [18]. The focus of this project is on microalgae which are currently considered to be valuable for energy and feedstock production due to their rapid growth rates, short life cycles and large biodiversity. In recent years importance of macroalgae has also increased as they require less complicated structures for the optimisation of light capture and are easy to harvest. Macroalgae tend to have low oil contents are therefore less suited for biodiesel production.

The biodiversity of photosynthetic microalgae is enormous and is as yet almost untapped. Only about 35,000 species are as yet described and these are divided into 24 taxonomic classes [19]. Due to the development of new taxonomic methods and modern molecular technologies, these classes are frequently revised. The most major classes of algae are list in table 1.1. To illustrate the relation and diversity between algae as well as fungi and protozoa, a phylogenetic tree of the eukaryotes is given in figure 1.2.

Table 1.1: Major classes of algae and examples of delegate species

Class	Examples of common species
Bacillariophyceae (Diatoms)	*Skeletonema, Thalassiosira, Phaeodactylum,*
Chlorophyceae (Green algae)	*Chlorella, Dunaliella, Scenedesmus, Haematococcus*
Rhodophyceans (Red algae)	*Porphyridium cruentum, Galdieria*
Haptophyceae	*Isochrysis, Pavlova*
Prasinophyceae	*Tetraselmis, Pyramimonas*
Cryptophyceae	*Chlamydomonas, Rhodomonas, Chroomonas*
Xanthophyceae	*Olistodiscus*
Eustigmatophyceae	*Nannochloropsis*
Dinophyceans	*Crypthecodinium, Alexandrium, Gymnodinium*
Euglenopyhceans	*Euglena*
Cyanophyceae (blue-green algae)	*Spirulina, Synechococcus, Synechocystis*

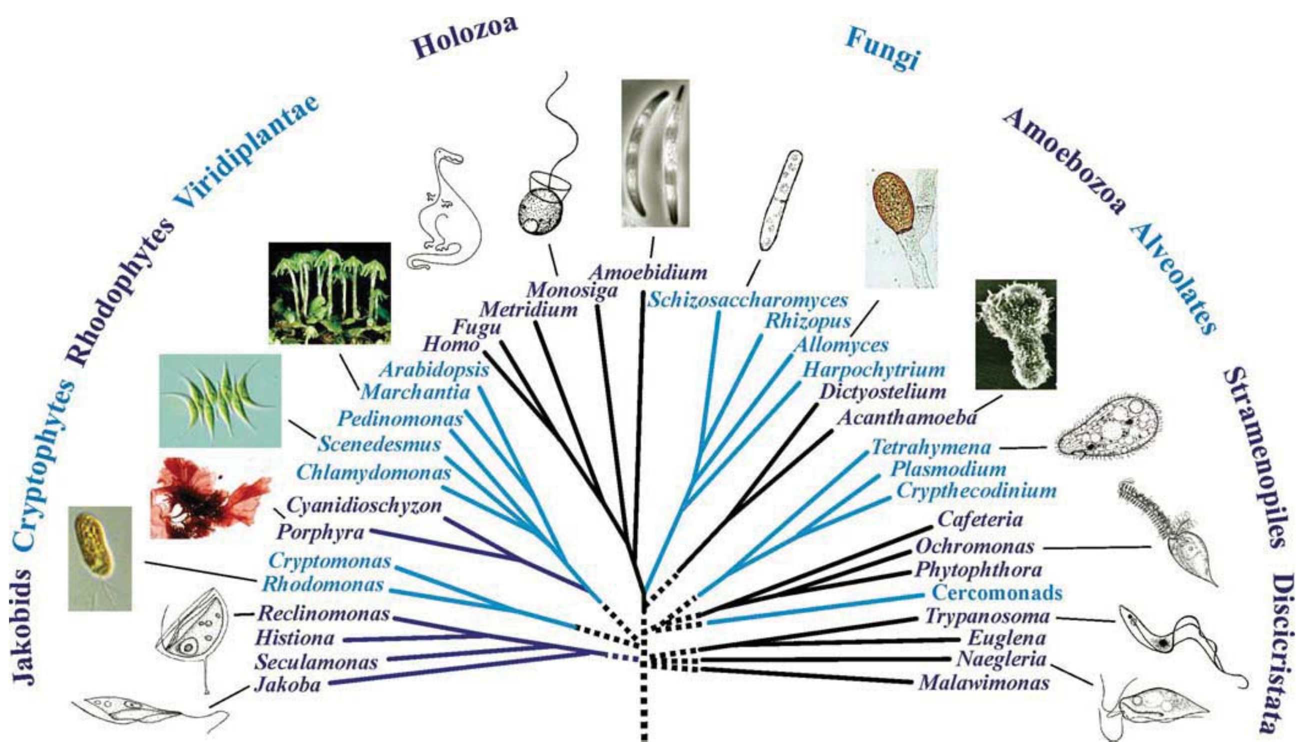

Figure 1.2: An eukaryotic phylogenetic tree among the protists (algae, fungi and protozoa, see 1.6) which is based on ultrastructural and molecular data (Gray *et al*. 2004)[20]. The lineage shows representative species corresponding to the main groups colour coded and written above.

The most commonly studied classes include the diatoms (*Bacillariophyceae*), green algae (*Chlorophyceae*), golden-brown algae (*Chrysophyceae*), *Prymnesiophytes*, *Eustigmatophytes* and cyanobacteria (blue-green algae) [21] and these are briefly described below.

Green Algae: Derived from an ancestral green flagellate (*Viridiplantae*), the lineage *Chlorophyta* (sister phylum to *Streptophyta* which contains land plants) includes the majority of described green algae species. *Chlorophyta* contains a large number of species and remarkable morphological diversity. Four classes are described within the *Chlorophyta* including the *Trebouxiophyceae* and *Chlorophyceae* (freshwater), the *Ulvophyceae* (coastal) and the *Prasiophyceae* (marine planktonic) [18, 22]. They contain chlorophyll a and chlorophyll b and use starch as the primary storage component. In certain species high lipid accumulation has been detected [21].

Diatoms: The diatoms probably include the greatest number of extant species (more than 100 000 species) of any group of microalgae [18]. Diatoms dominate salt water environments, but are also commonly found in fresh- and brackish-water habitats. They contain high levels of fucoxanthin, lower quantities of β-carotene, chlorophyll a and chlorophyll c. In terms of biofuel production one of the challenges in culturing diatoms is that they require large quantities of silica which is an expensive compound, and the resulting silica spicules can be problematic during processing. They do however produce relatively high levels of lipids [21].

Golden-Brown algae: This class is similar to the diatoms in pigments and biochemical composition (see above). Both diatoms and golden-brown algae, belong to the division *Chromophyta*. Approximately 1000 species have been identified, primarily in fresh water habitats. Lipids and chrysolaminarin are the major carbon storage form in this group [21].

Prymnesiophytes: This class includes around 500 species, which are primarily marine organisms. As in the diatoms and chrysophytes, fucoxanthin imparts a brown colour to the cells. Lipids and chrysolaminarin are, like golden-brown algae, the major storage products [21].

Eustigmatophytes: This class contains many picoplankton (only a few µm in diameter). Chlorophyll a is the predominant form of chlorophyll and several xanthophylls act as additional photosynthetic pigments [21].

Cyanobacteria: Cyanobacteria are gram-negative eubacteria, and therefore prokaryotic and very different from all other classes of 'microalgae'. Previous work classified them to the class *Phycophyta* (algae), leading to them being termed "blue green algae" although strictly speaking they are photosynthetic bacteria. Distinguishing features in this regard are their distinct gene structure and the absence of nuclei, chloroplasts and other organelles. Approximately 2500 species of cyanobacteria are described and include unicellular coccoid, colonial, and filamentous forms

[18]. Photosynthetic pigments include chlorophyll a and d, blue and red phycobilins and β-carotene. Starch is the primary storage product in blue green algae and some species produce more than 50 compounds which are toxic to vertebrates [23].

1.2.1 Classification of microalgae

Microalgae species were initially recognised and classified based on their phenotypic properties, such as whole organism morphology, cellular anatomy and ultrastructure, metabolism and physiology. However, a closer analysis led some microbiologists to conclude that more careful discrimination was required. This led for example to the classification of prokaryotic blue green algae and prochlorophytes as bacteria [24].

Correct identification of algae at least at the genus level is desirable to understand the ecology of aquatic ecosystems and global biogeochemistry, as well as for their successful use in biotechnological applications. Identification of algae provides the possibility of comparison between other strains of the same or a similar genus, which helps understanding metabolism and derivation of biochemical characteristics. Also in terms of metabolic engineering, identification using DNA sequencing assists future work, e.g. genetic manipulation, developing primers, and cloning.

1.2.2 Morphology and mortality

The algal body (thallus) exhibits a wide range of morphologies and the most common forms are briefly described below [25].

Unicellular and colony forming species: Algae species may occur as solitary cells separate from other identical cells (unicells) or as clusters of individual cells held together loosely or in highly organised structures. Unicellular microalgae can be further subdivided into motile and non-motile types. Motile cells possess one or more flagella for locomotion. This occurs also in colonial arrangements, which can consist of an assembly of individual cells in which there may be either a variable, or specific number of cells that remain constant throughout the life of the colony.

Coenocytic forms: Coenocytic algae are essentially unicellular, multinucleated algae. Such organisms basically consist of one large multinucleate cell, without cross walls. Examples include species of the order *Caulerpales*.

Coccoid forms: Coenobium algae are arranged as colonies consisting of a fixed number of cells with little or no specialisation. Reproduction occurs by a series of rapid cell divisions when the organism is first formed. Once the exact cell number is attained, the organism grows in size but not in cell number. The cells are often embedded in a mucilaginous matrix and may be motile or non-motile. Examples include *Scenedesmus* or *Pediastrum*.

Filamentous forms: The filament is a common growth form among algae. Daughter cells remain attached to each other following cell division and form a chain of cells which can remain unbranched or branched. The elongated assembly of cells may consist of uniseriate (single series of cells) or multiseriate, where individual filaments fuse together to form a larger, more complex structure. Some diatoms for example form linear colonies, and thus can be distinguished from true filaments. Diatoms possess their own individual walls, whereas adjacent cells of true filaments share a wall.

Capsoid forms: Chrysocapsa is an example of a capsoid organism. The numbers in these capsoids, which are embedded in a transparent gel, can vary and gradually increase in number over time.

Parenchymatous and pseudoparenchymatous forms: In this type of thallus (algae body) organisation, thalli are organised into true tissues composed of several different types of cells. Parenchymatous organisation is particularly common among the larger brown algae (e.g. observed in giant kelps). This results from cell divisions occurring in three directions, which gives rise to three-dimensional structures.

Flagella: Locomotion in algae is largely based on the action of flagella. A flagellum is a tail-like projection that protrudes from the cell body of the organism. The primary distinctions used for classification are the number of flagella, their location on the cell, and their morphology (e.g. *Euglenophyta* have one to three flagella, *Xanthophyta* (yellow-green algae) can have two or more for locomotion). Two major types of flagella are recognised; the smooth or acronematic and the hairy or pleuronematic types. The smooth flagella generally moves by whiplash motion (e.g. class of *Chlorophyceae*) and the hairy flagella move by a pulling motion (e.g. class of *Chrysophyta or Bacillariophyceae (Diatoms)*) [26-28].

Eyespot: Eyespots occur in many flagellate algae and are generally part of the chloroplast. Van Den Hoek (1995) described the presence of an eyespot (stigma) in the classes of *Chlorophyta* (green algae), *Phaeophyceae* (brown algae), *Chrysophyceae*, sometimes in *Cryptophyta* and *Haptophyta*.

The eyespot of *Euglenophyta* and *Eustigmatophyceae* lies in the cytoplasm as well as of the *Dinophyta* [29]. This eyespot can be seen in the living cell using the light microscope and appears as a small red spot at the anterior of the cell. Due to the eye spot motile algae are able to perceive light and to swim towards or away from it. Algae generally swim towards dim light but away from bright light [29, 30].

1.2.3 Metabolism

Eukaryotic algae, like all photosynthetic organisms, convert solar energy into chemical energy and biomass. In general, for such photoautotrophic growth they need an inorganic carbon source (CO_2) and solar energy to carry out photosynthesis resulting in biosynthesis, with cellular energy (ATP) and reducing power (NAD(P)H) also supplied by light (photophosphorylation). However, some algae can also utilise organic compounds as an energy source (i.e. heterotrophic growth) for example, by oxidation through the TCA cycle to produce ATP and NADH with CO_2 as a waste product, while others can use them both as an energy source and for the synthesis of new biomass [31, 32]. When a combination of light and organic carbon is used, growth is said to be mixotrophic. Therefore the efficiency of transforming organic and inorganic carbon sources into biomass is dependent on light intensity, the carbon source and algal growth phase and species. Some species such as *Chlorella vulgaris* [33], *Haemotoccus pluvialis* [34], or *Arthrospira platensis* (*Spirulina*) [32] are capable of growing under photoautotrophic, heterotrophic and mixotrophic conditions. They are thought to have developed this metabolic functionality as independent and simultaneous mechanisms [35]. However other strains such as *Scenedesmus acutus* [31]) only grow photoautotrophically.

Mixotrophic algae use photosynthesis as their main strategy to produce ATP and NADPH, but both organic compounds and CO_2 (for photosynthesis) may be essential. Amphitrophy, a subtype of mixotrophy, means that organisms are able to live either autotrophically or heterotrophically, depending on the concentration of organic compounds and light intensity available. Photoheterotrophic metabolism occurs where light is required to use organic compounds as a carbon source. Mixotrophic and photoheterotrophic metabolism are not well differentiated. A fine difference in energy source requirement distinguishes growth and specific metabolite production between both types [36].

In conclusion, complexity and inconsistency in trophic terminology of microalgae is a problem which is compounded by overlap of many types of metabolic program capability within one single algae that can be shifted simply by changing the environmental conditions [37]. Therefore it is

important to define what the desired outcome is and how the metabolic process can be tuned to achieve it. Whether conditions are favourable or adverse, the metabolic pathway of a cell is heavily influenced by its environment. Accordingly, the environment and conditions must be quantitatively defined when considering the use of microalgae for several desired products (lipid, biomass, hydrogen, etc.) [38].

1.2.4 Problems in the identification of algal species

Describing algal species is still far from a routine process and relatively few people are able to properly name or classify microorganisms such as algae and protozoa. Reasons for this include the fact that many algae are of microscopic size, and require skills in the use of dissecting and compound microscopes as well as further scanning or transmission electron microscopy in many cases. Furthermore sensitive algae often do not survive collection and chemical preservation procedures, or are altered after such procedures (e.g. loss of pigmentations, cell shrinkage, and detachment of distinctive flagella) making recognition difficult.

In some cases algae must be cultured to get a specific expression of a particular critical taxonomic character. For example, for identifying environmentally common coccoid green unicells, it is necessary to have information on zoospore characteristics.

Commonly used taxonomic or identification keys for algae are normally regional and it is uncertain to what degree such keys can be applied to more distant geographic areas. Taxonomic keys typically illustrate only very few species of a genus as samples of a wide array of diversity. Illustrations made by line drawing are problematic, due to their absence of colour and other details that would be useful to make proper classification. Even with high-quality colour images on websites or CD-ROM, more detail is often required [25]. Therefore, morphological classification is often only reliable to the genus level.

1.2.5 Molecular sequencing approaches

The most commonly used molecular approach to classify organisms is nucleotide sequencing which traditionally relies on the Sanger dideoxy chain-termination mechanism [39]. With sequencing methods, a large number of independently evolving characters of algae and their interrelatedness can be determined, with much greater sensitivity than is possible with classic morphological techniques. Full genome sequencing is still not routine, but a number of partial sequence methods are used for taxonomic classification [40].

Because of its redundancy, abundance and homology across all domains of life, ribosomal DNA (rDNA) sequencing has achieved enormous importance as a tool for discovering phylogenesis, evolution of life and the exploration of relation and connections between organisms. Today, the analysis of rDNA is a widely accepted method for classification of species in the universal genealogical tree of life and for identification of closely related species. Ribosomal genes evolved very slowly, making them useful for the molecular study of evolution more than 500 million years ago. There are nuclear-encoded genes for the small and large ribosomal subunits (SSU and LSU rRNA). Sequencing of the SSU like the *16S* RNA (from the chloroplast) or *18S* (from the nucleus, only in eukaryotes) subunits is more useful for the analysis of divergence over long periods because it is more highly conserved, as it is of integral importance in cellular processes. *16S* and *18S* subunits also contain regions of variability which can be used to identify divergence at lower taxonomic levels (including species-level investigations) [40]. The major advantage of rDNA genes is that they appear in both prokaryotic and eukaryotic taxa. Variations in these positions of bases in SSU genes can be treated as characteristics for specific algae species and phylogenetic trees. The University of Illinois has a large array of sequences for prokaryotes and eukaryotes in their Ribosomal Database Project (RDP) [41].

Very similar cell structures, such as coccoid, unicells or unbranched filaments are frequently the result of parallel evolution in widely divergent algal groups and challenge basic description. Potter *et al*. [42] described several coccoid picoplanktons, which appeared morphologically similar under the light microscope as “little brown balls”. By means of *18S* ribosomal DNA sequencing it was possible to define three eukaryotic lineages: heterokont algae, haptophyte algae and green algae, and at least four taxonomic classes [42]. The use of ribosomal DNA sequencing can also distinguish between bacterial and chloroplast ribosomes. The presence of chloroplast *16S* sequence confirms that DNA is algal in origin and will usually give a genus level identification but unfortunately the database of chloroplast rDNA sequences is still small and largely confined to commonly used industrial and scientific species and is therefore not yet as useful as it will eventually become for fine taxonomy until many more species have been examined.

Other molecular techniques are also important for the study of algal phylogeny and describing species, due to their enhanced sensitivity. RFLP (Restriction fragment-length polymorphisms) analysis which estimates DNA sequence dissimilarity, detects variation by nucleotide base substitutions, deletions, or insertions [43, 44]. The main advantage of RFLP approaches is the ability to discriminate between (and to enumerate) closely related strains.

Another approach used to study speciation is the use of Randomly Amplified Polymorphic DNA Analysis (RAPDs). This technique aims to synthesise many copies of anonymous DNA regions. Therefore a variety of DNA amplification primers are used in the polymerase chain reaction (PCR). Resolving these DNA regions by gel electrophoresis results in a fingerprint-like pattern, that is characteristic for each organism. In DNA databases, the patterns can be compared with those obtained from members of other species or populations. A high degree of similarity in banding patterns indicates a close relationship. Only a small amount of DNA is required to run RAPDs. A disadvantage of this procedure is that reproducibility can be difficult and the determination of band homology (degree of band relatedness) needs costly or time-consuming sequencing approaches [25].

One problem with nucleic acid PCR and sequencing is that non-target DNA may also be amplified in addition to target DNA. Amplification of contaminants can be avoided by either the use of monoculture samples or by usage of specific primers which are designed to amplify only the target DNA.

In the last decade the internal transcript spacer 2 (ITS2) was identified as a highly successful tool for DNA barcoding to identify plants and animals [45, 46]. Because *18S* or *28S* markers are only suitable for higher level classification (e.g. family and genus levels) it was proposed that the ITS2 marker could be used over a wider range of genus and species classification levels [47, 48]. This is beneficial for distinguishing even closely related species. Because it is short in length, PCR amplification and sequencing can be successful even for degraded DNA [49, 50].

As a part of the eukaryotic nuclear rDNA cistron, for eukaryotes it is located between the *5.8S* and *28S* rRNA genes [51] in the genome. The delimitation of ITS2 boundaries can be achieved by methods based on Hidden Markov Models (HMMs) [52, 53].

ITS2 for DNA barcoding is now a common tool to study algae diversity and phylogeny. Buchheim et al. used the approach to reconstruct phylum-level phylogenies of the green algae [54], whereas Moniz separated defined species of diatoms with a success rate up to 99.5% in the presence of bacterial contamination [55].

This shows the potential of the barcoding approach which can help to address complex problems in algal diversity, identification and taxonomic assessment.

1.3 Opportunities provided by screening wild type algal species

The development of any successful microalgal biofuel production system is heavily dependent upon the selection of the best microalgae strains and production conditions for the product of choice.

Microalgal strain collections are therefore an important resource [56]. Algae collections worldwide contain thousands of different algal strains that can be accessed. Typically sources of microalgae include existing collections of microalgae, commercially available either from universities or other national and international foundations (e.g. UTEX, CCAP, SAG (Sammlung von Algenkulturen – Georg-August Universitaet in Goettingen) and CAUP), but many companies have concluded that there are advantages to searching for new organisms. This is because it is estimated that there could potentially be hundreds of thousands of occurring microalgae species with a wide range of phenotypes which are often more competitive than their counterparts from established culture collections. In addition, local species are often adapted to local pathogens and conditions, and finally regulations (e.g. quarantine) and intellectual property legislation may restrict the cultivation and exploitation of non-native species, which is already the case in Australia.

The methodology of isolating microalgae from natural water sources, extensive analysis of individual strains and development of a local algal database provides the basis for selection and breeding for an expanded microalgal biofuels industry. It also has the advantage that collections:

- provide companies with independence from suppliers and generates in-house expertise that can be advantageous in the future
- provide new species or the development of new strains which represent a business opportunity, intellectual property and source of income from the possible royalties.

1.3.1 Growth characteristic for certain environments

Finding species with the right properties for specific conditions is challenging due to the large number of available strains and their limited characterisation [21]. For large-scale algal culture processes, a variety of desirable characteristics of algae are required some of which are shown below in table 1.2.

Table 1.2: Desirable characteristics of algae for mass culture [57]

Characteristic	**Advantages**
Rapid growth rate	Competitive advantage over non-target species; Reduces culture area required
High product content	Higher value of biomass (note: use of metabolic energy to generate product may lead to slower growth)
Growth in extreme environments	Reduces contamination and predation (note: Limited number of species can grow in extreme environments. Can be difficult to maintain conditions)
Large cell size, colonial or filamentous morphology	Reduces harvesting and downstream processing costs

Wide tolerance of environmental conditions	Less control of culture conditions required. Growth over range of seasons and ambient weather conditions
CO_2 tolerance and uptake	Greater potential for CO_2 sequestration and use of waste CO_2
Tolerance of shear force	Allows cheaper pumping and mixing methods to be used
Tolerance of contaminants	Potential growth in polluted water and on flue gases containing high CO_2, NOx and SOx
No excretion of autoinhibitors	Reduces autoinhibition of growth at high biomass densities

It is very unlikely that a single algae species will have all of the desired characteristics and a prioritisation of key features is required. For example, algae may be selected for fresh water or salt water conditions as well as temperature and high light tolerance or the production of specific high value products depending on the purpose for which they are required [21].

Fast-growing, productive strains, which are adapted or optimised for the local climatic conditions play an important role in the first generation of algal mass culture. They are already used particularly for the production of high value products as these are most commercially viable. Fast growth encourages high biomass productivity, increases yield per hectare and reduces harvesting costs, all of which are important for economic biofuels production [58].[59]. Also, a fast growth rate of microalgae assists in reducing contamination risks of large-scale microalgal production facilities.

The properties of algae in terms of harvesting are also important. Key features affecting harvesting include cell size, specific gravity and the ability to flocculate [60]. Some algae strains naturally flocculate reducing the requirement for expensive flocculation agents. Strains like the cyanobacteria *Spirulina* which form long spiral structures allow relatively low cost and energy-efficient microscreen harvesting methods, which is limited to filamentous or large colonial microalgae [58]. These properties will remain particularly important until more innovative strategies for the harvesting of dilute microalgae that have cell diameters of less than 20 µm are developed.

Griffiths and Harrrison (2009) pointed out the lack of available information on lipid productive microalgae species, especially needed to facilitate decision-making on species selection for biodiesel production. Only a handful of well-characterised species of algae are currently considered suitable for anything approaching scaled up conditions.

1.3.2 Metabolic engineering

As microalgal strain collections expand, it is expected that genetic manipulation will become increasingly important for the development of high-efficiency cell lines. Although routine genetic

manipulation currently remains limited to a few selected algal species (e.g. *Chlamydomonas reinharditii*, *Volvox carteri* or the diatom *Phaeodactylum tricornutum*), the expanding interest in algal biofuels will likely lead to the development of new techniques to engineer microalgae in order to develop organisms optimised for high productivity and energy content to achieve their full processing capabilities [38, 61].

Because microalgae represent a simpler system than higher plants (usually with no cell differentiation and shorter life cycles), genetic manipulations to increase the content of selected compounds should technically be simpler. Nevertheless, the optimisation of genetic techniques for microalgae has not until recently been considered to be of high priority [36]. In the future it is expected to play an increasingly important role in improving, biomass and oil, carbohydrate and protein production [61, 62] and increasing photosynthetic efficiency [63, 64].

1.4 Microalgal isolation and purification techniques

The aim of isolating and purifiying microalgae strains is to obtain cultures of single species free of other microorganism, whether different microalgae or contaminants (e.g. bacteria, fungi, viruses). If cultures have no detectable contaminants they are referred as pure or axenic. This is important for further screening, optimisation and identification of the cultures whose results would be influenced and falsified by non-target species.

Several techniques have been developed for the isolation and identification of microalgal species from natural water samples. Typically natural water samples have a mixture of different algal species with contamination of bacteria, fungi and multicellular organisms.

In addition, rare species may be present at very low frequency with one or two dominant species representing most of the biomass at a given time. Therefore isolation techniques need to provide the opportunity for low frequency algae to grow to higher frequency without interference from other organisms. Furthermore algal cells vary from their vulnerability to sudden, adverse chemical or physical changes and conditions which needs to be considered when replacing their natural environment with artificial growth medium. Analysing the composition of the original source water, or an adaption process to gradually increased full strength medium can overcome this hurdle. Light, temperature and pH sensitivity are further factors to be considered during isolation.

Conventional methods including dilution plating on solid media, liquid serial dilution followed by screening of coloured or morphological different colonies which provides the opportunity to pick manually a variety of species and at least separate them from other algae. Micromanipulation includes the use of a fine capillary syringe for controlled single cell selection (preferable by microscopic resolution) [65] [66] [17].

Bacterial and fungal growth can be discouraged by antibiotic treatment for example using Ampicillin, Kanamycin and/or Cefotaxime against gram-positive or gram-negative bacteria. However the use of antibiotics requires the optimisation of two variables: the dose/concentration of antibiotics and the time, (period of exposure until transfer to a medium with different type of antibiotic or without antibiotic). These variables needs to be carefully optimised to reduce the risks of harming and eventual losing target algal species [17]. As many algae are themselves sensitive to these agents, this approach also reduces biodiversity.

In the last two decades, flow cytometry has been recognised and used as a powerful tool for the study of phytoplankton ecology, especially for spatial and seasonal trends [67]. With addition of associated functions such as fluorescence and sorting, a new alternative and attractive isolation method has emerged, although as yet its application for sorting microalgae has been underutilised. Fluorescence activated cell sorting (FACS) might be a useful tool to isolate new algae species from diverse water sources or study natural population dynamics in a more efficient and time saving way, as long as the target species survive the relatively high shear and light stress involved. Subsequent steps can then focus on obtaining an axenic culture. Some of these techniques are explored in this thesis; in particular, FACS and micromanipulation have proved to be useful for obtaining axenic cultures. Once isolated, the properties and preferred growth conditions for the resultant strains need to be defined, and samples stored using cryopreservation if possible.

1.5 Culturing at large scale

1.5.1 Open and closed photobioreactor systems

Open pond photobioreactors: Microalgae cultivation requires large scale production for biomass; open ponds are the oldest and simplest systems for algal culturing and have been extensively studied. With outdoor cultivation in ponds the algae are exposed to the conditions of the external environment at the given area, which are largely uncontrolled (temperature, lighting). The most successful open pond systems which operate on commercial scales are high rate (raceway) ponds, circular ponds and unmixed ponds. These types vary in form, depth, water flow and mixing systems as well as productivity and economics. All of these types are widely used in large scale outdoor microalgal cultivation [68] because of their relatively low construction and maintenance costs relative to other algae production systems like complex photobioreactors (e.g. raceway pond \$250,000 ha^{-1} [69]). Furthermore open pond cultivation systems are suitable for scale up (increasing number of ponds) and in wastewater treatment processes.

Open ponds do however have several disadvantages. These include:

1. High contamination exposure which makes the maintenance of an axenic state impossible and complicates the management of population dynamics during long term cultivations.
2. Low algae concentrations per litre. This reduces yields ha^{-1} and increases harvesting and downstream processing costs.
3. Water evaporation losses as high as 10 L per m^2 per day, relative to closed PBR systems (especially in tropical or desert areas) over a large area remains a challenging problem [21, 68].

General optimisation work prior to more cost effective scale up on open ponds should be improvement of light distribution, in mixing efficiency, reducing the mixing cost and optimisation of the culture conditions to reduce contamination risks to improve overall biomass yield. Some open pond systems are able to achieve up to 20 g algae biomass dry weight m^{-2} d^{-1} on long term sustained basis (although higher rates have been reported for short term studies). This already makes them viable for some production systems involving the production of high value products [68, 70, 71]. The main problem with open pond systems is that few options are available for engineering improvements that alter the physics of light distribution for algal growth. Furthermore the open nature of the pond makes it difficult to prevent or limit contamination with other organisms.

Closed photobioreactors: In parallel with open pond development, closed photobioreactor (PBR) systems are being designed for microalgae mass production. Instead of being directly exposed to the atmosphere, the cultivated microalgae are enclosed in a transparent material.

Of the closed PBR designs, the ones most commonly used are tubular PBRs [72]. Their high flexibility of construction (straight vertical, horizontal, inclined or helical), control of gas transfer, large surface area to volume ratio and good biomass productivities (35 to 41 g m-^{2} d^{-1} [58, 72] and even 47 g m^{-2} d^{-2} [73]) makes them attractive as long as the additional cost can be justified.

Plate or flat panel PBRs consist of a transparent rectangular container (usually inclined or vertically aligned) with a light path between 1 and 30 cm [74, 75]. As an example of successful cultivation processes with these designs, a system in Italy used for the production of *Nannochloropsis* sp. and set up under several conditions, achieved a biomass productivity up to 30 g m^{-2} d^{-1} [76]. Because of improved mixing and sunlight capture, these systems provide higher biomass productivity and cell density [26], and contribute to significant reductions in algae harvesting and drying costs [77].

With a closed system, the contamination risks might be reduced but not eliminated. Furthermore cleaning and sterilisation of closed reactors after a culturing, stage remains difficult. Given improved control of culture conditions such as temperature, light, pH, and nutrients it is, however, possible to extend the growing season and enhance productivity. The reduction in CO_2 and water losses is also attractive.

Despite these advantages the high estimated construction cost still limits the scale up of these systems for biofuel manufacture. One estimated capital investment for PBR is \$180 m^{-2}, which is almost seven times that required for open ponds per unit area [78]. Engineering with low cost materials, low light-dilution, and high thermal isolation need to develop in order to decrease the PBR costs towards ~\$15 m^{-2}.

A comparative study of systems regarding efficiency was conducted by Fernandez et al. [79]. In this study parallel tests were conducted on open ponds, closed vertical plate and horizontal tubular PBRs, utilising the same algae strain (*Scenedesmus almeriensis*) and at the same location. This study concluded that a limiting factor for producing high biomass yield is light and therefore the surface area to volume ratio (SVR). The higher the SVR the higher the biomass productivity.
The recent review of Posten (2009) [80] summarises the current thinking on how productivity of PBRs can be driven up and their construction costs down.

Still both systems facing the significant challenge of separating and dewatering the algal biomass. The hurdles of harvesting and recent technologies are discussed more in depth under 1.7.

1.5.2 Monoculture vs. polyculture

In a polyculture, several algal species coexist. This may consist of a few tightly controlled target species, or more commonly, refers to a broad ecological mix of species that simply happen to find their way into the culture, typically airborne from nearly natural sources of algae, especially natural water bodies. If biomass is the final desired product, or if the culture is aimed at bioremediation (e.g. drawdown of nitrogen) then it may not be as important to know what algal species are present. If a particular strain is desired for some specific property it possesses, then an uncontrolled polyculture will not be satisfactory. Furthermore, since some natural algae are toxic (e.g. certain cyanobacteria) uncontrolled polycultures are not without risk. Therefore if a specific target strain is desired, a controlled polyculture may be a more desirable situation. Clear examples of side by side comparisons between monocultures and polycultures are difficult to find in the literature, but the proposed benefits of polycultures are largely related to robustness (since predators often target only one or a few algal species) while the disadvantages are largely related to the loss of control over which species are present. In this thesis, the use of controlled polycultures is explored, to see whether these offer the same benefits over monocultures that uncontrolled natural or "wild" polycultures are proposed to do [81]. If so, controlled polycultures may represent a viable and robust culture system for future biotechnology applications.

1.5.3 Contamination by foreign algae species and predators

In comparison to laboratory cultures of axenic microalgal strains, industrial scale culture systems inevitably contain other microorganisms or predators. These include other algal species which may compete with the desired strain, potential predators (multicellular organisms such as rotifers, protozoans, fungi or viruses) which eat or parasitise the target strain, and bacteria which compete for nutrients, and may produce either cofactors can be beneficial to the algae (e.g. vitamins B1 and B12) or allelopathic factors which can be inhibitory. The result is that the culture system becomes more complex and has lower predictability. Consequently, while it is not always necessary to eliminate other organisms, it is important to understand the effects of their presence.

In conclusion, even by providing the optimal production conditions (e.g. nutrition, temperature, pH, light etc.) a successful and productive algae cultivation in outdoor systems can’t be guaranteed due to contamination. Invasion and establishment of non-target organism can be highly diverse. In its presents it may influence the desired product quality, lower the aimed production, and in worst case

lead to costly culture crashes. Early detection and identification of invasive organisms, evaluation of possible impact and following the appropriate counteraction is of extremely value for industrial algae production. It is necessary to know which organism are common and likely to invade, their preferable circumstances and what impact may occur. Hence a detailed study of contaminating microorganism, their occurrence, warning signs, behaviour and environmental needs has to be conducted in the same extend as the desired algae species to guarantee predictable and controllable culture production. The following section provides informative background of the most common invasive pond organisms.

1.6 Pond water organisms: Protozoa and small animals (Rotifer)

1.6.1 Classification of protozoa

Protozoa ("first animals"; "primitive animals") are unicellular eukaryotes, most of them only detectable by microscopy. In the past (around 150 years earlier), Protozoa were treated taxonomically as a subset of the kingdom Animalia, however it is now abundantly clear that this definition does not reflect evolutionary phylogeny and that Protozoa are not simply animals, but contain organisms that are both separate from and included within other phyla [82].

The concept of species and phyla within the Protozoa has always been problematic and a subject of discussion. Classification of diverse microorganisms like Protozoa in the past and still now is mostly done via microscopy, however many crucial findings of structure of these species remained at the cellular levels and is not visible to the naked eye. With improvement in microscopy (light, electron) and the including use of molecular biological approaches the taxonomy (classification) and evolutionary interrelationships of major Protozoa groups has been refined in the past decades [82].

Generally described there are four major groups of Protozoa. The categories include ciliates (Ciliophora), flagellates (Mastigophora), amoebas (Sarcodina) and freshwater radiolarians (Heliozoa). Their division into several phyla is changing constantly and several systematics are proposed. This is mostly for convenience and ease of discussion amongst specialists. However arguments are continuing over the exact boundaries of recognised collections of protists and their division into subkingdoms and phyla [83].

More literature about the taxonomic schemes of Protozoa is provided by Margulis et al (1990) [84] and Corliss (2001) [82].

1.6.2 Protozoa characteristics and identification

For the sake of brevity and simplicity of morphological identification here we will discuss the 4 major groups of important Protozoa based on their locomotion:

- flagella (Zooflagellates)
- amoeboid (Sarcodina)
- radiolarian (Heliozoa)

- ciliate (Ciliophora)

Protozoa play an essential role in the aquatic and terrestrial food webs [85-87]. They are mainly feeders on bacteria, but can act as important herbivores [88] [89], detritivores [90], osmotrophs [91] [92] as well as mixotrophs [93]. Bacterial communities are not only grazed by Protozoa but are also structured by these grazers [94].

Flagellates (Zooflagellates):

The flagellates have been generally considered to be the most likely ancestors of the ciliates [95]. Flagellates vary in a wide size range between 1-450 µm. They are very tolerant to changes in salinity and apart from their distinction between marine (e.g. Euglenids, Cercozoans) and freshwater groups (e.g. dinoflagellates, choanoflagellates), the communities seem to be very similar [85].

Despite a number of structures in flagellates which are similar to the ciliates there are some which are not. The movement of flagellates is an important taxonomic character involving gliding, free-swimming and temporarily attaching to substrates [96]. These Protozoa move with whip-like extensions called flagella (similar to algae flagella described above). Flagella usually occur in pairs (but sometimes more) and are relatively long (often longer than the cell that carries them, and reaching lengths of up to 50 µm). In dinoflagellates, for example, a transverse flagellum is located in the girdle and surrounds the cell whereas the other flagellum is longitudinal and emerges from the cell. On the surface of both flagella, fibrillar hairs are present, which is not the case for ciliates [97].

An accessible guide to common heterotrophic freshwater flagellates is provided by Jeuck and Arndt, 2013 [98].

Amoebas: Sarcodina

These micro-aerobic amoebas have a plastic morphology using a protoplasmic flow as locomotion (pseudopodia), sometimes with flagella but when present these are restricted to developmental stages [99]. Most are free living in freshwater, feeding on bacteria, larger species being predatory to algae and other Protozoa. Encountered food is then surrounded by the cell (ectoplasm) and enveloped in a food vacuole [100].

Radiolarians: Heliozoa

The most noticeable characteristic of Heliozoa are the axopodia. This type of pseudopod is strengthened by tiny microtubules (axonome) that extend into solid protective rods surrounding the cell. Locomotion is a slow constant motion of axopods, reaching out and returning in on opposite sites. When food particles attach to the sticky surface of the tubules, it is transported to the cell body and is engulfed. Heliozoa ingest small organisms like other Protozoa and algae and reproduce asexually by binary fission [100].

Ciliates: Ciliophora

Ciliates are characterised by three major features: (1) the presence of cilia which are variable in number but distributed over the body surface and derived from kinetosomes with three fibrillar associates; (2) nuclear dimorphism – the large macronucleus and a small micronucleus which controls the physiological and biochemical functions as well as acting as germ-line reserves; (3) the sexual process of conjugation in which two cells fuse temporarily to exchange gametic nuclei [97] [101].

Ciliates are heterotrophic, often responsible for consuming the majority of organic material and bacteria in certain habitats [101]. Evolved from the flagellates, they display great diversity in shape (e.g. spheres, cones, spheroids, cylinders) and size (10-4500 µm). A complex microtubular, microfilamentous cytoskeletion keeps the body form relatively stable.

Generally cilia are short and densely packed and organised in parallel rows used for motility and food gathering. The precise arrangement of cilia on the cell membrane varies between species. Ciliary movement is faster than flagellar movement, creating a distinct 2 phase asymmetrical beat (stroke) cycle. The beats of the different ciliates around the cell are coordinated and propel the organism in specific directions. Movements can result in distinct, complicated patterns. For instance *Paramecium* normally swims forward, rotating around its axis simultaneously [102].

The ciliate *Tetrahymena*

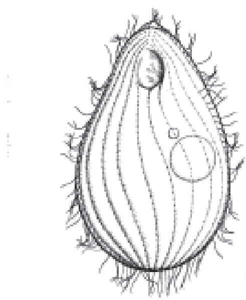

Figure 1.3: Drawing of a typical cell *Tetrahymena* [101].

The genus *Tetrahymena* (Figure 1.3), assigned to the ciliate family Tetrahymediae, includes at least 41 recognised species [101]. Its name derives from the oral apparatus made of four structures; the undulating membrane (paroral) and three membranelles (polykinetids). Cells are oval shaped, ranging in size between about 30-50 µm with numerous perfectly aligned ciliary rows longitudinal to the body. Like ciliates, in general *Tetrahymena* have a characteristic nuclear dimorphism and dimorphic life cycle. Because of these and other features, *Tetrahymena* (particular the species *T. thermophila* and *T. pyriformis*) has been an important model system for physiological, biochemical, and molecular research for many years [103]. It is fast and easy to grow, reaching a doubling time less than 2 hours under optimal conditions (37 °C) and can be maintained in a vegetative form over months and cryopreserved for years [104].

Dense core secretory granules located on the cell membrane near the cilia can be triggered to provide a sudden explosive release of mucopolypeptides in response to extracellular cues (exocytosis). Mucopolypeptides are also found in many higher organisms including in vertebrates including humans (e.g. in the human immune system). This remarkable feature is highly developed in ciliates making *Tetrahymena* a useful system to study this phenomena [105].

The freshwater cilated Protozoa are filter feeders mostly grazing on bacteria and metabolic waste from other microorganisms. Nevertheless feeding on algal cells has been observed (e.g. *Micractinium sp*), presumably as a last resort during food limitation since digestion of the algal cell wall is difficult. Studies indicate that long term survival solely by ingesting algae is not possible and *Tetrahymena* populations declined or became extinct in this case [106].

Factors affecting the distribution of Protozoa

Protozoa are found over a broad range of environmental habitats and conditions. Nevertheless some factors (physical, chemical) influence successful growth and distribution of protozoa. Despite the

supply of nutrition, the most important factor is temperature, and for autotrophic forms, light is also a crucial variable. Apart from organisms living in extreme environments, the majority of species increase their cell division and population at a temperature around 25 °C and can tolerate at least 30 °C, however species reproduction slows down with decreasing temperature and can stop below 6 °C [100].

Protozoa are highly tolerant to high levels of oxygen and some species can tolerate anaerobic conditions [107].

1.6.3 Rotifer (a multicellular animal)

These predatory microorganisms are multicellular and therefore do not belong to the kingdom Protozoa but rather the kingdom Animalia. The phylum Rotifera includes approximately 2000 species which are widely dispersed in all freshwaters habitats [108] [109] even including moist habitats such as mosses and lichens [110]. Typically rotifers are divided into three classes called Seisonidea, Bdelloidea and Monogomata [111] [112].

The class of Seisondiea is the smallest group of rotifers compromising only two marine species (*S. nebaliae* and *S. annulatus*). They are the only rotifers which reproduce strictly bisexually and show well developed males (similar in size and morphology to females). They are very large in size (2-3 mm) and show similarities to Bdelloidea. However they have striking morphological differences to the other two classes. Examples include a reduced corona (Latin, crown), the absence of copulatory organ, cilia in the digestive apparatus and absence of resistant stages [113].

The class Bdelloidea includes around 460 species [114] [115]. They are distinguished by their paired ovaries including vitellaria, pedal glands and branches in the trophi [116]. Feeding on minute particles or microorganism is performed using their corona of either two trochal disks or a ciliated field. Their external lorica (stiff body wall) structure remains in rings which permits shortening and lengthening of the body by telescoping. These organisms are often detected in sediments or among plant debris or crawling on the surfaces of aquatic plants and are capable of surviving desiccation and rehydration, so that they can use airborne dispersal. Male rotifers are absent in the class Bdelloidea.

The class Monogononta displays the largest group of rotifers with around 1450 species [115]. While most of them are free living and free swimming and only interacting solitarily as prey or mates, species of mongononts have been described to form permanent colonies [117]. Monogonont rotifers have only one gonad. Only in a few species males have been described which are, when compared to females, structurally reduced and short living. Most species of this class feed on microorganisms but a few are described as being parasitic. Besides the body division into three parts (head, trunk and foot) no true segmentation is present. The corona is a ciliary organ and is commonly modified into diverse functional types towards adaptation. In creeping forms the corona is enlarged for gliding over surfaces whereas in attached (sessile) forms the corona can be extended by the formation of lobes to increase the filtering efficiency [118].

Rotifers are well adapted to a variety of habitats. Their ability to form desiccated forms (anhydrobiosis) was described for the first time around 300 years ago (Hendrik van Bleyswijk, 1702). If the environment dries out, the organism contracts, encapsulates and enters a dormant state. When rehydrated they resume their activity within hours [119]. In the suspended state they can survive for months or even years. One of the longest authenticated periods of successful survival recorded was 9 years [120].

Most of the rotifers are raptorial predators or feeding/grazing on minute particles or microorganisms. When sufficient food is available rotifers can become very abundant and reach densities up to 5000 individuals per litre and in open water bodies like sewage ponds even as high as 12,000 per litre [121].

Because of their small size (ca 50-2000 µm) they are easily confused with Protozoa; however they are made of many cells and have organs like other members of the kingdom Animalia (e.g. Daphnia, Cypris or Cyclops). Some are free swimming, others are attached at surfaces (sessile). The typical body form of these animals is spindle-shaped or worm-like (elongated) and can be divided into three or four regions: head (corona), neck, body (trunk) and foot. However, depending on the species or sex, the neck and foot may be prominent or absent (Figure 1.4). With their foot rotifers can attach themselves to surfaces. Some species (e.g. sessile species) may also possess glands which secrete adhesives for temporary attachment [122, 123].

The skin (integument) of the rotifer can be thick and rigid like armour (loricate) or supple and flexible (illoricate). The integument is important for identification in some genera (e.g. *Brachionus, Euchlanis*) including its cuticular structures of bristles and fins. Several species show a mucilaginous envelope around them e.g. *Collotheca pleagica*, *Gastrophys stylifer* [112].

The special anatomical features of all these animals are the corona, and the muscular mastax. The corona located at the anterior end of rotifers is mostly (but not for all species) equipped with two concentric rings of cilia (trochus and cingulum). There are at least seven recognised types of corona varying in shape, placement of the mouth and distribution of cilia [124]. The corona is employed in all species to collect food; furthermore in some free living species it might be used for locomotion. Once the food is swept into the mouth, it will be processed by the chewing, grinding jaw-like trophi of the mastax [125] before it leads to the stomach. In some species the mastax is enlarged and serves as well as food storage organ.

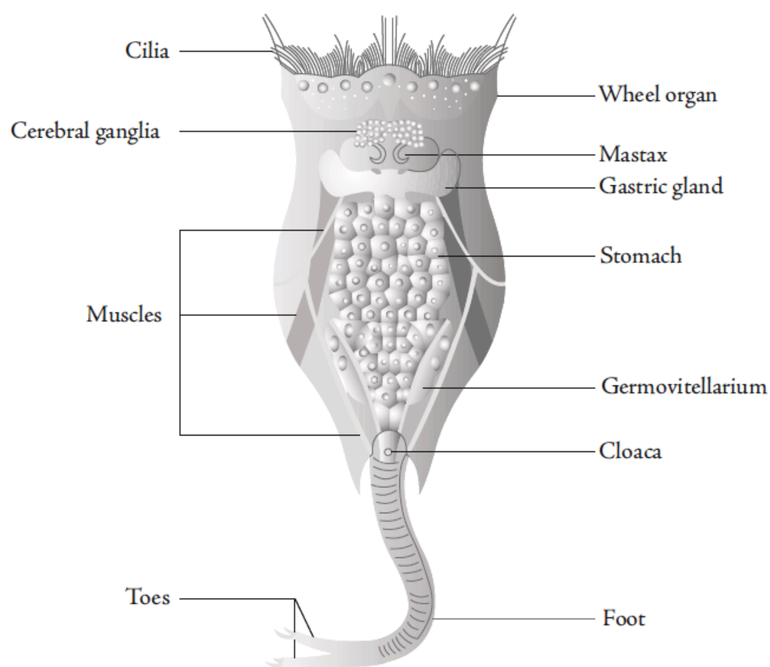

Figure 1.4: Schematic representation of a *Brachionus* rotifer [126].

Reproduction

As noted, each of the three classes of Rotifer employ different reproduction strategies. Seisonidea reproduce bisexually and gametogenesis takes place by ordinary meiosis [112]. Bdelloidea reproduce by asexual parthenogenesis and no males have been observed. The class of Monogononta have evolved by alternating generations. An asexual cycle is dominant but sexual reproduction occurs only after being triggered by specific environmental signals e.g. high population density [109]. Unlike the mature animals, the fertilised eggs of monogononts develop a thick wall and are capable of surviving harsh environmental conditions while resting. Furthermore resting eggs are more easily distributed by wind or animals. And when the right condition is supplied, parthenogenetic females hatch again.

The life cycle of rotifers is very short and without any larval stage. Within hours to a few days at the longest the development of the animals is complete after hatching. The life span of rotifers varies from a few days to about two to three weeks.

Foraging behavior and food source

Rotifers consume a wide variety of plant, protozoa and animal prey and may be described as generalist feeders. They play an important role in food webs of water bodies (lakes) because of their rapid turnover rate and metabolism. They feed largely on dead organic matter and bacteria besides

algae, and are important prey for small carnivores such as copepods, insects and fish larvae [111] [127].

Most of them are filter feeders or grazing on bacterial film or debris, however many are active predators or feed on large algae (e.g. *Asplanchna*). They either completely engulf their prey or suck out their contents [118]. As an example, *Brachionus* and *Ptygura*, which are usually herbivorous process many tiny particles in rapid succession but also have been seen to consume small ciliates [111].

Depending on the family, sessile rotifers capture food in two different ways. Water currents towards the mouth are created by members of the family Flosculariidae (e.g., *Floscularia*, *Ptygura*, *Sinantherina*). In contrast, collothecid rotifers are ambush raptors (*Collotheca*, *Stephanoceros*), which, once the prey comes close enough to the corona, fold over and trapping their prey using long bristles, [111].

During the screening process for food not all potential food items are consumed. Observations of the species *B. calyciflorus* in various densities of suspended food particles, revealed different mechanisms of the grazing process. Large particles are screened away from the mouth whereas suitable particles are collected within the corona, but may be rejected later. Even particles which are led to the oral cavity can be released [128]. This is clearly important in water bodies containing suspended inorganic material.

Therefore rotifers can have a high clearance rate on phytoplankton. A study showed that the rotifer *K. cochlearis* accounted for about 80% of the community-grazing pressure on small algae during the year [129].

Distribution and environmental factors

Water bodies are complex habitats with constantly fluctuating abiotic (dissolved-oxygen, light intensity, temperature, salinity and water movements) and biotic (presence or abundance of phytoplankton and predators) factors, which influence the distribution of rotifer communities [130].

Temperature is one of the key variables and abundance of rotifers varies with the season, for example increasing in summer [131]. High abundance of rotifers was found in waters around 17 to 30 °C [132] [133] however fail to establish at values above 30 °C [134] [135]. It has been reported that increasing salinity has a further impact on rotifer abundance [131] [136].

A correlation between grazing and oxygen availability was shown by Lair 1991, with rotifers preferring to feed in zones of higher oxygen levels [137].

Less is known of the specific metabolic responses of rotifers to pH, but in *B. plicatilis,* swimming activity and respiration rate did not significantly vary at pH values of 6.5–8.5 [138]. Nevertheless motility was reduced below pH 5.6 and above pH 9, with alkaline waters depressing swimming activity more than acidic conditions.

A high positive correlation is shown between rotifers and the presence of chlorophytes [132]. Furthermore a study showed that the presence of the algae *Chlorella* can aid hatching of the eggs of *Brachionus plicatitils* [139].

By knowing the different variables responsible for the presence or absence of rotifers, these organisms can be useful indicators for water quality. For example the presence of genus *Brachionus* can be indicative of moderate to high levels of organic pollution [140].

1.6.4 Impacts of predatory organisms on algae cultures

Contamination of algal cultures with non-target organisms (bacteria, protozoa, fungi, rotifers) is a known problem within industrial microalgae cultivation, especially in open pond outdoor cultivation systems. By grazing through the culture and competing for nutrients they can significantly reduce algal growth and productivity and in the worst case lead to destruction of the algal culture ("culture crash") [141].

Apart from the type of algae and environmental conditions, the impact of microorganisms on the cultivation system depends on the type and species itself. Protozoa are widely known as bacterivores, feeding on bacteria trapped on surfaces or particles (e.g. debris or flocs). They are abundant in active sludge and used for wastewater treatment. The larger the protozoa, the more likely it will feed on algae and other Protozoa (algivores). When starved, Protozoa may ingest algae even when they represent a poor food source.

As described above, rotifers are in most cases raptorial predators feeding on microorganism and phytoplankton. They are able to alter the species composition of algae in water systems. It was reported that the intense feeding of the rotifer *Brachionus rubens* caused a shift in the dominant algal species from *Scenedesmus* to the spined algae, *Micractinium* [141]. This shift occurred most

likely due to the inability of the rotifer to consume algae with "protective" spines which greatly increase the effective diameter of the organism.

This and other types of defense mechanisms in algae against feeding pressure has been observed, eventually altering the cell morphology (e.g. protective spines) or the whole community. For instance the presence of grazing predatory organism forces some algae to initiate colony formation as a stress response and defense mechanism. Small algae bundled together are more difficult to tear apart or to ingest. This occurs in members of the *Scenedesmaceae* (Chlorophyta) [142] [143]. Mechanisms behind this concerted colony formation are not well understood but assumed to be via chemical components released into the environment. A demonstration of *Scenedesmus obliquus* was given during the exposure to a test medium previously incubated with *B. calyciflorus.* The algae were shown to respond to the biological compounds released by grazing by exhibiting a logistic dose of response chemicals, also called infochemicals [143].

Algal defenses mechanism can influence and stabilise the population fluctuations, and by doing so, the long-term survival of both rotifer and algal populations.

Furthermore, in artificial systems of predator and prey cultivations a rapid evolution of the prey organism has been documented resulting from the strong selection of genotypes which are more digestion-resistant [144] [145].

In some circumstances, both rotifers and protozoa can be useful. Rotifers are themselves feed for higher organisms and can act as a high value product [146]. In particular, as long as the target alga is not eaten they can actually be useful in restricting bacterial numbers. As protozoa are large organisms relative to bacteria they are easily identified and quantified. There is also the possibility that Protozoa may contribute useful nutrients or cofactors to the culture or otherwise change the physical factors to benefit the algal system.

1.7 Downstream processing and harvesting

1.7.1 Hurdles in dewatering of algae

Although some algae production systems use immobilised cells [147] [148], most require suspension of the algae in a nutrient medium. Since the mixing required for this is energy intensive, it represents a substantial drain on the energy balance of the culture if bioenergy/biofuels are a desired product. Consequently, algae possessing small cells which stay in solution easily are highly desirable in the production scenario. However, once an algal culture has grown successfully, this trait becomes problematic because harvesting small cells is more energy intensive. Harvesting is a key issue for a systems energy balance and is considered by some to be the most problematic area of algal biofuel production, thereby limiting the commercial use of microalgae. It is estimated that 20-30% of the costs of producing microalgal biomass is due to harvesting costs [149] [36], some estimations are as high as 50% [150].

Algae suspensions, even at culture completion are very dilute at a cell density of around 0.25 g L^{-1} to 1.5 g L^{-1} [151] [149]. Algae cells are very small (most algae cells below 30 µm, often ~5-10 µm and some as small as 1-2 µm). Typically they often have a similar density to the growth medium, together with a negative surface charge on the algae, which results in a stably dispersed algal suspension, especially during growth phase. An important parameter which describes the ability for a colloid to stay in suspension is the zeta potential and this is dependent on several factors including pH and ionic strength. Further background can be found from Borowitzka and Moheimani (2013) [152].

Sawayama *et al*. (1999) estimate that conventional methods (sedimentation and centrifugation) have a harvesting energy requirement of 1 MJ kg^{-1} of dry biomass, which is greater than the energy cost of harvesting wood at 0.7–0.9 MJ kg^{-1} [153]. Apart from energy balance, effective harvesting systems must be able to process large volumes, be highly reliable, flexible for different species and growing systems, and be cost effective. They must also be suitable to the species (e.g. size, surface charge etc.) [154].

Harvesting of biomass typically requires one or more solid-liquid separation steps [149] [155]:

- Step 1: Bulk harvesting - separation of biomass form the bulk suspension, the aim is to reach up to 2-7% solid matter, depending on biomass concentration technologies e.g. flocculation, flotation or gravity sedimentation

- Step 2: Thickening - Further concentration of the slurry by centrifugation, filtration or ultrasonic aggregation. This is a more energy intensive step, so minimising the volume treated is a key requirement.

1.7.2 Current algae harvesting methods

Most biological work on microalgae system has been on species selection for biofuel production, with a particular focus on yield and biomass composition, rather than on recovery.
The main current methods of harvesting are summarised in Table 1.3:

Table 1.1: Comparison of microalgal harvesting methods [156].

	Advantages	Disadvantages	Dry solids output conc' (%)
Centrifugation	Can handle most algal types with rapid efficient cell harvesting	High capital and operational costs	10–22
Filtration	Wide variety of filter and membrane types available	Highly dependent on algal species; best suited to large algal cells. Clogging or fouling an issue	2–27
Ultrafiltration	Can handle delicate cells	High capital and operational costs	1.5–4
Sedimentation	Low cost, potential for use as a first stage to reduce energy input and cost of subsequent stages	Algal species specific, best suited to dense non-motile cells. Separation can be slow. Low final concentration	0.5–3
Chemical flocculation	Wide range of flocculants available, price varies although can be low cost	Removal of flocculants, chemical contamination	3–8
Flotation	Can be more rapid than sedimentation. Possibility to combine with gaseous transfer	Algal species specific. High capital and operational cost	7

No single method is suited for all microalgae, especially since the design and operation of the downstream process in a microalgal biofuel production process has to be considered. These technologies are well summarised by Molina Grima *et al*. (2003) and Christenson *et al*. (2011) [149] [157]. Here, only flocculation is discussed in detail, since the work in this thesis examines flocculation as a harvesting strategy.

Flocculation is a particularly suitable method of harvesting for algal cultures because it enables either airlift (dissolved air flotation) followed by skimming or else utilises gravity thickening to concentrate the biomass. It is potentially applicable to large quantities and to a wide range of species and is best suited to be used as a dewatering or initial concentration step. It is also applicable both to batch and continuous harvesting approaches [158].

Flocculation requires aggregation of algal cells which were previously well separated and which, in suspension, have similar surface charge and therefore generally tend not to approach each other

closely nor aggregate. It increases the particle size and therefore the rate of settling or flotation. The act of bringing together the algal cells into aggregates or flocs therefore requires a means to reduce the electrostatic repulsion between cells and the substitution of some physical or chemical attraction between them.

Types of flocculation

Flocculation can be induced chemical, physical or biological.

Chemical flocculants are typically either highly charged inorganic ions (Al^{3+}, Zn^{2+}, Fe^{3+}) which eliminate surface charge and encourage ionic interactions, or else synthetic (polyacrylamide) or organic polymers (chitosan, cationic starch), which not only neutralise, but entangle and draw the cells together [157] [159].

Such flocculants are typically added anew for each harvest and usually remain in the biomass after it is harvested. There is an expense associated with their storage and distribution and with any removal which is required [160]. Especially inorganic flocculants can be toxic preventing the recycling of growth media and the use for further feedstock applications (e.g. aquaculture) [161] [162].

Changing the pH to low or high values can also contribute to flocculation (e.g. increasing pH to 11-12 induces flocculation in *Chlorella* [163]. This works for some, but not all species, for example it did not produce flocculation in *Chlamydomonas* [164]. It may require the addition of considerable amounts of pH-altering compounds to alter the pH (e.g. relatively large amounts of NaOH) which is considered as uneconomic and can cause cell damage and death which can harm the quality of the biomass [162].

A purely physical flocculation method (electro-coagulation-flocculation) uses electric current to generate Al^{3+} or Fe^{3+} ions to act similarly to exogenously added salts, but with much lower material requirements. This has a much lower power consumption than centrifugation and utilises less chemical mass than exogenous aluminium or iron. Since it works best under high salt conditions, it is particularly attractive for marine algal strains [165].

Electrolytic flocculation is also possible using non-sacrificial anodes; here algae which move towards the anode lose their negative charge and aggregate. It has the disadvantage that the electrodes are prone to fouling [158].

Biological flocculation ("bio-flocculation") involves using one organism to flocculate another. In the case of auto-flocculation, there is only one species involved, but this requires the potential for self-flocculation to be present in the target algal species and is therefore not a generally applicable method. Microalgae may flocculate in response to environmental stress e.g. changes in nitrogen, pH and dissolved oxygen, and if possible this may provide a viable route for recovery of a specific strain. An auto-flocculating strain can be used as a flocculant for a non-flocculating strain [166], but this requires a substantial input of biomass from the flocculating strain, which may not necessarily be of any other interest as a product (thereby diluting value of final product), nor may it be a rapidly growing strain. However, it should be mentioned that a simultaneous cultivation of both non-flocculating and flocculating algae species might overcome this hurdle and improve harvesting [167], as the presence of strain diversity can cause aggregation as defense mechanism against predatory organisms.

In addition to using algae, other microorganisms, typically bacteria or fungi, can be used as flocculants. This is usually because they produce filamentous hyphae in the case of fungi, or else because they secrete extracellular polymeric substances (EPS) which act as a naturally produced polymer, acting similarly to exogenously added polymers [168] [169]. These systems typically take a long time for flocculation to occur (e.g. overnight) which is problematic for algal harvesting where biomass losses due to techno-economic considerations as well as the potential for extended respiration to impact on the quality of the recovered biomass.

Finally, other macro-organisms (e.g. crustaceans or fish e.g. brine shrimp or tilapia [157]) can be used to harvest algae, especially if the biomass represented by the harvesting organism is more valuable in its own right than the raw algal biomass. The inevitable losses in energy caused by progress up the food chain however, is not likely to make these methods viable for large scale biofuel production. However, naturally occurring predatory microorganisms (protozoa, rotifers) in open outdoor system may be useful for biomass aggregation when algae forming aggregates to avoid grazers [170] [171].

1.8 References

1. Jakob, G., et al., *Triggered exocytosis of the protozoan Tetrahymena as a source of bioflocculation and a controllable dewatering method for efficient harvest of microalgal cultures.* Algal Research, 2016. **13**: p. 148-158.
2. (CIA), C.I.A. *The World Factbook*. [website] 2015 June 2015]; Available from: https://www.cia.gov/library/publications/the-world-factbook/geos/xx.html.
3. Stephens, E., et al., *Algae Fuels as an Alternative to Petroleum.* Journal of Petroleum and Environmental Biotechnology, 2013. **4**(4): p. 148.
4. International Energy Agency, *World Energy Outlook 2010*, in *IEA publications*. 2010: Paris.
5. *How to Feed the World 2050*. 2009, Food and Agriculture Organisation of the United Nations.
6. *Environmental Outlook to 2050*. 2014, Organisation for Economic Co-operation and Development.
7. Intergovernmental Panel on Climate Change (IPCC). *Climate Change 2014*. Fifth assessment report (AR5) 2014 [cited 2014 November 2014]; Available from: http://www.ipcc.ch/report/ar5/.
8. Food and Agriculture Organisation of the United Nations, *The State of Food and Agriculture 2008: Biofuels: Prospects, Risks and Opportunities*. 2008, Rome: FAO. xi+128.
9. King, D., et al. *Global Apollo Programme - To combat climate change*. 2015 June 2015]; Available from: http://globalapolloprogramme.org.
10. Vigani, M., et al., *Food and feed products from micro-algae: Market opportunities and challenges for the EU.* Trends in Food Science & Technology, 2015. **42**(1): p. 81-92.
11. Ciamician, G., *THE PHOTOCHEMISTRY OF THE FUTURE.* Science (New York, N.Y.), 1912. **36**(926): p. 385-94.
12. Gregory, J.A., et al., *Alga-Produced Cholera Toxin-Pfs25 Fusion Proteins as Oral Vaccines.* Appl Environ Microbiol, 2013. **79**(13): p. 3917-3925.
13. Tran, M., et al., *Production of unique immunotoxin cancer therapeutics in algal chloroplasts.* P Natl Acad Sci USA, 2013. **110**(1): p. E15–E22.
14. Radzun, K.A., et al., *Automated nutrient screening system enables high-throughput optimisation of microalgae production conditions.* Biotechnol Biofuels, 2015. **8**: p. 65.
15. Bui, T.V.L., et al., *Impact of Procedural Steps and Cryopreservation Agents in the Cryopreservation of Chlorophyte Microalgae.* Plos One, 2013. **8**(11).
16. Wolf, J., et al., *Integrated medium design and microbial screening for high biomass production.* Algal Research, submitted.

17. Anderson, R.A., *Algal Culturing Techniques*. 2005: ACADEMIC PRESS.
18. Metting, F.B., *Biodiversity and application of microalgae.* Journal of Industrial Microbiology and Biotechnology, 1996. **17**(5): p. 477-489.
19. *Research on microalgae* Retrieved 2009-05-18; Available from: http://www.algae.wur.nl/uk/.
20. Gray, M.W., B.F. Lang, and G. Burger, *Mitochondria of protists.* Annual Review of Genetics, 2004. **38**: p. 477-524.
21. Sheehan J, D.T., Benemann J, Roessler P, *A look back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae. Close-Out report.* National Renewable Energy Lab, Department of Energy, Golden, Colorado, U.S.A., 1998. **Report number NREL/TP-580-24 190**.
22. Leliaert, F., et al., *Phylogeny and Molecular Evolution of the Green Algae.* Critical Reviews in Plant Sciences, 2012. **31**(1): p. 1-46.
23. Carmichael, W.W., *Cyanobacteria Secondary Metabolites - The Cyanotoxins.* Journal of Applied Bacteriology, 1992. **72**(6): p. 445-459.
24. Castenholz, R.W., *Species Usage, Concept, and Evolution in the Cyanobacteria (Blue-Green Algae).* Journal of Phycology, 1992. **28**(6): p. 737-745.
25. Graham, L.E. and L.W. Wilcox, *Algae*. 2000: Prentice-Hall, Inc.
26. Chisti, Y., *Biodiesel from microalgae.* Biotechnology Advances, 2007. **25**(3): p. 294-306.
27. Dick, M.W., *Fungi, flagella and phylogeny.* Mycological Research, 1997. **101**(4): p. 385-394.
28. Kugrens, P., R.E. Lee, and R.A. Andersen, *Ultrastructural Variations in Cryptomonad Flagella.* Journal of Phycology, 1987. **23**(4): p. 511-518.
29. Hoek, C.V.D., D.G. Mann, and H.M. Jahns, *Algae: An Introduction to Phycology*. 1995: Cambridge University Press.
30. Hegemann, P., *Vision in microalgae.* Planta, 1997. **203**(3): p. 265-274.
31. Ogawa, T. and S. Aiba, *Bioenergetic Analysis of Mixotrophic Growth in Chlorella-Vulgaris and Scenedesmus-Acutus.* Biotechnology and Bioengineering, 1981. **23**(5): p. 1121-1132.
32. Marquez, F.J., et al., *Growth-Characteristics of Spirulina-Platensis in Mixotrophic and Heterotrophic Conditions.* Journal of Fermentation and Bioengineering, 1993. **76**(5): p. 408-410.
33. Zhang, X.W., F. Chen, and M.R. Johns, *Kinetic models for heterotrophic growth of Chlamydomonas reinhardtii in batch and fed-batch cultures.* Process Biochemistry, 1999. **35**(3-4): p. 385-389.

34. Kobayashi, M., et al., *Growth and Astaxanthin Formation of Haematococcus-Pluvialis in Heterotrophic and Mixotrophic Conditions.* Journal of Fermentation and Bioengineering, 1992. **74**(1): p. 17-20.
35. Wood, B.J.B., et al. *Photoheterotrophy in the production of phytoplankton organisms*. 1998. Noordwijkerhout, Netherlands: Elsevier Science Bv.
36. Mata, T.M., A.A. Martins, and N.S. Caetano, *Microalgae for biodiesel production and other applications: A review.* Renewable & Sustainable Energy Reviews, 2010. **14**(1): p. 217-232.
37. Katarzyna Chojnacka and F.-J. Marquez-Rocha, *Kinetic. Stoichiometric relationships of the energy and carbon metabolism in the culture of microalgae.* Biotechnology 2004. **3**(1): p. 21-43.
38. Rosenberg, J.N., et al., *A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution.* Current Opinion in Biotechnology, 2008. **19**(5): p. 430-436.
39. Zimmermann, J., et al., *Automated Sanger Dideoxy Sequencing Reaction Protocol.* Febs Letters, 1988. **233**(2): p. 432-436.
40. Buchheim, M.A., J.A. Buchheim, and R.L. Chapman, *Phylogeny of Chloromonas (Chlorophyceae): A study of 18S ribosomal RNA gene sequences.* Journal of Phycology, 1997. **33**(2): p. 286-293.
41. Maidak, B., et al., *The RDP (Ribosomal Database Project).* Nucl. Acids Res., 1997. **25**(1): p. 109-111.
42. Potter, D., et al., *Convergent evolution masks extensive biodiversity among marine coccoid picoplankton.* Biodiversity and Conservation, 1997. **6**(1): p. 99-107.
43. Manhart, J.R., et al., *Molecular-Data and Species Concepts in the Algae.* Journal of Phycology, 1992. **28**(6): p. 730-737.
44. Goff, L.J., et al., *Tracing Species Invasion in Codium, a Siphonous Green-Alga, using Molecular Tools.* American Journal of Botany, 1992. **79**(11): p. 1279-1285.
45. Gao, T., et al., *Identification of medicinal plants in the family Fabaceae using a potential DNA barcode ITS2.* Journal of Ethnopharmacology, 2010. **130**(1): p. 116-121.
46. Yao, H., et al., *Use of ITS2 Region as the Universal DNA Barcode for Plants and Animals.* Plos One, 2010. **5**(10): p. 9.
47. Coleman, A.W., *ITS2 is a double-edged tool for eukaryote evolutionary comparisons.* Trends in Genetics, 2003. **19**(7): p. 370-375.
48. Schultz, J., et al., *The internal transcribed spacer 2 database - a web server for (not only) low level phylogenetic analyses.* Nucleic Acids Research, 2006. **34**: p. W704-W707.
49. Chase, M.W., et al., *A proposal for a standardised protocol to barcode all land plants.* Taxon, 2007. **56**(2): p. 295-299.

50. Han, J.P., et al., *The Short ITS2 Sequence Serves as an Efficient Taxonomic Sequence Tag in Comparison with the Full-Length ITS.* Biomed Research International, 2013: p. 7.
51. Raue, H.A., J. Klootwijk, and W. Musters, *EVOLUTIONARY CONSERVATION OF STRUCTURE AND FUNCTION OF HIGH MOLECULAR-WEIGHT RIBOSOMAL-RNA.* Progress in Biophysics & Molecular Biology, 1988. **51**(2): p. 77-129.
52. Keller, A., et al., *5.8S-28S rRNA interaction and HMM-based ITS2 annotation.* Gene, 2009. **430**(1-2): p. 50-57.
53. Eddy, S.R., *Profile hidden Markov models.* Bioinformatics, 1998. **14**(9): p. 755-763.
54. Buchheim, M.A., et al., *Internal Transcribed Spacer 2 (nu ITS2 rRNA) Sequence-Structure Phylogenetics: Towards an Automated Reconstruction of the Green Algal Tree of Life.* Plos One, 2011. **6**(2).
55. Moniz, M.B.J. and I. Kaczmarska, *Barcoding of Diatoms: Nuclear Encoded ITS Revisited.* Protist, 2010. **161**(1): p. 7-34.
56. Singh, A., P.S. Nigam, and J.D. Murphy, *Mechanism and challenges in commercialisation of algal biofuels.* Bioresource Technology, 2011. **102**(1): p. 26-34.
57. Griffiths, M. and S. Harrison, *Lipid productivity as a key characteristic for choosing algal species for biodiesel production.* Journal of Applied Phycology, 2009. **21**(5): p. 493-507.
58. Schenk, P.M., et al., *Second generation biofuels: high-efficiency microalgae for biodiesel production.* BioEnergy Research, 2008. **1**(1): p. 20-43.
59. Borowitzka, M.A., *Algal Biotechnology Products and Processes - Matching Science and Economics.* Journal of Applied Phycology, 1992. **4**(3): p. 267-279.
60. Borowitzka, M.A., *Microalgae for aquaculture: Opportunities and constraints.* Journal of Applied Phycology, 1997. **9**(5): p. 393-401.
61. Beer, L.L., et al., *Engineering algae for biohydrogen and biofuel production.* Current Opinion in Biotechnology, 2009. **20**(3): p. 264-271.
62. Wu, L. and R.G. Birch, *Doubled sugar content in sugarcane plants modified to produce a sucrose isomer.* Plant Biotechnology Journal, 2007. **5**(1): p. 109-117.
63. Mussgnug, J.H., et al., *Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion.* Plant Biotechnology Journal, 2007. **5**(6): p. 802-814.
64. Beckmann, J., et al., *Improvement of light to biomass conversion by de-regulation of light-harvesting protein translation in Chlamydomonas reinhardtii.* Journal of Biotechnology, 2009. **142**(1): p. 70-77.
65. Beijerinck, B.W., *Culturversuche mit Zoochlorellen, Lichenengonidien und anderen niederen Algen.* Bot. Zeitung, 1890. **48**: p. 725-39, 741-54, 757-68, 781-85.

66. Miquel, P., *De la culture artificielle des Diatomees.* C. R. Acad. Sci. Paris, 1892. **114**: p. 780-2.
67. Shapiro, H.M., *Practical Flow Cytometry*. 4th ed. 2003: Wiley Liss, New York.
68. Shen, Y., et al., *Microalgae Mass Production Methods.* Transactions of the Asabe, 2009. **52**(4): p. 1275-1287.
69. Ben-Amotz, A., *Biofuel and CO_2 capture by algae*, in *Presented at the 2008 Microalgae Biomass Summit*. 2008: Seattle, Wash.: Algal Biomass Organisation.
70. Lee, Y.K., *Microalgal mass culture systems and methods: Their limitation and potential.* Journal of Applied Phycology, 2001. **13**(4): p. 307-315.
71. Walker, T.L., et al., *Microalgae as bioreactors.* Plant Cell Reports, 2005. **24**(11): p. 629-641.
72. Pulz and O. Pulz, *Photobioreactors: production systems for phototrophic microorganisms.* Applied Microbiology and Biotechnology, 2001. **57**(3): p. 287-293.
73. Carlozzi, P., *Dilution of solar radiation through ldquoculturerdquo lamination in photobioreactor rows facing south-north: A way to improve the efficiency of light utilization by cyanobacteria.* Biotechnology and Bioengineering, 2003. **81**(3): p. 305-315.
74. Hu, Q., H. Guterman, and A. Richmond, *A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs.* Biotechnology and Bioengineering, 1996. **51**(1): p. 51-60.
75. Moheimani, N.R., *The culture of Coccolithophorid algae from carbon dioxide bioremediation.* PhD diss., 2005.
76. Rodolfi, L., et al., *Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor.* Biotechnology and Bioengineering, 2009. **102**(1): p. 100-112.
77. Hu, Q. and M. Sommerfeld, *Photobioreactor: System and Process*, in *Presented at the 2008 Microalgae Biomass Summit*. 2008: Seattle, Wash.: Algal Biomass Organisation.
78. Dimitrov, K., *GreenFuel Technologies: A case study for industrial photosynthetic energy capture.* Available at: http://www.nanostring.net/Algae/CaseStudy.pdf 2007.
79. Fernández F.G, A., J.M. Fernández-Sevilla, and E.M. Grima, *Challenges in microalgae biofuels.* New Biotechnology, 2009. **25**: p. S268-S268.
80. Posten, C., *Design principles of photo-bioreactors for cultivation of microalgae.* Engineering in Life Sciences, 2009. **9**(3): p. 165-177.
81. Neori, A., et al., *Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modem mariculture.* Aquaculture, 2004. **231**(1-4): p. 361-391.

82. Corliss, J.O., *Protozoan Taxonomy and Systematics*, in *eLS*. 2001, John Wiley & Sons, Ltd.
83. Cavalier-Smith, T., *Deep phylogeny, ancestral groups and the four ages of life.* Philosophical Transactions of the Royal Society B-Biological Sciences, 2010. **365**(1537): p. 111-132.
84. Margulis, L., J.O. Corliss, and M. Melkonian, *Handbook of protoctista: The structure, cultivation, habitats, and life histories of the eukaryotic microorganisms and their descendants exclusive of animals, plants, and fungi : a guide to the algae, ciliates, foraminifera, sporozoa, water molds, slime molds, and the other protoctists*. 1990: Jones & Bartlett Pub; illustrated edition edition.
85. Arndt, H., et al., *Functional diversity of heterotrophic flagellates in aquatic ecosystems.* Flagellates: Unity, Diversity and Evolution, 2000. **59**: p. 240-268.
86. Bonkowski, M., *Protozoa and plant growth: the microbial loop in soil revisited.* New Phytologist, 2004. **162**(3): p. 617-631.
87. Azam, F., et al., *The Ecological Role of Water-Column Microbes in the Sea.* Marine Ecology Progress Series, 1983. **10**(3): p. 257-263.
88. Arndt, H. and J. Mathes, *Large heterotrophic flagellates form a significant part of protozooplankton biomass in lakes and rivers.* Ophelia, 1991. **33**(3): p. 225-234.
89. Sherr, E.B. and B.F. Sherr, *Bacterivory and Herbivory - Key roles of phagotrophic protists in pelagic food webs.* Microbial Ecology, 1994. **28**(2): p. 223-235.
90. Scherwass, A., Y. Fischer, and H. Arndt, *Detritus as a potential food source for protozoans: utilization of fine particulate plant detritus by a heterotrophic flagellate, Chilomonas paramecium, and a ciliate, Tetrahymena pyriformis.* Aquatic Ecology, 2005. **39**(4): p. 439-445.
91. Sherr, E.B., *Direct use of high molecular-weight polysaccharide by heterotrophic flagellates.* Nature, 1988. **335**(6188): p. 348-351.
92. Christoffersen, K., et al., *Measurement of bacterivory by heterotrophic nanoflagellates using immunofluorescence labelling of ingested cells.* Aquatic Microbial Ecology, 1997. **13**(1): p. 127-134.
93. Sanders, R.W., *Mixotrophic protists in marine and fresh-water ecosystems.* Journal of Protozoology, 1991. **38**(1): p. 76-81.
94. Boehme, A., U. Risse-Buhl, and K. Kuesel, *Protists with different feeding modes change biofilm morphology.* Fems Microbiology Ecology, 2009. **69**(2): p. 158-169.
95. Taylor, F.J.R., *On Dinoflagellate Evolution.* Biosystems, 1980. **13**(1-2): p. 65-108.
96. Fenchel, T., *Ecology of protozoa: the biology of free-living phagotrophic protists*. Ecology of protozoa: the biology of free-living phagotrophic protists. 1987. x + 197pp.-x + 197pp.

97. Lee, R.E. and P. Kugrens, *Relationship between the Flagellates and the Ciliates.* Microbiological Reviews, 1992. **56**(4): p. 529-542.
98. Jeuck, A. and H. Arndt, *A Short Guide to Common Heterotrophic Flagellates of Freshwater Habitats Based on the Morphology of Living Organisms.* Protist, 2013. **164**(6): p. 842-860.
99. Levine, N.D., et al., *A newly revised Classification of the Protozoa.* Journal of Protozoology, 1980. **27**(1): p. 37-58.
100. Taylor, W.D. and R.W. Sanders, *Chapter 3 - Protozoa*, in *Ecology and Classification of North American Freshwater Invertebrates (Third Edition)*, J.H.T.P. Covich, Editor. 2010, Academic Press: San Diego. p. 49-90.
101. Lynn, D.H., *The Ciliated Protozoa: Characterization, Classification, and Guide to the Literature*. 3rd ed. 2008: Springer Dordrecht.
102. Andrivon, C., *Membrane control of ciliary movement in ciliates.* Biology of the Cell, 1988. **63**(2): p. 133-142.
103. Collins, K., *Chapter 1 - Perspectives on the Ciliated Protozoan Tetrahymena thermophila*, in *Methods in Cell Biology*, C. Kathleen, Editor. 2012, Academic Press. p. 1-7.
104. Cassidy-Hanley, D.M., *Chapter 8 - Tetrahymena in the Laboratory: Strain Resources, Methods for Culture, Maintenance, and Storage*, in *Methods in Cell Biology*, C. Kathleen, Editor. 2012, Academic Press. p. 237-276.
105. Turkewitz, A.P., *Out with a bang! Tetrahymena as a model system to study secretory granule biogenesis.* Traffic, 2004. **5**(2): p. 63-68.
106. Nakajima, T., et al., *Exploitation or cooperation? Evolution of a host (ciliate)-benefiting alga in a long-term experimental microcosm culture.* Biosystems, 2013. **113**(3): p. 127-139.
107. Finlay, B.J., et al., *Some factors controlling the distribution of 2 pond-dwelling ciliates with algal symbionts (frontonia-vernalis and euplotes-daidaleos).* Journal of Protozoology, 1987. **34**(4): p. 349-356.
108. Segers, H., *Annotated checklist of the rotifers (Phylum Rotifera), with notes on nomenclature, taxonomy and distribution.* Zootaxa, 2007(1564): p. 1-104.
109. Wallace, R.L., *Rotifers: Exquisite metazoans.* Integrative and Comparative Biology, 2002. **42**(3): p. 660-667.
110. Ricci, C., L. Vaghi, and M.L. Manzini, *Desiccation of Rotifers (Macrotrachela-Quadricornifera) - Survival and Reproduction.* Ecology, 1987. **68**(5): p. 1488-1494.
111. Wallace, R.L. and T.W. Snell, *Chapter 8 - Rotifera*, in *Ecology and Classification of North American Freshwater Invertebrates (Third Edition)*, J.H.T.P. Covich, Editor. 2010, Academic Press: San Diego. p. 173-235.

112. Shiel, R.J., *A guide to identification of rotifers, cladocerans and copepods from Australian inland waters*. 1995: Co-operative Research Centre for Freshwater Ecology Canberra.
113. Ricci, C., G. Melone, and C. Sotgia, *Old and new data on Seisonidea (Rotifera).* Hydrobiologia, 1993. **255**: p. 495-511.
114. Ricci, C.N., *Ecology of Bdelloids - how to be successful.* Hydrobiologia, 1987. **147**: p. 117-127.
115. Segers, H., *The nomenclature of the Rotifera: annotated checklist of valid familyand genus-group names.* Journal of Natural History, 2002. **36**(6): p. 631-640.
116. Fontaneto, D., et al., *On the reality and recognisability of asexual organisms: morphological analysis of the masticatory apparatus of bdelloid rotifers.* Zoologica Scripta, 2007. **36**(4): p. 361-370.
117. Wallace, R.L., *Coloniality in the Phylum Rotifera.* Hydrobiologia, 1987. **147**: p. 141-155.
118. Segers, H., *Rotifera: Monogononta.* Freshwater Invertebrates of the Malaysian Region, 2004: p. 112–126.
119. Ricci, C., *Anhydrobiotic capabilities of bdelloid rotifers.* Hydrobiologia, 1998. **387**: p. 321-326.
120. Guidetti, R. and K.I. Jonsson, *Long-term anhydrobiotic survival in semi-terrestrial micrometazoans.* Journal of Zoology, 2002. **257**: p. 181-187.
121. Seaman, M.T., et al., *Brachionus-Calyciflorus pallas as agent for the removal of Escherichia-Coli in sewage ponds.* Hydrobiologia, 1986. **135**(1-2): p. 55-60.
122. Fontaneto, D., G. Melone, and R.L. Wallace, *Morphology of Floscularia ringens (Rotifera, Monogononta) from egg to adult.* Invertebrate Biology, 2003. **122**(3): p. 231-240.
123. Wallace, R.L., *Ecology of sessile rotifers.* Hydrobiologia, 1980. **73**(1-3): p. 181-193.
124. Koste, W. and R.J. Shiel, *Rotifera from Australian inland waters. Bdelloidea (Rotifera, Digononta).* Australian Journal of Marine and Freshwater Research, 1986. **37**(6): p. 765-792.
125. Barnes, R.D., *Invertebrate Zoology*. Invertebrate Zoology. 1987. 893pp.-893pp.
126. Krista, G., *Freshwater Invertebrates in Central Europe*. 2014: Springer-Verlag Wien.
127. Walz, N., *Rotifer Populations in Plankton Communities - energetics and life-history strategies.* Experientia, 1995. **51**(5): p. 437-453.
128. Gilbert, J.J. and P.L. Starkweather, *Feeding in Rotifer Brachionus-Calyciflorus .1. Regulatory Mechanisms.* Oecologia, 1977. **28**(2): p. 125-131.
129. Bogdan, K.G. and J.J. Gilbert, *Seasonal patterns of feeding by natural-populations of Keratella, Polyarthra, and Bosmina - clearance rates, selectivities, and contributions to community grazing.* Limnology and Oceanography, 1982. **27**(5): p. 918-934.

130. Makarewicz, J.C., et al., *A decade of predatory control of zooplankton species composition of Lake Michigan.* Journal of Great Lakes Research, 1995. **21**(4): p. 620-640.

131. Aube, C.I., A. Locke, and G.J. Klassen, *Zooplankton communities of a dammed estuary in the Bay of Fundy, Canada.* Hydrobiologia, 2005. **548**: p. 127-139.

132. Sellami, I., et al., *Abundance and biomass of rotifers in relation to the environmental factors in geothermal waters in Southern Tunisia.* Journal of Thermal Biology, 2009. **34**(6): p. 267-275.

133. Yoshida, T., et al., *Top-down control of population dynamics of the dominant rotifers in two mesotrophic lakes in Hokkaido, Japan.* Archiv Fur Hydrobiologie, 2000. **148**(4): p. 481-498.

134. Work, K. and M. Gophen, *Environmental variability and the population dynamics of the exotic Daphnia lumholtzi and native zooplankton in Lake Texoma, USA*. Hydrobiologia, 1999. **405**: p. 11-23.

135. Rothschild, L.J. and R.L. Mancinelli, *Life in extreme environments.* Nature, 2001. **409**(6823): p. 1092-1101.

136. Park, G.S. and H.G. Marshall, *Estuarine relationships between zooplankton community structure and trophic gradients.* Journal of Plankton Research, 2000. **22**(1): p. 121-135.

137. Lair, N., *Grazing and assimilation rates of natural-populations of planktonic Cladocerans in a eutrophic lake.* Hydrobiologia, 1991. **215**(1): p. 51-61.

138. Epp, R.W. and P.W. Winston, *The effects of salinity and pH on the activity and oxygen consumption of Brachionus plicatilis (rotatoria).* Comparative Biochemistry and Physiology Part A: Physiology, 1978. **59**(1): p. 9-12.

139. Minkoff, G., E. Lubzens, and D. Kahan, *Environmental-factors affecting hatching of rotifer (Brachionus-plicatilis) resting eggs.* Hydrobiologia, 1983. **104**(AUG): p. 61-69.

140. Duggan, I.C., J.D. Green, and R.J. Shiel, *Distribution of rotifers in North Island, New Zealand, and their potential use as bioindicators of lake trophic state.* Hydrobiologia, 2001. **446**: p. 155-164.

141. Schluter, M., J. Groeneweg, and C.J. Soeder, *Impact of rotifer grazing on population-dynamics of green microalgae in high-rate ponds.* Water Research, 1987. **21**(10): p. 1293-1297.

142. Van der Stap, I., M. Vos, and W.M. Mooij, *Inducible defenses and rotifer food chain dynamics*. Hydrobiologia, 2007. **593**: p. 103-110.

143. Verschoor, A.M., et al., *Inducible colony formation within the Scenedesmaceae: Adaptive responses to infochemicals from two different herbivore taxa.* Journal of Phycology, 2004. **40**(5): p. 808-814.

144. Yoshida, T., et al., *Rapid evolution drives ecological dynamics in a predator-prey system.* Nature, 2003. **424**(6946): p. 303-306.
145. Yoshida, T., N.G. Hairston, and S.P. Ellner, *Evolutionary trade-off between defence against grazing and competitive ability in a simple unicellular alga, Chlorelia vulgaris.* Proceedings of the Royal Society B-Biological Sciences, 2004. **271**(1551): p. 1947-1953.
146. Rainuzzo, J.R., K.I. Reitan, and Y. Olsen, *The significance of lipids at early stages of marine fish: a review.* Aquaculture, 1997. **155**(1-4): p. 103-115.
147. Lam, M.K. and K.T. Lee, *Immobilization as a feasible method to simplify the separation of microalgae from water for biodiesel production.* Chemical Engineering Journal, 2012. **191**: p. 263-268.
148. Gonzalez, L.E. and Y. Bashan, *Increased growth of the microalga Chlorella vulgaris when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium Azospirillum brasilense.* Applied and Environmental Microbiology, 2000. **66**(4): p. 1527-1531.
149. Grima, E.M., et al., *Recovery of microalgal biomass and metabolites: process options and economics.* Biotechnology Advances, 2003. **20**(7-8): p. 491-515.
150. Greenwell, H.C., et al., *Placing microalgae on the biofuels priority list: a review of the technological challenges.* Journal of the Royal Society Interface, 2010. **7**(46): p. 703-726.
151. Li, Y., et al., *Biofuels from Microalgae.* Biotechnology Progress, 2008. **24**(4): p. 815-820.
152. Borowitzka, M.A. and N.R. Moheimani, *Algae for Biofuels and Energy*. Vol. 5. 2013, Dordrecht: Springer Netherlands.
153. Sawayama, S., T. Minowa, and S.Y. Yokoyama, *Possibility of renewable energy production and CO2 mitigation by thermochemical liquefaction of microalgae.* Biomass & Bioenergy, 1999. **17**(1): p. 33-39.
154. Olaizola, M., *Commercial development of microalgal biotechnology: from the test tube to the marketplace.* Biomolecular Engineering, 2003. **20**(4-6): p. 459-466.
155. Brennan, L. and P. Owende, *Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products.* Renewable and Sustainable Energy Reviews, 2010. **14**(2): p. 557-577.
156. Milledge, J. and S. Heaven, *A review of the harvesting of micro-algae for biofuel production.* Reviews in Environmental Science and Bio-Technology, 2013. **12**(2): p. 165-178.
157. Christenson, L. and R. Sims, *Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts.* Biotechnology Advances, 2011. **29**(6): p. 686-702.

158. Uduman, N., et al., *Dewatering of microalgal cultures: A major bottleneck to algae-based fuels.* Journal of Renewable and Sustainable Energy, 2010. **2**(1).
159. Rashid, N., S.U. Rehman, and J.I. Han, *Rapid harvesting of freshwater microalgae using chitosan.* Process Biochemistry, 2013. **48**(7): p. 1107-1110.
160. Papazi, A., P. Makridis, and P. Divanach, *Harvesting Chlorella minutissima using cell coagulants.* Journal of Applied Phycology, 2010. **22**(3): p. 349-355.
161. Harith, Z.T., et al., *Effect of different flocculants on the flocculation performance of microalgae, Chaetoceros calcitrans, cells.* African Journal of Biotechnology, 2009. **8**(21): p. 5971-5978.
162. Vandamme, D., et al., *Flocculation of microalgae using cationic starch.* Journal of Applied Phycology, 2010. **22**(4): p. 525-530.
163. Ras, M., et al., *Experimental study on a coupled process of production and anaerobic digestion of Chlorella vulgaris.* Bioresource technology, 2011. **102**(1): p. 200-6.
164. Schlesinger, A., et al., *Inexpensive non-toxic flocculation of microalgae contradicts theories; overcoming a major hurdle to bulk algal production.* Biotechnology Advances, 2012. **30**(5): p. 1023-1030.
165. Vandamme, D., et al., *Evaluation of Electro-Coagulation-Flocculation for Harvesting Marine and Freshwater Microalgae.* Biotechnology and Bioengineering, 2011. **108**(10): p. 2320-2329.
166. Salim, S., et al., *Harvesting of microalgae by bio-flocculation.* Journal of Applied Phycology, 2011. **23**(5): p. 849-855.
167. Park, J.B.K., R.J. Craggs, and A.N. Shilton, *Recycling algae to improve species control and harvest efficiency from a high rate algal pond.* Water Research, 2011. **45**(20): p. 6637-6649.
168. Gutzeit, G., et al., *Bioflocculent algal-bacterial biomass improves low-cost wastewater treatment.* Water Science and Technology, 2005. **52**(12): p. 9-18.
169. Lee, A.K., D.M. Lewis, and P.J. Ashman, *Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel.* Journal of Applied Phycology, 2009. **21**(5): p. 559-567.
170. Gonzalez-Fernandez, C. and M. Ballesteros, *Microalgae autoflocculation: an alternative to high-energy consuming harvesting methods.* Journal of Applied Phycology, 2013. **25**(4): p. 991-999.
171. Young, K.D., *The selective value of bacterial shape.* Microbiology and Molecular Biology Reviews, 2006. **70**(3): p. 660-+.

Chapter 2

The development of any successful microalgal production system is heavily dependent upon the selection of the best microalgae strains and production conditions for the product of choice. Microalgal strain selections are therefore an important resource.

Sources of microalgae include existing collections, but there are advantages to searching for new organisms because many more strains of microalgae exist than have been identified and classified in the literature to date.

The identification of local strains with suitable characteristics for mass production which are often more competitive than their counterparts from established culture collections is recognised as an important strategy for obtaining cost-effective algal products. In addition, local species are often adapted to local pathogens and conditions.
This chapter describes the development of a high throughput pipeline for the rapid isolation and purification of a wide range of Australian wild type strains, their subsequent maintaining by cryopreservation, taxonomic identification and the characterisation of top biomass production candidates for further scale up.

The work is presented in the word format that it was published in the *Journal of Petroleum & Environmental Biotechnology*, 2013.

Surveying a Diverse Pool of Microalgae as a Bioresource for Future Biotechnological Applications

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Abstract

Resource limitation is an escalating concern given human expansion and development. Algae are increasingly recognised as a promising bioresource and the range of cultivated species and their products is expanding. Compared to terrestrial crops, microalgae are very biodiverse and offer considerable versatility for a range of biotechnological applications including the production of animal feeds, fuels, high value products and waste-water treatment. Despite their versatility and capacity for high biomass productivity on non-arable land, attempts to harness microalgae for commercial benefit have been limited. This is in large part due to capital costs and energy inputs remaining high, the necessity of identifying 'suitable' land with proximal resource and infrastructure availability and the need for process and strain optimisation. Microalgae represent a relatively unexplored bioresource both for native and engineered strains. Success in this area requires (1) appropriate methods to source and isolate microalgae strains, (2) efficient maintenance of motherstocks, (3) rapid strain characterisation and correct matching of strains to applications, (4) ensuring productive and stable cultivation at scale, and (5) ongoing strain development (breeding, adaptation and engineering). This article illustrates a survey and isolation of over 150 local microalgae strains as a bioresource for ongoing strain development and biotechnological applications.

Keywords: Microalgae, Bioresource, Motherstocks

2.1 Introduction

The global population is projected to increase from its current level of ~7 billion up to ~9 billion by 2050 [1]. This, together with unprecedented levels of lifestyle change in developing countries and policies designed to alleviate poverty (though global effect on addressing hunger appears to have recently stalled [2]), is by 2050 forecast to result in the requirement of ~70% more food [3] and ~50% more fuel [4], as well as ~50% more fresh water [5] and an increasing amount of chemical feedstocks. To supply these resources while simultaneously reducing global CO_2 emissions requires a transition away from fossil fuels, and towards renewable systems. The scale of this challenge should not be underestimated, given the urgent need for a very significant CO2 emission reduction in this decade if we are to stay within the so called 'safe limit' (2°C) defined by the Intergovernmental Panel on Climate Change [6]. This is an ambitious target given recent claims that 80% of remaining fossil fuels must be left in the ground to prevent progressing past this threshold [7].

Fuel, food and water resources are all inextricably connected within our production-consumption cycles. For example, high levels of fertiliser use and water desalination are already required to support our existing population and will likely have to increase to provide food and water security. This in turn requires increased fuel consumption. More efficient means for utilising biological systems as sustainable bioresources to produce food, fuel chemical feedstocks and high value products are becoming increasingly important as consuming ancient fossil fuels becomes more controversial, and the necessity of CO_2 emission reductions becomes more widely represented in global policy.

Microalgae production systems are positioned at the nexus of these challenges as many species have high efficiencies relative to conventional crops in terms of using solar energy to drive the conversion of CO_2 to biomass (stored chemical energy). This biomass can subsequently be used to produce a broad range of downstream products. It has been widely stated that microalgae have the advantage that they can be produced on a proportion of non-arable land (non- arable land is ~25% of global surface area vs. ~3% arable land area [8,9]) and in many cases can use saline and waste water streams. This theoretically opens up the opportunity to extend global photosynthetic capacity beyond arable lands and assist with a transition from the current *food vs fuel* position [10,11] to a more sustainable 'food and fuel' future. However the simplicity of the concept has not progressed to commercial reality despite a significant international research effort. This is primarily due to the many interconnected challenges of optimising biology and engineering parameters for high

efficiency production and integrating these into commercially viable systems. Newly emerging strategies for high efficiency microalgae production [12,13] may contribute significantly to a food and fuel future but they are not the panacea that some have promoted. Opposing opinions that microalgal production systems lack the appropriate production strains suitable to overcome the challenges of economic and environmental sustainability for competitively priced biofuel production may be valid at the present time, but such arguments are insubstantial given the early stage of technology maturity, the rapid ongoing development in the field currently, and the large microalgae biodiversity (~350,00 species) and advanced genetic engineering techniques that can be tapped for strain optimisation [14-16]. Exploiting such a large biological resource is clearly an advantage but also presents a considerable undertaking, and high-throughput processes for strain isolation and maintenance are certainly required to increase the efficiency of traditionally laborious methods. This article describes the establishment of native Australian microalgae collections in terms of bioresource potential, and summarises the purification and cryopreservation protocols developed to efficiently isolate over 150 native strains from a range of water sources for ongoing strain development in a broad range of applications.

Founding a microalgae strain library

International microalgae collections such as the Culture Collection of Algae and Protozoa (CCAP), Culture Collection of Algae at Göttingen University (SAG) and the University of Texas (UTEX) algae collection already offer a valuable resource for the provision of microalgae reference, research and breeding stocks. However international strain collections have their limitations and would benefit from augmentation with complementary local native strain collections which can offer a number of advantages. First, indigenous species are less likely to trigger local quarantine regulations (e.g. some imported strains are considered invasive 'weed' species or contain compounds undesirable for introduction into natural ecosystems). Second, indigenous species are generally more adapted to local climate conditions (e.g. light and temperature) and local biology (e.g. competitors and predators). Third, if correctly maintained and preserved (e.g. cryopreserved) wild type collections of indigenous species can be prevented from adapting to laboratory conditions (i.e. low selection pressures) which over time can result in a loss of culture robustness and suitability for large scale outdoor mass cultivation. Fourth, many strain collections are encumbered with intellectual property restrictions which specific local strain collections can avoid, although governments, national parks and private land owners can exert certain rights over commercially interesting strains isolated in such owned areas. The establishment of a phenotypically broad collection of local strains provides both a motherstock suited for further strain development and optimisation, and an improved understanding of competitor species that can

invade aspiring monocultures of local or imported species. The aim of this article is to assist others with the establishment of similar local collections.

2.2 Methods

Capturing a broad range of phenotypic diversity from natural water sources requires collection from a broad range of environmental conditions. In this study, saline and fresh water sources, as well as photoautotrophic and mixotrophic environments were sampled. Sampling from extreme environmental conditions is possible and can reveal extremophile species which continue to yield significant potential. 'Moderate' extremophiles like *Dunaliella* or some *Tetraselmis* strains (growing in hyper saline ponds) or *Arthrospira* (growing in alkaline ponds) are relatively easy to cultivate using these methods, but 'extreme' extremophiles generally require more advanced facilities (e.g. 60°C cultivation systems) that are not discussed here.

Isolation of strains from water samples is indelibly influenced by the isolation process design, and furthermore both passive analytical screens (e.g. productivity and compositional monitoring) and active biological response screens (e.g. selection pressure applied through cultivation) can be used to guide the strain selection processes and the subsequent development of databases of strain characteristics. A flow diagram of the strategies used for microalgae isolation is shown in Figure 2.1.

In the strategy presented here the collection of crude water samples was followed by microscopic analysis (Figure 2.1 Native water samples) and subsequent incubation of the sampled species both in 'sterile source water' (to maintain species diversity) and in 'nutrient enriched water' samples supplemented with artificial medium for selection of the most adaptable species (Figure 2.1 Pretreatment). Following incubation several isolation techniques were employed including micromanipulation (Figure 2.1 Microman.), fluorescence activated cell sorting (Figure 2.1 FACS) and dilution (Figure 2.1 Dilution). Once isolated the method of choice for long-term storage was cryopreservation (Figure 2.1 Cryo) while serial cultivation on agar plates and in liquid media (Figure 2.1 Serial) was used for storage of sensitive strains. These isolates were identified via 18S [17,18] and 16S ribosomal sequencing [19] in conjunction with morphological classification (Figure 2.1 Identification) [20,21]. They were subjected to further screening to improve cultivation conditions and identify species for specific traits of interest (Figure 2.1 Screening) and to evaluate commercial cultivation capacity (Figure 2.1 Scale-up) to assist with strain selection and development for specific biotechnological applications. Each method step is described below.

Water samples

500 mL samples were collected from a broad range of local water sources in the east and south of Australia (Table 2.1). At the location site, samples were taken between the water surface and 10 cm depth. Samples from biofilms on plant and rock surfaces were also obtained. The samples were processed immediately after arriving in the laboratory, however storage time between collection and processing ranged between 20 min to one day, depending on the distance. Filtration through a coarse strainer (1 mm mesh size) was performed as a pre-treatment to remove larger dirt and debris particles but avoiding any algal species alteration.

Microscopic analysis (Nikon Ti-U fitted with a Nikon Digital Sight DS- U2, 5mp colour head; 200x and 400x magnification) was performed prior to further treatment to record microorganism diversity and provide an initial basis for morphological classification (e.g. Figure 2.1 Native water samples).

Pre treatment

Sterile source water cultivation: The 'sterile source water' strategy was used to maintain maximum biodiversity. Although original water samples were non-sterile, the source water was sterilised (0.2 µm Supor® Membrane Syringe Filter, Acrodise® 32 mm, Pall Life Sciences) to produce a natural water supply for subculture. Sterile technique was practiced throughout the purification process to preserve initial biodiversity and prevent further contamination. The microalgae were cultivated (100 rpm, C10 Platform Shaker, New Brunswick Scientific; illumination at 10 to 100 µE m^{-2} s^{-1} cool white fluorescent light, relative to cell density) to increase the microalgae concentration.

Nutrient enriched water based cultivation: In this scenario water samples were enriched with nutrients to favour the selection of strains capable of fast nutrient uptake and fast growth. For nutrient enrichment, TP medium (TAP media [22] without acetate) was added to base water at a 1:3 enrichment ratio with subsequent cultivation for 4-7 days. Following initial enrichment and isolation, strains were transitioned to a range of fully artificial media including TP, TP +250 mM NaCl, TP +500 mM NaCl, TP + vitamins (3.9 µM thiamine, 7.5 nM cyanocobalamin, and 0.16 µM biotin, and these same vitamin concentrations were maintained as constant for all vitamins included media in this work, denoted as +V), TAP+V, 3NBBM+V [23], BG11+V for cyanobacteria [23], and DM+V for diatoms [23]. TAPY (TAP + 0.35% yeast extract) was used to encourage growth of contaminating microorganisms to confirm establishment of axenic cultures. Reagents were supplied by Sigma-Aldrich, Chem-Supply and Amresco.

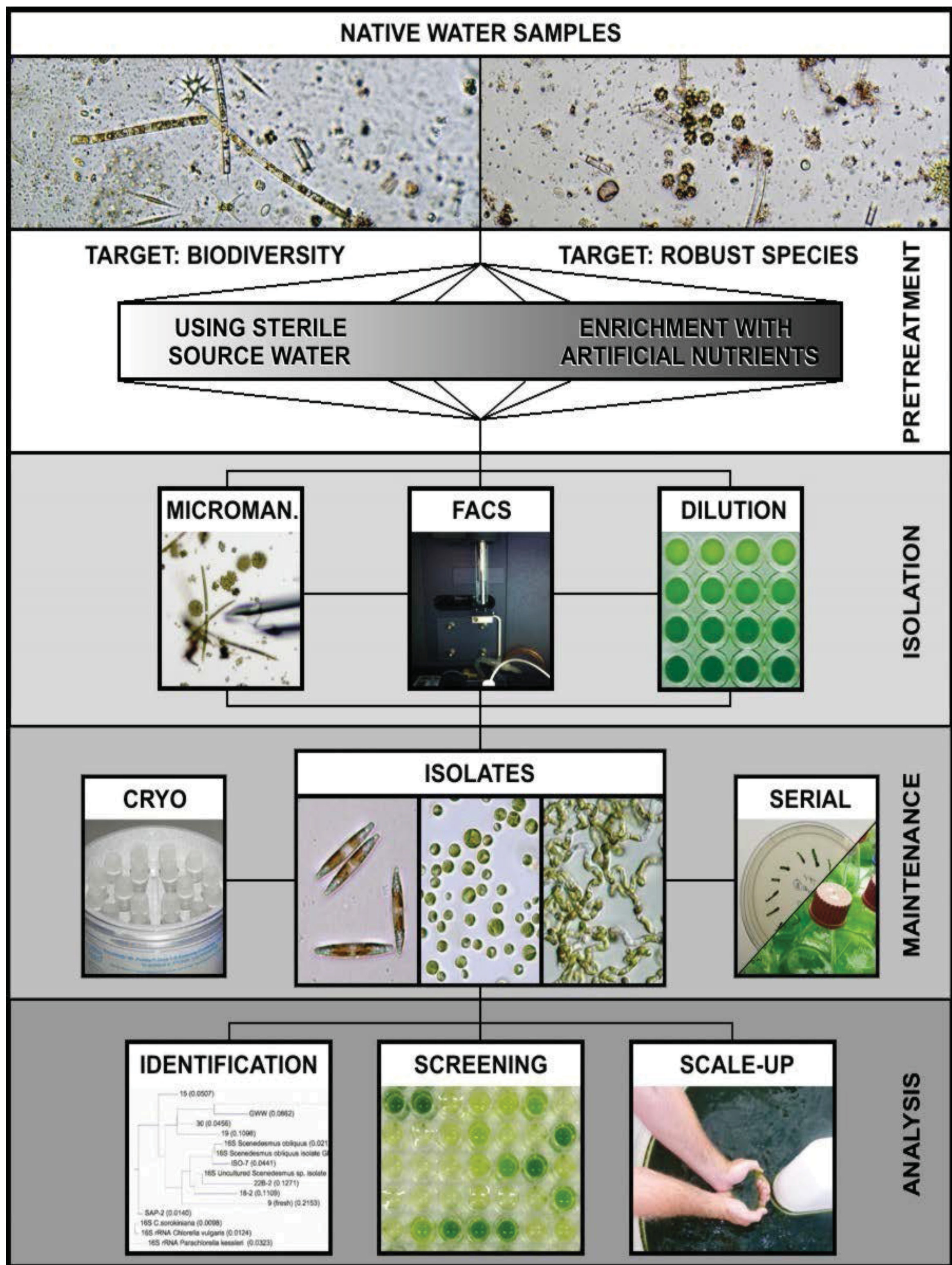

Figure 2.1: Flow chart of the isolation, maintenance and analysis connected to the establishment of a mid-size microalgae strain collection for biotechnological applications

Isolation

Fluorescence activated cell sorting (FACS): FACS offers a rapid isolation technique to purify microalgae from the original sample or from contaminants. FACS has become increasingly popular in freshwater and marine ecology studies [24-26], and for these isolation procedures [27,28] due to the efficacy and high throughput aspects of this process. Success in this approach relies on several factors including the algal cell density and composition of the sample. Dominating species are more likely to be successfully obtained, and therefore the algal diversity of purified cells can be compromised. The size and shape of individual algae cells also has an influence on the success rate of sorting, and the survival rate differs from species to species because of sensitivity to physical stress. Fragile diatoms for example had lower survival rates than chlorophytes. For FACS analysis 5 mL samples of the *sterile source water* and *nutrient enriched water* based cultivations were pre-filtered (40 µm, Nylon Cell strainer, BD Falcon) into a FACS tube and analysed in a BD FACS Aria unit (BD Biosciences). The samples were then probed with a laser to detect individual 'events' corresponding to specific particles (e.g. algae cells or bacteria). The resultant dot plots present individual algae cells as population clusters (Figure 2.2a) which can be analysed in terms of parameters such as forward and side scatter (which represent cell size and granularity). In addition chlorophyll fluorescence was monitored (488 nm excitation wavelength, 695 ± 40 nm transmitting filter) to distinguish between bacteria and dead/stressed algal cells (low fluorescence) and healthy algae cells (high fluorescence). This is achieved through the application of gating thresholds (Figure 2.2a delineated regions) which define different subpopulations based on size and fluorescence (e.g. P1-P6).
In order to maximise species diversity it is important to select cells and discrete regions to avoid oversampling dominant species, a process that is simplified by FACS.

Single or multiple events (e.g. individual or multiple cells) with different cell size and chlorophyll content were sorted into 96 well plates at a gating setting of one or more events per well (Figure 2.2b), containing 150 µL of solid agar media, 150 µL of liquid media, or 150µL solid agar media topped with 50 µL liquid media (media as defined above). After sorting, microalgal growth was monitored via inverted microscope (Figure 2.2c), and success rates were ranked for each strain on the basis of colony formation and contamination status. Using a setting of three sorting events per well into liquid media yielded the highest success rate of single species recovery (>63%). Using a lower events/well setting or sorting on solid agar media resulted in a lower success rate (<10%) and less diverse algae populations constant with [29] who also reported a relationship between sorting success, culture media and culture vessel size of the micro well plate.

Collection sites	Salinity	Collected samples	Isolation technique	Non-axenic isolates	Axenic isolates	Acetate utilising isolates	Isolates lost	Isolates cryopreserved	Identified isolates
Australia, QLD, Brisbane, rain water tank	fresh	2	TAP Enrichment + FACS	12	12	12	0	12	*Chlorella* sp., *Scenedesmus* sp., *Desmodesmus* sp., *Desmodesmus intermedius*
Australia, QLD, Brisbane, Nursery 1	fresh	3	TAP Enrichment + FACS	12	12	10	2	2	*Chlorella* sp., *Chlorella sorokiniana*, *Micractinium pusillum*
Australia, QLD, Brisbane, Nursery 2	brackish/ fresh	3	TAP Enrichment + FACS	18	18	14	4	14	*Chlorella* sp., *Chlorella sorokiniana*, *Micractinium pusillum*, *Scenedesmus* sp.
Australia, QLD, Brisbane, Nursery 3	fresh	1	TAP Enrichment + FACS	6	6	6	1	2	*Chlorella* sp., *Micractinium* sp.
Australia, NSW, rainforest waterfall	fresh	1	TAP Enrichment + FACS, Micro-manipulation	15	15	15	0	6	*Chlorella* sp., *Chlorococcum* sp., *Chlamydomonas* sp., *Desmodesmus* sp.
Australia, QLD, SE Townsville, port, seaside pond	salt	1	Micro-manipulation, FACS	2	2	1	2	0	No confirmed identifications
Australia, QLD, Townsville, river outlet	salt	4	Micro-manipulation, FACS	27	20	18	3	8	*Chlorella* sp., *Chlorella sorokiniana*,
Australia, QLD, NE Townsville, pond	salt	1	Micro-manipulation, FACS	13	4	4	9	0	*Chlorella* sp.
Australia, QLD, Townsville, lake	brackish	2	FACS in Liquid	9	3	5	3	0	*Chlorella* sp., *Micractinium* sp., *Navicula pelliculosa* sp.
Australia, QLD, Gold coast, fish tank	fresh	2	Dilution, FACS in Liquid	18	11	4	3	7	*Stichococcus* sp., *Merismopedia* sp., *Elakatothrix* sp., , *Ankistrodesmus* sp., *Chlorella* sp.
Australia, QLD, UQ, pond	fresh	3	Dilution, Micro-manipulation	9	5	9	0	3	*Chlorella* sp., *Scenedesmus* sp.
Australia, QLD, D'Aguilar, river	fresh	1	Micro-manipulation	7	7	0	1	3	*Ankistrodesmus* sp., *Chlorella* sp., *Scenedesmus abundans*
Australia, QLD, Central coast lake (1)	fresh	1	Micro-manipulation	3	1	0	0	0	*Chamydomonas* sp., *Chlorella* sp.
Australia, QLD, Central coast lake (2)	fresh	1	Micro-manipulation	4	1	0	1	0	*Euglena* sp., *Chlamydomonas* sp., *Chlorella* sp.
Australia, SA, Waikerie, Murray River	fresh	1	Micro-manipulation	5	3	0	1	0	No confirmed identifications
Australia, NSW, Yanga, storm water	fresh	1	Micro-manipulation	4	1	0	1	0	*Anabaena* sp., *Staurastrum* sp., *Coleastrum* sp., *Nannochloris* sp.
Australia, QLD, Goondiwindi, creek	fresh	1	Micro-manipulation	3	0	0	0	0	*Aulacoseira* sp., *Closterium* sp.,

Table 2.1: Statistical analysis of algae isolation success from crude water samples. Collection sites, water characteristics, the number of water samples and strains isolated to increasing levels of purity are provided, together with their ability to utilise acetate, storage characteristics and species identification.

Micromanipulation: Micromanipulation is a laborious but powerful technique which allows the manual targeting of specific cells within a complex mixture. This is useful for sensitive strains and to increase biological diversity. Individual target cells were identified by microscopy (Olympus BX 41, 100x magnification) and extracted with a micromanipulator MM33 (Maerzhauser Wetzlar). Replaceable glass capillaries (Drummond Scientific, length 3.5", outer diameter 1.14 mm, inner diameter 0.53 mm) were used to select and transfer the cell into either *sterile source water* or *nutrient enriched water* as defined above. Individual cells were directly selected from these water samples (Figure 2.3). An alternative strategy involved spreading the microalgae sample (50µl) onto agar and selecting cells after they had settled [29].

Dilution technique: Dilution either in liquid or solid media can be used as an alternative technique to resolve and purify individual algae strains. Achieving effective dilution on solid media [30] involves streaking of a small volume of the original sample onto agar plates (TP or original sterile water source media) with an inoculation loop in a three- or four phase streaking pattern. Plates were then incubated (conditions as above) until colonies appeared (some originating from a single isolated cell) which could then be manipulated individually. Re-streaking was repeated until pure cell colonies were observed. In parallel, liquid serial dilution was performed using 96 well plates.

Enriched as well as untreated water samples were serially diluted (4:1) through 48 wells filled with 500 µl of the appropriate medium. Samples were incubated under low light conditions (~50 µE m^{-2} s^{-1} cool white fluorescent light) and examined daily (Nikon Ti-U inverted microscope).

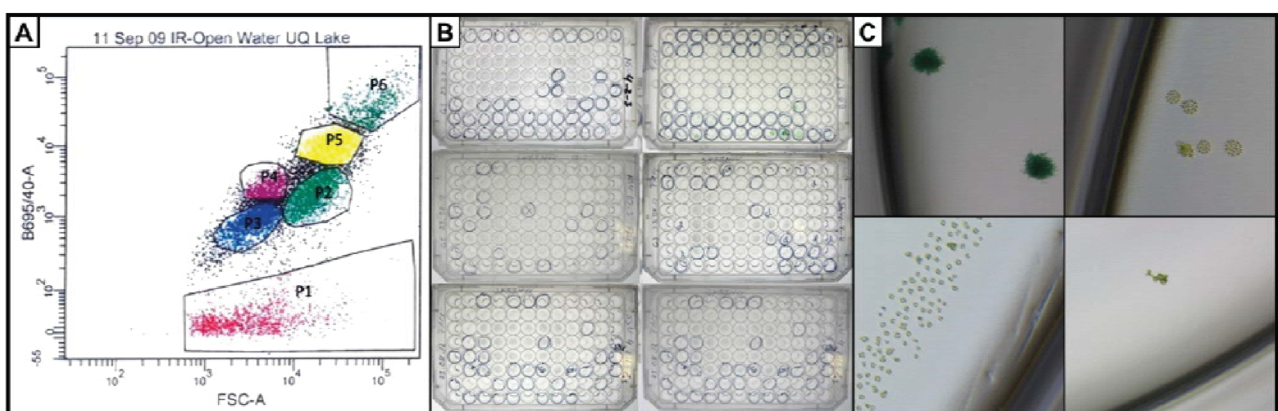

Figure 2.2: FACS isolation process. (A) FACS dot plot based upon laser excitation (y-axis) and forward scatter (x-axis) of cell mixtures which facilitates population analysis and selection of defined regions (P1-P6) for sorting; (B) 96 well plates containing purified algae; (C) microalgal isolate growth in individual wells monitored at 100x magnification.

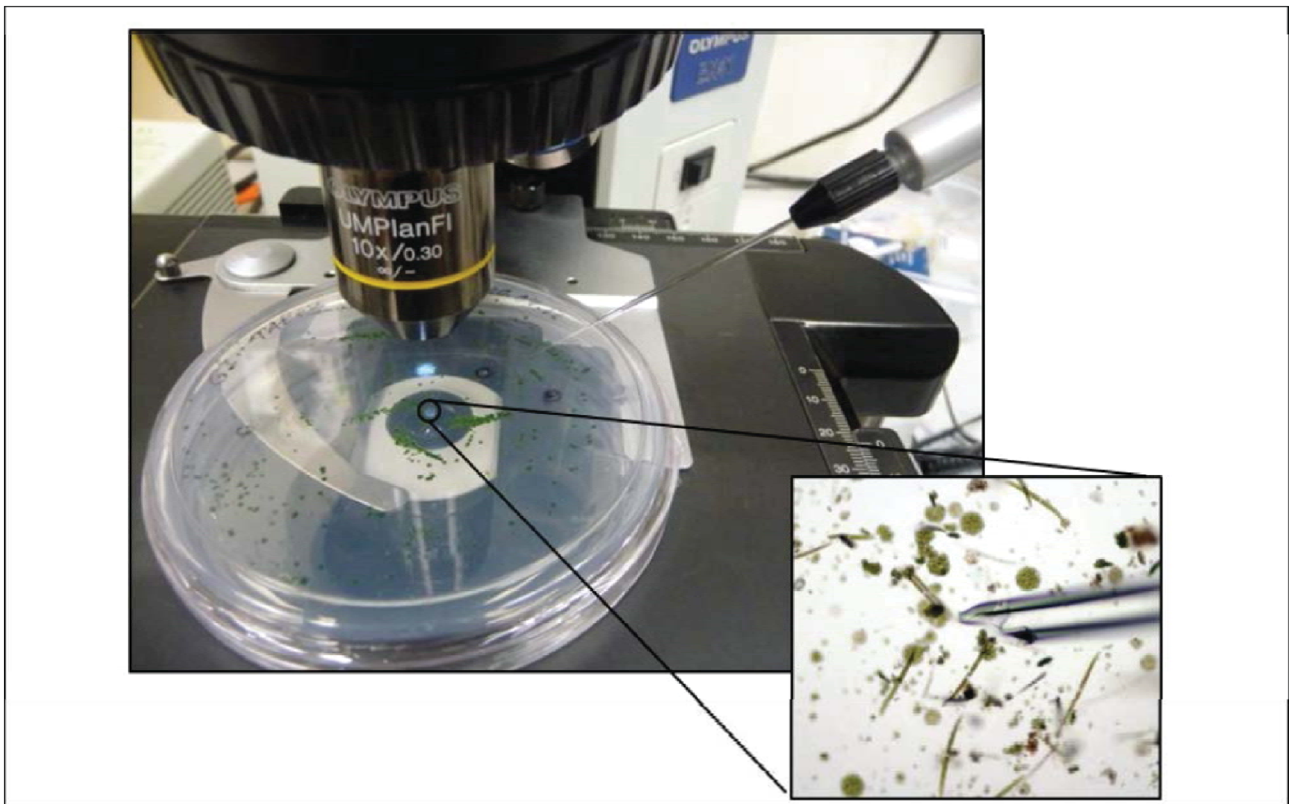

Figure 2.3: Micromanipulation setup. Cells are imaged on agar plates. The insert shows individual cells at the tip of the needle used for aspiration (needle ID 0.53 mm)

Maintenance

Enrichment and maintenance of established isolates: Established isolates were enriched further with artificial media and incubated in larger volumes (10 mL) to increase cell number and concentration. For some microalgal isolates a stepwise increase of the concentration of artificial medium was found to be beneficial and was applied, with growth monitored microscopically and by optical density (OD_{750}) measurements. For long term storage triplicate samples of each isolate were cryopreserved using 3-5 x 10^6 cells per cryo-vial using a refined two-step freezing protocol developed for microalgae [31]. The final volume (1 mL containing 6.5 % DMSO and 0.2 M sucrose (*Sigma-Aldrich*, *Chem-Supply*)) was stored at -80°C for at least 4 hours before being transferred to -196°C for long-term storage in liquid N_2 vapour phase. Strains that could not be efficiently cryopreserved were maintained through serial cultivation using both liquid and solid media.

Analysis

Screening: Screening for desirable properties is an ongoing process that can be repeated once a microalgal collection has been established. The isolates can be re-screened for a variety of

applications, and where breeding is not possible, rapidly advancing methods for engineering microalgae can enable further advancement. The screening characteristics used here are therefore illustrative only. The principles, however are universal – very specific screens are usually time consuming so early rapid screening for indicative traits can be utilised first, followed by specific screening on a smaller subset of parameters. The isolates obtained in this work were initially screened on the basis of biomass productivity, and have already been subjected to a rigorous set of secondary screens and this work will be reported in the near future.

Identification: Only a subset of ~20% of strains, which performed well in early screens, were selected for full identification (though this is clearly flexible). Identification consisted of morphological investigation (Olympus BX42 and Nikon Ti-U, 200x and 400x magnification) [20,21] and molecular classification by rDNA analysis. For the latter, DNA was isolated according to [32] though a 10 min sonication step was required to break open the cell walls of numerous wild type strains. Both18S and 16S ribosomal DNA analysis was performed. The amplification of 18S rDNA and its sequencing was outsourced to the Australian Genome Research Facility (AGRF). The analysis of 16S rDNA was performed in house using two 'universal' primers [19] that specifically target cyanobacteria and eukaryotic photosynthetic plastids. PCR amplicons were sequenced at AGRF. Sequences were aligned using nucleotide BLAST (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the 'nucleotide collection (nr/nt)' database.

2.3 Results and Discussion

To establish this subset of our local microalgae strain library, water samples were sourced from 17 locations which included rainforest water sources, creeks, ponds and rivers, brackish/saline seaside ponds and river outlets, as well as artificial systems such as rainwater tanks, plant nurseries and fish tanks (Table 2.1). Clearly the biodiversity recovered from any biodiscovery program is dependent upon the biodiversity of the original water samples. The number of species recovered from a given water sample may be related to the trophic status of the source (i.e. oligo-, meso- or eutrophic) [33]. It has been previously reported [34] that oligotrophic conditions can have a higher level of species biodiversity for algae and while eutrophic water sources may contain more algae, species biodiversity is often lower as fewer species tend to dominate. Our observations supported this, and isolation processes yielded more isolates from eutrophic water sources. To prevent the isolation of multiple isolates of the same strain, no more than one species was taken from each water sample unless it was morphologically distinct. It is possible, however, that the same species was isolated from different water samples taken across SE Qld.

In this study the 17 locations sampled, initially yielded a total of 167 non-axenic isolates. Of these ~95% were Chlorophytes (e.g. *Chlorella*, *Chlorococcum*, *Scenedesmus* and *Chlamydomonas*), ~4% cyanobacteria (e.g. *Anabaena* and *Merismopedia*) and diatoms (e.g. *Navicula*), and ~1% were unidentified cell types. 104 strains were recovered using the sterile source water approach and 63 were obtained using the nutrient enrichment water method. Although source and treatment specific, the fact that the sterile source water approach generally yielded a greater biodiversity suggests that it may be the better standard method for the establishment of bio-diverse local microalgae culture collections. For the isolation of fast growing strains higher levels of nutrient enrichment were beneficial.

120 of the initial non-axenic strain samples were recovered using FACS, 42 using micromanipulation, and 5 by dilution. This clearly shows the benefit of using FACS as a platform for developing local microalgae strain collections as it could, in our case, sort and dispense single cells into ~500 individual plate wells per hour, a variable percentage of which can grow into axenic populations. Along with flow cytometry approaches, isolation of more fragile strains using micro manipulation gives a biodiverse population of isolated strains from a given environmental sample. Using a combination of FACS and micromanipulation yielded an average of approximately 10 ±7 strains per water sample. Using a FACS setting of 3 sorting events instead of 1 per well resulted in only slightly higher bacterial contamination levels, but increased the success rates of recovering algal cell isolates. Despite this it was noted that the survival rate of sorted algae cells rose when 3 events per well were used and so this is suggested as a sensible starting point for FACS purification. It was also noted that the use of 96 well plates instead of 384 well plates improved species recovery, with 150 µL solid agar media topped with 50 µL liquid media being the preferred media configuration.

Of the 167 non-axenic isolates, 121 were purified to the axenic level. This was confirmed by microscopic investigation of cultures supplemented with acetate and yeast extract as a carbon source to encourage heterotrophic growth and demonstrating the absence of contamination. Antibiotic treatment in some cases was able to assist with the production of axenic cell lines, but in many cases proved toxic to the algae themselves and so was of limited utility. Overall approximately 90% of the 121 axenic strains were purified from bacteria simply by using FACS or through repeated subcultivation on carbon-free agar media. The remaining 46 non-axenic isolates could not be successfully purified from contaminating bacteria. This may indicate the presence of either strong adhesion of the bacteria to the algae cells or the presence of endogenous bacteria. The observation that most of the non-axenic algae cultures visibly exhibited a white

biofilm around the cells, suggests that the former was predominantly the case. Furthermore certain species having complex shapes (e.g. constricted symmetrical arrangements, spiral twisted, colonial or filamentous) such as the Chlorophyte *Staurastrum* proved more difficult to purify from bacterial contamination. Whether these strong interactions between the bacteria and algae are simply physical or represent a form of symbioses remains to be established, however it is commonly noted in our open pond trials and by others that in healthy and relatively stable raceway pond systems many bacteria and algae can coexist effectively. Indeed one benefit to their presence may be that the bacteria use the dissolved oxygen in the culture produced through the photosynthetic reactions of microalgae. The importance of this is that dissolved oxygen levels become increasingly inhibitory to algae photosynthetic processes. A further benefit of bacterial interactions might be the synthesis of essential vitamins required by certain algae (e.g. Vitamin B12 [35]), as well as some other beneficial compounds [36].

Of the 121 axenic cultures 57 were successfully cryopreserved using the method of Bui et al (47% success rate) [31]. Strains having a diameter of 3 to 50 μm were effectively recovered although some of the very large strains proved difficult. While acceptable this step of the process would clearly benefit from improvement. Critical parameters include the optimisation of light level as high light can result in oxidative damage, as well as in the optimisation of nutrient conditions for specific strains.

Of the 64 strains that could not be cryopreserved 24 were lost during serial subcultivation. One reason for this is that the standard media used may not be sufficiently specific to the needs of individual species. Ongoing research is therefore required to optimise media composition.

Initial species identification was based on morphological classification but was refined through ribosomal sequencing, which was carried out primarily to obtain an approximate idea of the algal genus, and to differentiate different strains from each other (i.e. that they truly represented different species or at least different strains). The aim of this work was not to undertake a complete phylogenetic description of the isolates, which is a task that awaits further study.

Ribosomal sequence analysis can be based on 18S rDNA and 23S rDNA analysis (derived from the nuclei of eukaryotes), or on 16S rDNA analysis (derived from chloroplasts and mitochondria) present in both eukaryotic microalgae and prokaryotic cyanobacteria. In this study 18S rDNA analysis was used as the primary rDNA analysis method but was supplemented with 16S rDNA analysis, contributing to the expansion of this resource. The 18S rDNA sequencing approach has

the advantage that corresponding databases (e.g. NCBI) are more advanced than those for 16S rDNA, making it possible to achieve a higher quality of strain identification. Furthermore the 18S rDNA approach can currently enable identification to the species level in many cases. In practice our analysis typically yielded sequence identities of >95% but less than 100%, suggesting that while closely related to some strains in the online database, many of these wild isolates have not been previously catalogued.

Exact matches occurred at low frequency and in some cases two or more hits with a similar identity greater than 95% were noted. Theoretically the combined use of 18S and 16S rDNA sequence analysis may facilitate improved identification and could also resolve the origin of specific plastids within a given species, contributing not only to species identification but the evolutionary relationships between specific nuclear and plastid genomes.
The rDNA sequences of a selection of strains which were intended for further study (chapter 3) is given in table 2.2. Because these sequences were not added to the public database, the corresponding author and supervisor Prof Ben Hankamer (b.hankamer@imb.uq.edu.au) can be contacted for any strain inquiry.

Strain	rDNA Sequence (forward)
Chlorella sp. (20_G10)	CCAGATTAGCCTGCATGTCTAAGTATAAACTGCTTTATACTGTGAAAC TGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTACT ACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCC GACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCTC TGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTC GTGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGG TAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGG TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGA AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTG ACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGAATG AGTACAATCTAAACCCCTTAACGAGGATCAATTGGACGAAGATGTCT GCTGGGAAAAAA
Chlorella sorokiniana (15_E4)	TTAGATTAGCATGCATGTCTAAGTATAAACTGCTTTATACTGTGAAAC TGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTACT ACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCC GACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCTC TGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTT GCGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGG TAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGG TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGA AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTG ACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGAATG AGTACAATCTAAACCCCTTAACGAGGATCAATTGGAGAGAAAAGTCC TGGTATAATA

Chlorella sorokiniana (12_A9)	TAAGATTAGCCTGCATGTCTAAGTATAAACTGCTTTATACTGTGAAAC TGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTACT ACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCC GACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCTC TGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTT GCGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGG TAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGG TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGA AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTG ACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGAATG AGTACAATCTAAACCCCTTAACGAGGATCAATTGGAGGGCAAGTCTG GTAAAA
Chlorella sp. (11_H5)	ATAGATTAGCATGCATGTCTAAGTATAAACTGCTTTATACTGTGAAAC TGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTACT ACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCC GACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCTC TGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTT GTGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGG TAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGG TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGA AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTG ACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGAATG AGTACAATCTAAACCCCTTAACGAGGATCAATTGGAGGGAGAAATTC TGGGTAATATT
Chlorella sp. (10_B9)	GGGCTAGATGTACTCGTTCTTTTACCTTACCTGATAAGGCCCAGTATT GTTATTTATTGTCACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGC GCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCACGCTCCCTCTCC GGAATCGAACCCTAATCCTCCGTCACCCGTTACCACCATGGTAGGCCT CTATCCTACCATCGAAAGTTGATAGGGCAGAAATTTGAATGAAACAT CGCCGGCACAAGGCCATGCGATTCGTGAAGTTATCATGATTCACCGC GAGTCGGGCAGAGCCCGGTCGGCCTTTTATCTAATAAATACGTCCCTT CCAGAAGTCGGGATTTACGCACGTATTAGCTCTAGATTTACTACGGGT ATCCGAGTAGTAGGTACCATCAAATAAACTATAACTGATTTAATGAG CCATTCGCAGTTTCACAGTATAAAGCAGTTTATACTTAGACATGCATG GCTTAATCTTTGAGACAAGCATATGACTACTGGCAGGATCAACCAGT AGAGACACGCTGAGTCGGAGACACGCAGGGATGAGATGGGCTGGAA AAAAATC
Chlorococcum sp. (12_02)	ATTTGATGGTACCTCCTACTTGGATAACCGTAGGAAATCTAGAGCTAA TACATGCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATAAA AGGCCAGCCGGGCTTGCCCGACCTTAGGCGAATCATGATAACTTCAC GAATCGCATGGCCTTGTGCCGGCGATGTTTCATTCAAATTTCTGCCCT ATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGT GACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGG CTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCG ACACGGGGAGGTAGTGACAATAAATAACAATACTGGGCATTTATGTC TGGTAATTGGAATGAGTACAATGTAAATATCTTAACGAGTATCCATTG GAGGGCAAGTCTGGTAAAA

Chlorella sorokiniana (8_C4)	GCCAGATTCGCATGCATGTCTAAGTATAAACTGCTTTATACTGTGAAA CTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTAC TACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCC GACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCTC TGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTC GCGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGG TAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGG TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGA AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTG ACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGAATG AGTACAATCTAAACCCCTTAACGAGGATCAATTGGAGGGCAAAGTGA CTGGTAAAAAA
Chlorella sorokiniana (7_B6)	CCTAGATTAGCATGCATGTCTAAGTATAAACTGCTTTATACTGTGAAA CTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACCTAC TACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAATCCC GACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCTC TGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTT GCGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGG TAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGG TTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGA AGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTG ACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGAATG AGTACAATCTAAACCCCTTAACGAGGATCAATTGGACGAAGATAACA TCTGTTGTAAAA
Micractinium pusillum (5_H4)	GGTCCAGATTAGCCATGCATGTCTAAGTATAAACAGCTTTATACTGTG AAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTACC TACTACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGCGTAAAT CCCGACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGG CTCTGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGC CTCGTGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGAT GGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAG GGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAG GAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTA GTGACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGA ATGAGTACAATCTAAACCCCTTAACGAGGATCATTCGACGTTTTTTTA AAAAAAAATA
Scenedesmus sp. (1_C4)	GTAGATTAGCATGCATGTCTAAGTATAAACTGCTTATACTGTGAAACT GCGAATGGCTCATTAAATCAGTTATAGTTTATTTGGTGGTACCTTCTT ACTCGGAATAACCGTAAGAAAATTAGAGCTAATACGTGCGTAAATCC CGACTTCTGGAAGGGACGTATATATTAGATAAAAGGCCGACCGGGCT CTGCCCGACCCGCGGTGAATCATGATATCTTCACGAAGCGCATGGCCT TGCGCCGGCGCTGTTCCATTCAAATTTCTGCCCTATCAACTTTCGATG GTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGG GTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGG AAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACGGGGAGGTAGT GACAATAAATAACAATACCGGGCATTTCATGTCTGGTAATTGGAATG AGTACAATCTAAATCCCTTAACGAGGATCCATTGGACGAGTTTTCGGT AAAATCTC

Table 2.2: Molecular classification of the 10 top biomass production candidates. The table shows the result of the 18S ribosomal DNA analysis which was aligned against the 'nucleotide collection (nr/nt)' database and the strain with the closest match identified.

2.4 Conclusion

In this paper we have demonstrated a streamlined process for microalgae recovery from a broad range of water sources and used this to conduct a mid-scale survey of species native to Australian waters. Typically the water sources collected yielded ~10 strains of microalgae per sample, of which approximately half could be effectively cryopreserved to minimise maintenance costs and genetic drift, with most of the remainder being amenable to traditional subculture. Through the use of rDNA sequence analysis and morphological examination the resultant isolates were identified, either to the genus or species level providing a solid basis to assist the international research community with the establishment of multiple local strains collections to maximise microalgae species recovery as a breeding stock for cell lines beneficial for a wide range of biotechnological applications including the production of food, fuel, chemical feedstocks, high value products and for applications for wastewater treatment and bioremediation. Although there are already large international algae collections, the benefit of local strains collections include the establishment of robust, well adapted and locally derived breeding stocks that are often without the IP encumbrance associated with commercial strain collections. These can be used for the development of improved cell lines for a wide range of biotechnological applications. At a time when the global population is expanding from ~7 to ~9 billion people by 2050 and food, fuel and water demands are predicted to increase by 70%, 50% and 30% respectively the importance of establishing such diverse stocks becomes apparent. The ongoing exploration of the diversity of microalgal biology is already yielding advances in high performance wild types with commercial potential and genetic characteristics that could enable improvements for engineered strains. Initial screens focused on biomass productivity as a primary criteria (being a critical economic driver for commercialisation) but ongoing strain development will require further screens for a range of other useful characteristics including oil composition and profile, predator resilience, flocculation and other traits that enhance harvestability (e.g. floatation or sedimentation), and capacity for wastewater systems and bioremediation.

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2.5 References

1. Population Reference Bureau (2009) World Population Data Sheet.
2. Food and Agriculture Organization of the United Nations (2012) Economic growth is necessary but not sufficient to accelerate reduction of hunger and malnutrition. The State of Food Insecurity in the World.
3. Food and Agriculture Organization of the United Nations (2009) How to Feed the World in 2050.
4. International Energy Agency (2010) World Energy Outlook 2010
5. OCED (2012) OECD Environmental Outlook to 2050: The Consequences of Inaction.
6. Stocker TF (2013) The Closing Door of Climate Targets. Science 339: 280-282.
7. Climate Commission (2013) The Critical Decade 2013: Climate Change Science, Risks and Responses.
8. Food and Agriculture Organization of the United Nations (2010) FAO Statistical Yearbook 2010.
9. Central Intelligence Agency (2013) The World Factbook.
10. Pimentel D, Marklein A, Toth MA, Karpoff MN, Paul GS, et al. (2009) Food Versus Biofuels: Environmental and Economic Costs. Hum Ecol 37: 1-12.
11. Gomiero T, Paoletti MG, Pimentel D (2010) Biofuels: Efficiency, Ethics, and Limits to Human Appropriation of Ecosystem Services. J Agric Environ Ethics 23: 403-434.
12. Vasudevan V, Stratton RW, Pearlson MN, Jersey GR, Beyene AG (2012) Environmental Performance of Algal Biofuel Technology Options. Environ Sci Technol 46: 2451-2459.
13. Stephens E, Ross IL, Hankamer B (2013) Expanding the microalgal industry-- continuing controversy or compelling case? Curr Opin Chem Biol 17: 444-452.
14. Norton TA, Melkonian M, Andersen RA (1996) Algal biodiversity. Phycologia 35: 308-326.
15. Larkum AW, Ross IL, Kruse O, Hankamer B (2012) Selection, breeding and engineering of microalgae for bioenergy and biofuel production. Trends Biotechnol 30: 198-205.
16. Georgianna DR, Mayfield SP (2012) Exploiting diversity and synthetic biology for the production of algal biofuels. Nature 488: 329-335.
17. Huss VAR, Frank C, Hartmann EC, Hirmer M, Kloboucek A, et al. (1999) Biochemical Taxonomy and Molecular Phylogeny of the Genus Chlorella Sensu Lato (Chlorophyta). J Phycol 35: 587-598.

18. de-Bashan LE, Trejo A, Huss VA, Hernandez JP, Bashan Y (2008) Chlorella sorokiniana UTEX 2805, a heat and intense, sunlight-tolerant microalga with potential for removing ammonium from wastewater. Bioresour Technol 99: 4980-4989.
19. Stiller JW, McClanahan (2005) Phyto-specific 16S rDNA PCR primers for recovering algal and plant sequences from mixed samples. Molecular Ecology Notes 5: 1-3.
20. Entwisle TJ, Sonneman JA, Lewis SH (1997) Freshwater Algae in Australia A Guide to Conspicuous Genera. Sainty & Associates Australia.
21. Hallegraeff GM, Bolch CJS, Hill DRA, Jameson I, Leroi JM, et al. (2010) Algae of Australia: Phytoplankton of Temperate Coastal Waters. ABRS, Canberra & CSIRO Publishing Melbourne, Canberra, Melbourne 432.
22. Gorman DS, Levine RP (1965) Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi. Proc Natl Acad Sci USA 54: 1665-1669.
23. CCAP (2012) Culture Collection of Algae and Protozoa.
24. Reckermann M (2000) Flow sorting in aquatic ecology. Sci Mar 64: 235-246.
25. Cellamare M, Rolland A, Jacquet S (2010) Flow cytometry sorting of freshwater phytoplankton. J Appl Phycol 22: 87-100.
26. Crosbie ND, Teubner K, Weisse T (2003) Flow-cytometric mapping provides novel insights into the seasonal and vertical distributions of freshwater autotrophic picoplankton. Aquat Microb Ecol 33: 53-66.
27. Andersen RA (2005) Algal Culturing Techniques. Academic Press London UK.
28. Pienkos, PT (2008) Update on Algal Biofuel Research at NREL. 2nd Algae Biomass Summit. Seattle, WA, USA.
29. Sieracki M, Poulton N, Crosbie, N (2005) Automated Isolation Techniques for Microalgae, in Algal Culturing Techniques, Anderson RA Elsevier Academic Press London UK 101-116.
30. Anderson RA, Kawachi M (2005) Traditional Microalgae Isolation Techniques. Algal Culturing Techniques Anderson RA Elsevier Academic Press London 83-100.
31. Bui TVL, Ross IL, Jakob G, Hankammer B (2013) Impact of Procedural Steps and Cryopreservation Agents in the Cryopreservation of Chlorophyte Microalgae. Plos One, 8(11).
32. Oey M, (2009) Chloroplasts as bioreactors: high-yield production of active bacteriolytic protein antibiotics. Potsdam, Univ., Dissertation.

33. Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, et al. (2008) Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. BioEnergy Research 1: 20-43.
34. Rawson DS (1956) Algal Indicators of Trophic Lake Types. Limnol Oceanogr 1: 18-25.
35. Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438: 90-93.
36. Gonzalez LE, Bashan Y (2000) Increased Growth of the Microalga Chlorella vulgaris when Coimmobilized and Cocultured in Alginate Beads with the Plant- Growth-Promoting Bacterium *Azospirillum brasilense*. Appl Environ Microbiol 66: 1527-1531.

Chapter 3

Pilot scale cultivation of microalgae mono and polycultures

3.1 Introduction

The previous chapter described the isolation and characterisation of over 150 microalgae strains. In parallel studies these were subjected to extensive nutrient optimisation trials which were designed to identify the best production conditions for each strain and identify those strains yielding the highest growth rates [1]. Although mainly Chlorophytes, the isolated species encompass a range of cell size, morphology and presumably, metabolic diversity. All strains in this collection can be used freely in outdoor bioreactor cultures in Australia, free of quarantine restrictions, while the presence of the nutrient dataset enables a choice of different nutrient regimes to be studied. Initial experiments were focussed primarily on growth rate. Based on the laboratory data, the top 8 strains in terms of growth rate were selected for further evaluation regarding their suitability for outdoor culture under pilot scale conditions. The focus of work presented in this chapter was to test their performance in high rate ponds (HRPs) and to identify key parameters affecting their production. This is important as under operational pilot scale conditions algae are exposed to a different range of stresses relative to lab conditions, including fluctuations in temperature, pH, nutrient supply, predation and cross contamination.

The experiments described here examine very few characteristics of importance to large scale algal culture. The SBRC facility should enable, in the future, examination of many other variables of interest, including complex polyculture work to assess the impact of specific organisms on the mix of algal species across the seasons. Metagenomic methods would be particularly appropriate as culture complexity increased. As the necessary regulatory controls are put in place, it is expected that genetically modified organisms may provide opportunities for large scale production of natural products and recombinant proteins.

The first set of experiments described here focus on single species trials using the best nutrient conditions identified by Wolf et al [1] for their production. This allows species-specific growth responses to the environmental conditions to be monitored and evaluated under outdoor production conditions. The second set of experiments focus on evaluating the performance of mixed populations of these species (polycultures) and to test the performance of the individual species within them. This approach aims to identify key variables that require optimisation for high performance production

conditions both in HRPs and more advanced flat panel on tubular photobioreactors (see figure 3.2). Furthermore the trials identified potential factors affecting flocculation which is an important strategy with potential for dewatering processes and is discussed further in chapter 4.

3.2 Material and Methods

Choice of algae

Our previously established microalgae strain collection contains over hundred microalgae strains and was recently increased with over 150 native Australian species collected from eastern and southern Australia [2]. The development of an advanced high-throughput screening system [3] [1] enabled the classification of these native strains according to their growth performance and nutrient preference. From this classification, 8 strains exhibiting the highest biomass productivities, based on maximum growth rate (μ_{max}) and morphologic diversity, were chosen for further experiments in medium scale HRP systems (Figure 3.1, a-h).

Indigenous Microalgae strain Pinjarra 001

A local microalgae species indigenous to the Pinjarra Hills site was isolated by nutrifying an HRP system and running it without adding inoculum. Over 2 weeks several microorganisms established themselves in the system and a particular microalgae species was found to be dominating the culture. The alga was isolated and inoculated in a new HRP system and re-nutrified every 3 to 4 weeks. The culture was monitored over one year by weekly optical density measurements (OD_{750} and OD_{680}) as well as pH and conductivity measurements (Figure 3.1, i).

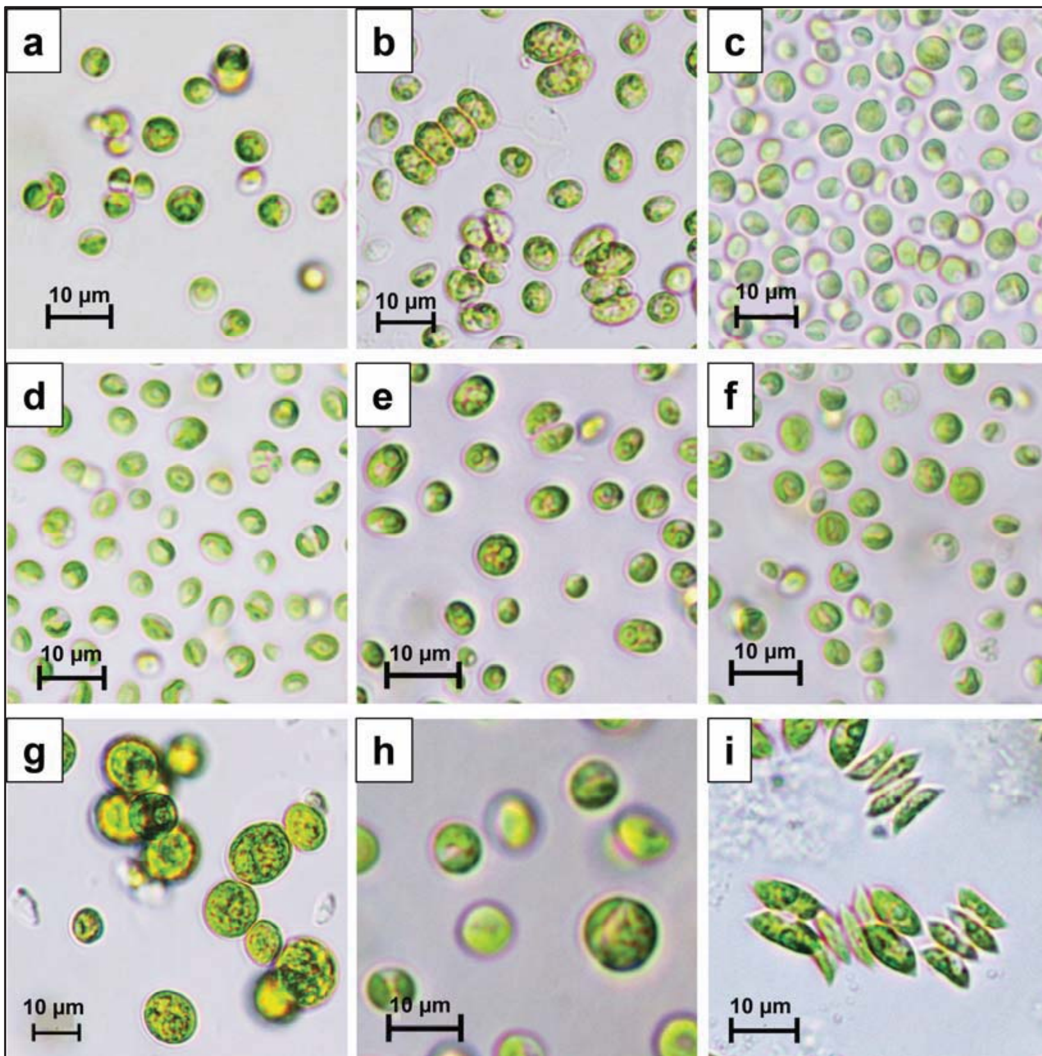

Figure 3.1: Microscopic imaging (x400) of the highest biomass producing strains, *Micractinium pusillum* (5_H4) (a), *Desmodesmus intermedius* (1_C4) (b), *Chlorella pyrenoidosa* (10_B9) (c), Chlorella sp. (11_H5) (d), *Chlorella sorokiniana* (12_A9) (e), *Chlorella sorokiniana* (8_C4) (f), *Chlorococcum sp.* (12_02) (g), *Chlorella sorokiniana* (15_E4) (h) and the indigenous microalgae strain *Scenedesmus sp.* (Pinjarra 001) (i).

Design of Nutrient Media

The individual optimal nutrient requirements as determined by the automated screening [1] were reformulated on the basis of elemental equivalence for the use of agricultural grade nutrients as would be necessary in commercial scale systems. For polyculture experiments, a medium representing a compromise between each of the determined optimum media was determined and urea was used as the nitrogen source (Table 3.1).

Table 3.1: Optimised media compositions for each algal strain used for the monoculture open pond cultivation experiments and the compromised medium for polyculture cultivations.

Fertiliser chemical		*Desmodesmus intermedius* (1_C4)	*Micractinium pusillum* (5_H4)	*Chlorella sorokiniana* (8_C4)	*Chlorella pyrenoidosa* (10_B9)	*Chlorococcum sp.* (12_02)	*Chlorella sorokiniana* (12_A9)	*Chlorella sorokiniana* (15_E4)	*Chlorella sp.* (11_H5)	Compromised media (polyculture)
Formula	MW [g/mol]	c [mM]	c [mM]	c [mM]	c [mM]	c [mM]	c [mM]	c [mM]	c [mM]	c [mM]
$(NH_4)_2SO_4$	132.14		3.3209		5.9995	14.9623	9.1281	0.6282		
CH_4N_2O	60.07	2.8439							3.3397	2.8361
$Ca(NO_3)_2$	164.1	1.0941	1.0941	1.0941	0.5471	0.5471	0.5471	0.5471	0.5471	1.0941
KNO_3	101.11			11.5290	0.0158	0.0788	2.0119		0.0158	0.0158
K_2SO_4	174.26		0.0423	0.0085				1.0802		
KH_2PO_4	136.09	9.9563	9.9563	1.9913	1.9913	9.9563			1.9913	1.9913
$NH_4H_2PO_4$	115.04						2.0280	2.0280		
$MgSO_4$	120.37	1.5838	1.5838	1.5838	3.1675	1.5838	1.5838	1.5838	1.5838	1.5838
$FeSO_4$	151.91	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019
$MnSO_4$	151	0.0303	0.0303	0.0606	0.0303	0.0303	0.0303	0.0303	0.0303	0.0303
$CuSO_4$	159.61	0.0102	0.0102	0.0102	0.0102	0.0102	0.0102	0.0102	0.0102	0.0102
$ZnSO_4$	161.45	0.1415	0.2830	0.1415	0.1415	0.1415	0.1415	0.2830	0.1415	0.1415
$Na_2B_8O_{13}$	340.46	0.0139	0.0278	0.0278	0.0278	0.0278	0.0278	0.0278	0.0278	0.0278
Na_2MoO_4	205.92	6.83E-03	6.83E-03	6.83E-03	6.83E-03	6.83E-03	6.83E-03	6.83E-03	0.0068	0.0068
NaCl	58.44	1.7112	1.7112	1.7112	1.7112	1.7112	1.7112	4.2779	1.7112	1.7112
$CoCl_2\,6H_2O$	237.929	1.66E-03	1.66E-03	1.66E-03	1.66E-03	1.66E-03	1.66E-03	1.66E-03	0.0017	1.66E-03
Na_2SeO_4	172.94	4.18E-05	4.18E-05	4.18E-05	4.18E-05	4.18E-05	4.18E-05	4.18E-05	0.000042	4.18E-05
$VOSO_4\,H_2O$	163	2.30E-06	2.30E-06	2.30E-06	2.30E-06	2.30E-06	2.30E-06	2.30E-06	0.000002	2.30E-06
$Na_2SiO_3\,5H_2O$	212.14	3.60E-02	3.60E-02	3.60E-02	3.60E-02	3.60E-02	3.60E-02	3.60E-02	0.036036	3.60E-02

Medium precipitation

In large scale algal cultivation expensive chemicals like EDTA were greatly minimised relative to the artificial conditions in the automated screening process, to provide a scenario that was as economically realistic as possible. Therefore media precipitation could not be completely avoided, and this has an effect upon nutrient availability (e.g. phosphate) although this is arguably not at a level significant to experimental results.

Pre-cultivation of inoculum in hanging bag systems

Inoculation volumes (up to 20 L), for inoculating the open ponds, were established from motherstock cultures (maintained on agar TAP medium at 120 μE m^{-2} s^{-1}, 23 ºC, sub-cultivated to fresh media on a monthly basis) into liquid TP medium (Tris-Phosphate medium, 25 mL) and grown to approximately 2 L volumes of dense culture (OD_{750} = ~2). The culture was subsequently transferred into gamma sterilised hanging bag culturing systems (Pure Biomass, USA) growing for one to two weeks up to 20 L culture volume, targeting an OD_{750} of up to 3 (constant illumination, 310 mE m^{2} s^{-1}, ~25 ºC using TLD 58W/840, cool white, Philip fluorescent lights, RT, ~0.5 L min^{-1} sterile air sparging). The pH was monitored and regulated to maintain neutrality on a daily basis by addition of filter sterilised NaOH (5 M). 48 hours before inoculation into open pond systems, the hanging bags were transferred to a partially shaded outdoor area (~0.5 L min^{-1} air sparging, pH 7), to acclimatise

cells to outdoor environmental conditions i.e. day and night cycles, fluctuating temperature and sunlight-irradiation.

Pond design and Control Systems

The Solar Biofuels Research Centre (SBRC, www.solarbiofuels.org/sbrc) provides an advanced system testing facilities in a subtropical climate (University of Queensland, Brisbane, Australia) (Figure 3.2) System monitoring, controlling and basic culture analysis was performed in the on-site laboratory.

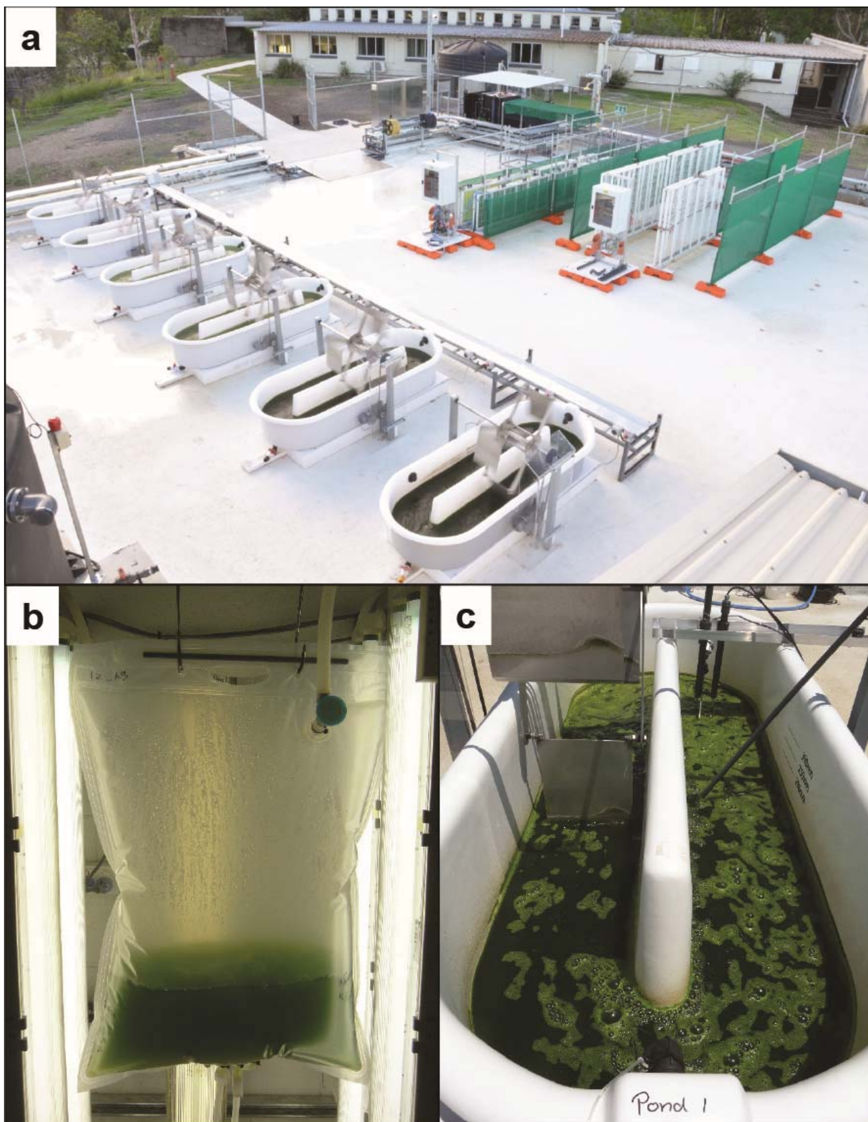

Figure 3.2: SBRC Pilot plant overview with algae bioreactor in the front and the laboratory facility in the back (a), hanging bag pre-cultivation system for algae inoculum (b), open pond cultivation system (c).

High Rate Ponds (HRPs) were designed by Kellogg Brown & Root Pty Ltd Australia (KBR) through consultation with Professor Ben Hankamer and Doctor Evan Stephens. Continuous mixing of the algae cultivation solution was performed mechanically by a non-conventional paddle wheel system (four swinging blades, adjustable frequency), resulting in standardised water flow velocity of ~ 20 cm s^{-1}, culture depth dependent), gas sparger (perforated PVC T-piece, 0.5 mm hole diameter) and micro-bubble diffuser (MBD 600, Point Four Systems Inc., bubble size 100-500 micron). Due to the shape of ponds, a volumetric equation (Equation 3.1) was established to allow pond volume to be simply determined from culture depth (measured at centre point of longitudinal length).

Equation 3.1:
Depth (mm) = (0.4953 × Volume (L)) + 6.052
or
Volume (L) = (Depth (mm) – 6.052) / 0.4953

The HRPs were physically aligned along a north south axis to ensure the maximum solar energy illumination to reactor surface area during the day.

Set up of sensors, probes for pond culture

All HRPs were equipped with sensors as required for online measurements, mounted on the south facing end of the HRPs to minimise shading. Sensors for culture monitoring included temperature (temperature sensor, WKU-361-00DU Electrotherm (Germany), pH (pH sensor POLILYTE Plus ARC 120, Hamilton, Switzerland) and dissolved oxygen (O_2 sensor, VISIFERM DO, Hamilton, Switzerland). The individual values were recorded on a 1-minute timescale.

Weather station

During the time of cultivation a weather station containing a pyranometer (Pyranometer CMP11, Kipp & Zonen, Germany), a solar irradiance sensor (Sunshine sensor BF5, Delta-T Devices Ltd, United Kingdom) and a climatic sensor unit (Clima Sensor US, Adolf Thies GmbH & Co. KG, Germany) was used to monitor and record global radiation (W m^{-2}), PAR total and diffuse (μE m^{-2} s^{-1}), wind speed and direction (m s^{-1}, º), ambient temperature (ºC), humidity (%), atmospheric pressure (hPA) and precipitation (mm h^{-1}).

Control units (CUs) and Software

Two control units and software used for controlling and monitoring the different parameters were designed at the Karlsruhe Institute of Technology (KIT). A process control unit (SIMATIC S7-1200,

Siemens, Germany) provided the interface between all sensors connected to the HRPs, the mass flow controllers and magnetic valves to an external desktop computer. The maximum capacity of a CU was 16 sensors (6 using analogue and 10 using digital data communication). Sensor signals and other process parameters were sent to LabView based bioprocessing software (BioProCon, Karlsruhe Institute of Technology, Germany) in the on-site laboratory for data recording and operator control.

Each control unit was provided with mass flow controllers (1179B and 1579A, MKS Instruments, USA) and a 'set up agent box' equipped with magnetic valves (5 in total, 1 valve per HRP) for the control of pH by the addition of alkali. One mass flow controller for air and two for CO_2 were used for each set of two HRPs (airflow divided between two reactor systems). When opened, a magnetic valve of the 'set up agent box' regulated the inlet of alkali which was connected and supplied via tube from a storage container (2 L, pressurised with approximately 0.5 bar) to the culturing system.

pH control

The pH of the HRPs in these experiments was maintained through the sparging of an air mixture with variable CO_2 concentration for lowering pH as well as liquid ammonium hydroxide (8 % v/v, Industrial Cleaners Pty Ltd) for raising pH (setpoint: pH 7, upper/lower limits: + 0.2/ - 0.5). The pump in the set up agent box was switched on for 10 s per pH unit deviation from the setpoint, before being switched off for 100 s to allow for mixing and culture equilibration. CO_2 was supplied at a working concentration of 1 % CO_2 and 99 % air mixture when pH value was within the operational range and to a maximal concentration of 3%. CO_2 input concentrations were allowed to drop to 0 % when pH levels decreased.

Inoculation

The starting optical density of the inoculated HRP system was targeted to an optical density of OD_{750} = 0.1 (0.25 for polyculture) in a volume of ~ 192 L (100 mm depth). For the highly flocculating culture of *Chlorococcum sp.* (12_02) optical density was difficult to measure, so as dense a culture as practical was targeted.

All chemicals were separately pre-dissolved in RO (reverse osmosis) water prior to addition into larger volumes. Previous to the algae inoculation, the media was mixed in the HRP in approximately 3/4 of the final volume using RO water. The mixing speed was adjusted (35-50 Hz dependent upon culture depth in order to attain a stable mixing velocity), and the sequence of nutrient chemicals added. After approximately 10 minutes of mixing and dissolving, the pH of the medium was adjusted with ammonium hydroxide (ammonium 8% (v/v)) and the aeration with the CO_2/air mixture was

established until a stable value of ~ 7 was achieved. The EDTA chelated iron sulphate mixture which had been previously neutralised was added last. The pond volume was topped up to its final amount after inoculation with the algae broth. Inoculation of the open ponds was performed in the afternoon between ~ 3-5 pm to mitigate photoinhibition during the adaptation period of the highly diluted cultures. The amount of inoculum was calculated from its OD_{750} value of the pre-cultivation and added to the pond to achieve a desired starting OD.

For the polyculture experiment inoculation of an equal cell number of each strain was the aim. Therefore cells per mL were counted in duplicates (heamocotymeter, Mallassez bright line, 0.2 mm depth) for each of the five strains and the cell number per unit volume was calculated. Because of its strong flocculation, cell counting of the strain *Chlorococcum sp.* (12_02) was unreliable and generally low compared to the other species. Therefore the whole inoculum volume available was distributed between the ponds and flasks, on the percentage of the original value needed. The volumes of the strains were pre-mixed before adding into the pond bioreactor systems to ensure time-simultaneous inoculation for all algae species. 2 litres of polyculture from each pond was taken after 10 minutes mixing for indoor flask cultivation (this minimised variability between pond and flask experiments).

Regular Troubleshooting of airflow, pressure, valves and pH tubes

PH setup agent tubes degraded over time due to ammonium usage and resulted in an outage of pH control during the experiment until fixed. BioProCon software occasionally experienced issues with the main server and control unit connection which consequently had an influence on the control of air and CO_2 flow and ammonium hydroxide regulation. Periodic heavy storm weather in the area during the storm season also occurred and these storm events were responsible for unpredictable power outages which had a similar influence on the experiments (especially when occurring during periods when the facility was unattended).

Sampling and Measurements

Water depth level in pond cultures was taken to monitor evaporation and precipitation. The depth level was maintained as constant as possible by topping up with RO water after evaporation, and where culture volume increased (and was consequently diluted) due to rainfall, measurements were recorded based on 'actual' volume and also calculated to a value for a 'standard' volume equivalent. Careful sampling due to cross contamination between ponds (contaminated gloves disposed, hands washed and disinfected before each sampling) was a standard operating procedure. Conductivity was measured manually from the collected samples (Sharp EC WP Waterproof C66). pH of starter

cultures and of the polyculture flask experiment was measured by using a pH probe (MARTINI Instrument pH 55).

Microscopy

Algae culture samples were microscopically observed once daily (Olympus BX41 microscope, 200-400x magnification) and pictures were taken by using an inverse-Microscope (Nikon Ti-U (magnification x200, x400). Visual analysis of the culture gives information about culture behaviour in terms of cell morphology and general health, aggregation, and the presence of contaminants like predators or foreign algae species.

Growth parameter determination

Absorbance (λ_1= 680nm and λ_2= 750nm) of 1 mL algae culture (diluted at higher cell densities up to a 1:20 ratio) were measured in triplicates twice a day for single strain experiments and three times daily in polyculture experiments, using 1 cm path length plastic cuvettes (PG Instruments T60 UV-Visible Spectrophotometer– Software: UV Win).

Dry-weight biomass

The volumetric biomass yield Y_{vol} (g L^{-1}) was determined via triplicate sampling of 5 mL filtered culture (100u mesh size filter, Millpore) for non-flocculating algae, coarse strainer ~ 1 mm mesh size for flocculating algae) into pre-weighed tubes (5 mL Polystyrene Round-Bottom Tube, BD Falcon™) and centrifuged (10 min x 2890 g). Pre-filtration of the samples was done to remove larger non-algae biomass which could lead to falsification of the results. After carefully discarding the supernatant, the pellet was dried in the oven for 3 days at 70 °C (Labec oven ODWF36, LABEC Laboratory Equipment Pty Ltd, Australia) and cooled down to room temperature overnight. Tubes including dry biomass were weighed on a precision balance (Shimadzu AUW220D) to 6 decimal places and dry weight biomass (DW) was determined by subtracting the weight of the empty tube.

Determination of growth rates and productivity

The specific growth rate μ expressed in unit time (t^{-1}) was calculated based on the optical density values (OD_{750}) during the logarithmic state (Equation 3.2) determined for each cultivation individually [4]. The maximum specific growth rate (μ_{max}) represents the maximum specific growth rate over the most productive 24-hour period [4].

Equation 3.2: $$\mu = (\ln OD_{750,n+1} - \ln OD_{750,n}) \times (t_{n-1} - t_n)^{-1}$$

To determine volumetric P_{vol} (g L^{-1} d^{-1}) and areal P_{areal} (g m^{-2} d^{-1}) biomass productivities, the volumetric biomass yields Y_{vol} (g L^{-1}) over the time of the cultivation were used (equation 3.3 and 3.4). The values represent the daily biomass increase of the pond reactor volume or pond reactor illuminated culture surface area (m^2).

Equation 3.3: $P_{vol} = (Y_{vol, t2} - Y_{vol, t1}) \times (t_2 - t_1)^{-1}$

Equation 3.4: $P_{areal} = (Y_{vol, t2} - Y_{vol, t1}) \times (t_2 - t_1)^{-1} \times (A_{illuminated\ area})^{-1}$

3.3 Results

Table 3.2 provides the primary results from both singles species (monoculture) and mixed species (polyculture) cultivation experiments, including productivity values as maximum specific growth rate per hour considering illumination period only (μ_{max} hr^{-1} Day only), maximum specific growth rate considering light and dark periods i.e. daily average (μ_{max} hr^{-1} Day/Night), and maximum specific growth rate per day (μ_{max} day^{-1}), as well as maximum volumetric (P_{vol}) and areal (P_{areal}) productivities. As cultivations were performed as batch experiments, maximum productivity values are the most important values to serve as target values (under similar conditions) for future investigation in semi-continuous production regimes. Most relevant parameters for each cultivation experiment are also listed.

It should be noted that due to sharing of facilities with other staff, that equipment limitations did impact upon data acquisition in some cases. Also, due to site specific issues including power outages and network disruptions, some interruption to both measurement and control capacity was experienced during the course of the project.

To compare the energy input for each cultivation the mean value of global radiation and PAR total was calculated of the first 14 days (~336 hrs) and presented in the table. For the duration of the polyculture trial the data of daily solar exposure was obtained online from the closest available weather station data of Brisbane (www.bom.gov.au).

Additional detail to that presented in table 3.2 is provided in figures 3.3-3.50 which are associated with each of the subsequent sections describing the cultivated strains individually.

Table 3.2: Single species and combined species experimental cultivations. Productivity values and primary production parameters.

Strain Cultivated	Season of Cultivation	Global radiation mean [MJ m^{-2} day^{-1}]	PAR total mean [µE m^{-2} s^{-1}]	Ambient Temp. [°C]	Culture Temp. [°C]	pH	CO_2 input avg. [L min^{-1}]	Conductivity [mS]	pO_2 [%]	μ_{max} [hr^{-1}] Day only	μ_{max} [hr^{-1}] Day/Night	μ_{max} [day^{-1}]	P_{vol} [g m^{-3} d^{-1}]	P_{areal} [g m^{-2} d^{-1}]
Chlorococcum sp. (12_O2)	Autumn (9-28 April)	15.85 ± 5.3	385.94 ± 172.4	13-33	14-31	6.8-7.3	0.078	-	-	0.2367† N/A	0.0739† 0.0315*	1.775† 0.7555*	233.00	21.19
Scenesdesmus sp. (Pinjarra 001)	Autumn (9-29 April)	15.85 ± 5.3	385.94 ± 172.4	13-33	15-30	6.9-7.1	0.238	1.8-2.2	11-150	0.0280	0.0175	0.4209	234.00	21.28
Micractinium pusillum (5_H4)	Autumn (16-30 April)	16.71 ± 3.1	404.84 ± 74.9	12-33	14-29	6.9-7.2§	-	4.01-4.43	68-149	0.0799	0.0400	0.9590	234.00	21.28
Chlorella sp. (11_H5)	Autumn (6-26 May)	14.52 ± 6.0	257.93 ± 73.2	10-28.5	14-25	6.9-7.0	0.214	2.3-2.7	92-120	0.0440	0.02998	0.7196	228.67	20.80
Desmodesmus intermedius (1_C4)	Autumn (9-23 May)	11.62 ± 2.1	279.81 ± 51.7	10-29	13-25	6.9-7.1	0.281	2.34-2.81	68-125	0.0451	0.0228	0.5708	125.60	11.42
Chlorella sorokiniana (8_C4)	Autumn (9-23 May)	11.62 ± 2.1	279.81 ± 51.7	10-29	12-27	6.8-7.2	0.125	1.35-1.71	75-113	0.0678	0.0270	0.6491	239.30	22.63
Chlorella pyrenoidosa (10_B9)	Winter (13-30 June)	11.45 ± 3.6	270.01 ± 81.8	3-26	6.5-24.5	6.9-7.1	0.143	-	-	0.0376	0.0263	0.6315	-	-
Chlorella sorokiniana (15_E4)	Winter (13-30 June)	11.45 ± 3.6	270.01 ± 81.8	3-26	6.5-23.5	7.0-8.0§	0.386	-	-	0.0509	0.0254	0.6102	-	-
Chlorella sorokiniana (12_A9)	Winter (13-30 June)	11.45 ± 3.6	270.01 ± 81.8	3-26	5.5-24	6.9-7.1	0.182	-	-	0.1060†	0.0237	0.5690	-	-
Polyculture Pond 1	Spring (31 Oct-13 Nov)	23.95 ± 8.1	-	13-43	14-35	7.0-8.0§	0.203	0.92-1.18	85-260	0.0722	0.0293	0.7022	266.67	24.25
Polyculture Pond 2	Spring (31 Oct-13 Nov)	23.95 ± 8.1	-	13-43	14-35	7.0-8.0§	0.240	0.86-1.0	75-160	0.0936	0.0310	0.7432	260.00	23.65
Polyculture flasks (indoor)	Spring (31 Oct-13 Nov)	400 µE continuous		24.8-28.5	24.8-28.5	6.9-7.8	N/A	0.93-1.08	-	0.1395†	0.0169	0.4052	320.00	N/A

* specific growth rate values calculated from biomass dry weight due to the effect of auto-flocculation upon OD measurement

§ temporary fault of pH regulation system (see individual graph for more information)

† anomaly values

3.3.3 Single species experiments

Autumn Cultivations:

***Chlorococcum sp.* (12_02)** cultivation data and conditions are illustrated in figure 3.3 A and 3.3 B. The cultivation was inoculated at a starting OD_{750} of 0.1, and the resulting growth curves based upon optical density and dry weight measurements are presented in figure 3.3 A (a-c). After an adaptation period of approximately 18 hours, this species transitioned into a rapid growth stage with a short logarithmic phase before entering the stationary phase at ~90 hours under the conditions tested.

For figure 3.3 A (a-b) both OD-based growth curves have a similar pattern, and the $OD_{750/680}$ ratio was relatively stable which suggests that cellular chlorophyll levels remained relatively constant. Significant OD fluctuation events were detected at 66 and 138 hours as can be seen in figure 3.3 A (a) which impact upon growth results. Given that this strain is generally observed in a largely auto-flocculating state, this affects the reliability in OD values being representative. Additionally, for the dry weight values used to calculate biomass g L^{-1} (Figure 3.3 A, c), error margins were substantial and the high early values are counterintuitive. Consequently the confidence in these values is low. In light of these points, both actual recorded maximum values and values averaged over the logarithmic phase are presented in table 3.2 for μ, but for dry weight values averaged over the logarithmic phased the values were negative.

There was a singular significant rainfall event which occurred around 90 hours (Figure 3.3 B, d) at the end of the log phase. This did have a short effect upon pH which otherwise remained entirely within the range of 6.8-7.3 and predominantly was well maintained within 7.0-7.1 (Figure 3.3 B, c). The predicted pattern of CO_2 input was observed with a maximum of ~ 0.65 L min^{-1} (Figure 3.3, c). No dissolved oxygen and conductivity data are available for this cultivation of *Chlorococcum sp.* (12_02) due to equipment limitations.

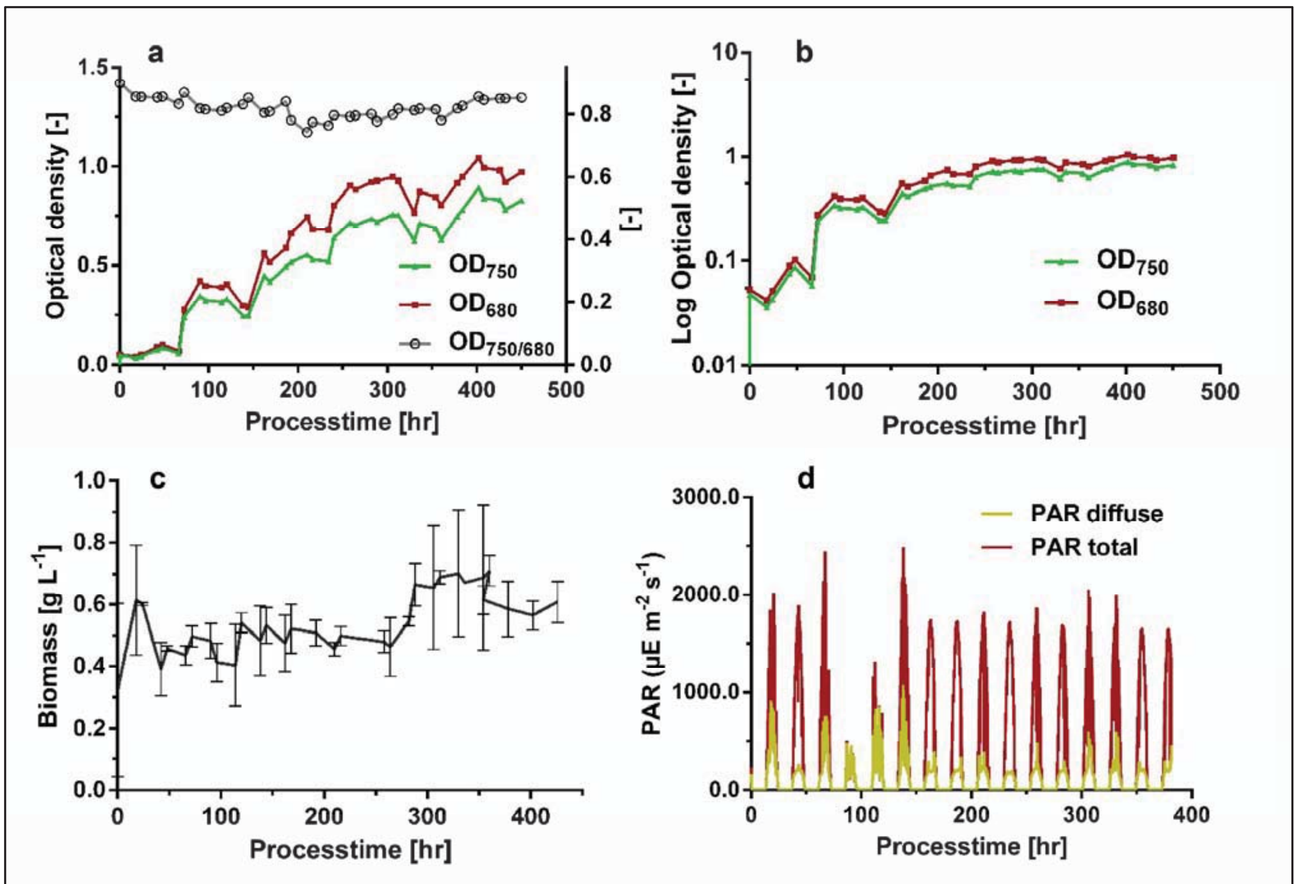

Figure 3.3 A: Culture performance and cultivation conditions of *Chlorococcum sp.* (12_02): display of the growth curve based on optical density a) linear and b) logarithmic. c) growth curve of *Chlorococcum sp.* (12_02) based on biomass density. d) total and diffuse photosynthetically active radiation easured over the time course of the experiment.

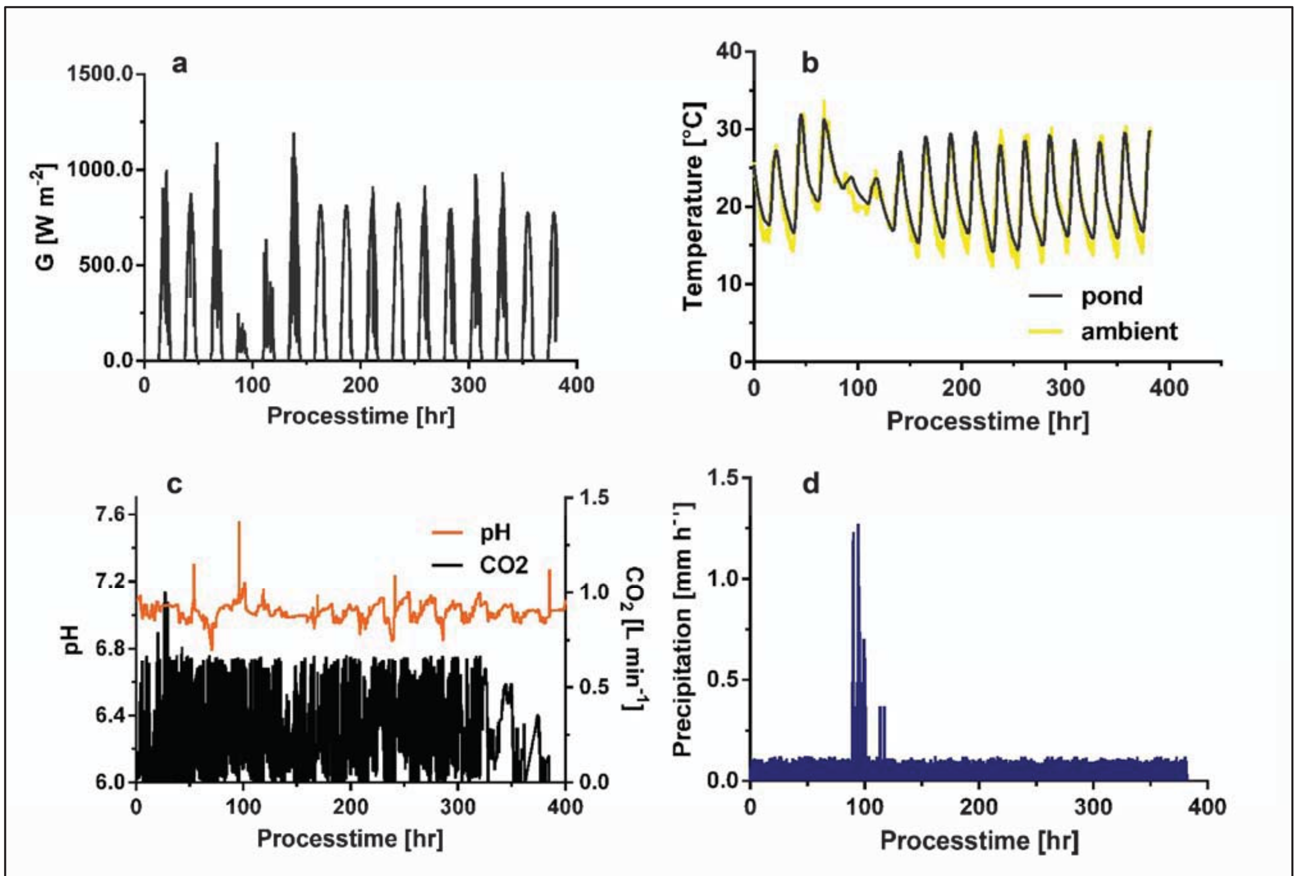

Figure 3.3 B: Culture performance and cultivation conditions of *Chlorococcum sp.* (12_02): display of the a) global solar radiation. b) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle. c) measured pH of the algal culture and dependent CO_2 input. d) precipitation occurrence measured over the time course of the experiment.

Microscopic monitoring during the cultivation of *Chlorococcum sp.* (12_02) revealed the cell development and culture shifts over the time course of the cultivation. Images are provided at 48, 240, and 360 hrs at low (x200) and high (x400) magnification.

Microscopic images on day 2 (48 hrs) showed some single cell algae (with low numbers that were motile), interspersed with algae flocs. Debris and fungi contamination was clearly visible (Figure 3.4).

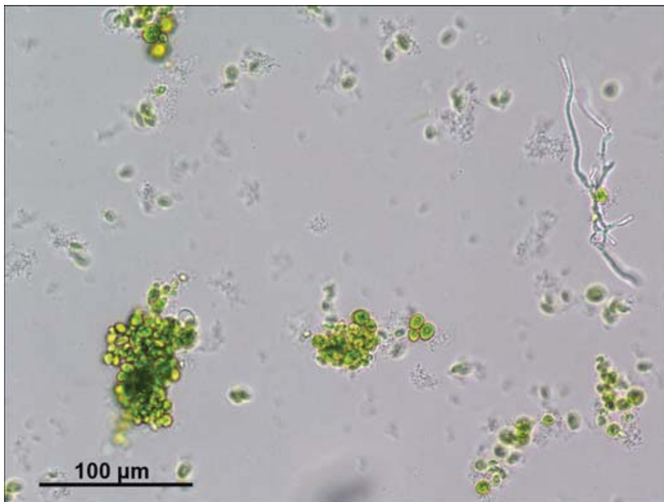

Figure: 3.4: *Chlorococcum sp.* (12_02) pond culture 48 hours after inoculation showing algae flocs and single cells. Fungi contamination is indicated by a red arrow.

Microscopic images on day 10 (240 hrs) showed a dramatic increased number of algae flocks and single cells including motile fast swimming cells (daughter cells, emerging out of bigger non motile algae cells) (Fig. 3.5) (supplementary data video 3.1). A decreased fungi population was visible with no further evidence of impact on the culture. Besides a few small motile *Paramecium* like predator organisms (not visible in the picture) no other contaminants were detected.

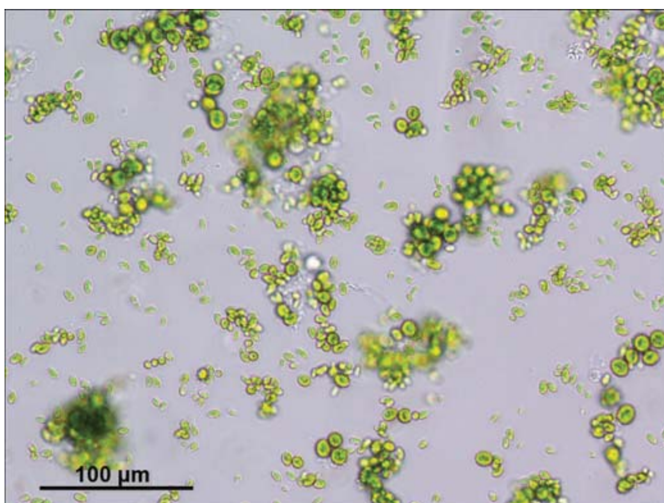

Figure 3.5: *Chlorococcum sp.* (12_02) pond culture 240 hours after inoculation showing increased density of alga cells and flocs.

Microscopic images on day 15 (360 hrs) shows numerous large sized algae cells which are accumulated into flocks up to 100 µm in size (example shown in supplementary data video 3.2). An increased number of small oval shaped (motile and non-motile) cells are distributed throughout the culture (Fig. 3.6, a, b). Besides the few small motile *Paramecium* like predator organisms, rod shaped bacteria were detected (not shown in pictures).

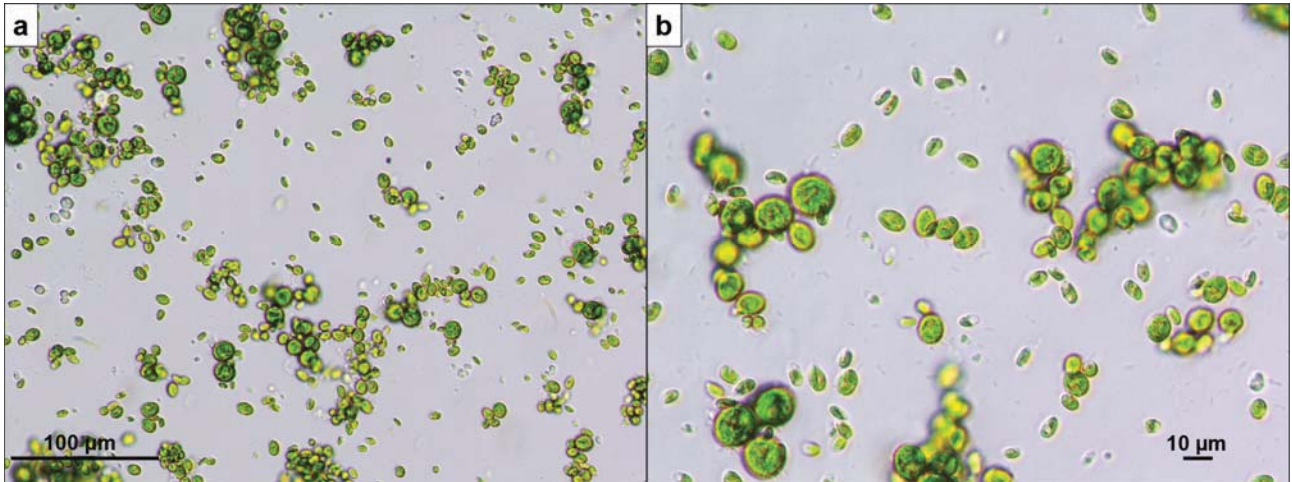

Figure 3.6: *Chlorococcum sp.* (12_02) pond culture 360 hours after inoculation a) x200 and b) x400 magnification.

In summary: *Chlorococcum sp.* (12_02) appeared to be a highly auto-flocculating strain. Two two cell morphologies were observed; large round cells mostly aggregated in flocs and small motile cells dispersed throughout the culture. *Chlorococcum sp.* (12_02) proved to be robust in that it exhibited low levels of predatory organisms which seemed to have no major influence on the culture. Only bacterial contamination was observed during the time of the cultivation.

***Scenedesmus sp.* (Pinjarra 001):** Cultivation data and conditions are illustrated in figure 3.7 A and figure 3.7 B. The cultivation was inoculated at a starting OD_{750} of 0.2, and the resulting growth curves based upon optical density and dry weight measurements are presented in figure 3.7 A (a, b) and figure 3.7 B (a). This species did not exhibit any observable lag phase and transitioned immediately into a logarithmic growth phase up until ~90 hours (similarly to *Chlorococcum* sp. 12_O2). Subsequently this strain tended to continue to grow, albeit slowly, and an actual stationary phase was not observed during the cultivation period. From previous cultivations it has been observed that this strain is capable of slowly increasing in cell density towards OD_{750} values of between 3-4 however light penetration becomes severely limiting and cells tend to settle to the bottom of the HRP where respiration ensues (data not shown). Thus while this strain is not the fastest growing of those cultivated during this project, it is extremely robust to both local environmental conditions and biological challenges.

The pH of the system remained mostly stable at 7 except for a sustained but small increase at ~17-46 hours (pH = 7.2) which was accompanied by an increase in CO_2 delivery to amend the pH (Figure 3.7 B, c). The dissolved oxygen shows a distinct circadian rhythm which decreased constantly in value from 140 hours until the end of the cultivation (Figure 3.7 B, d).

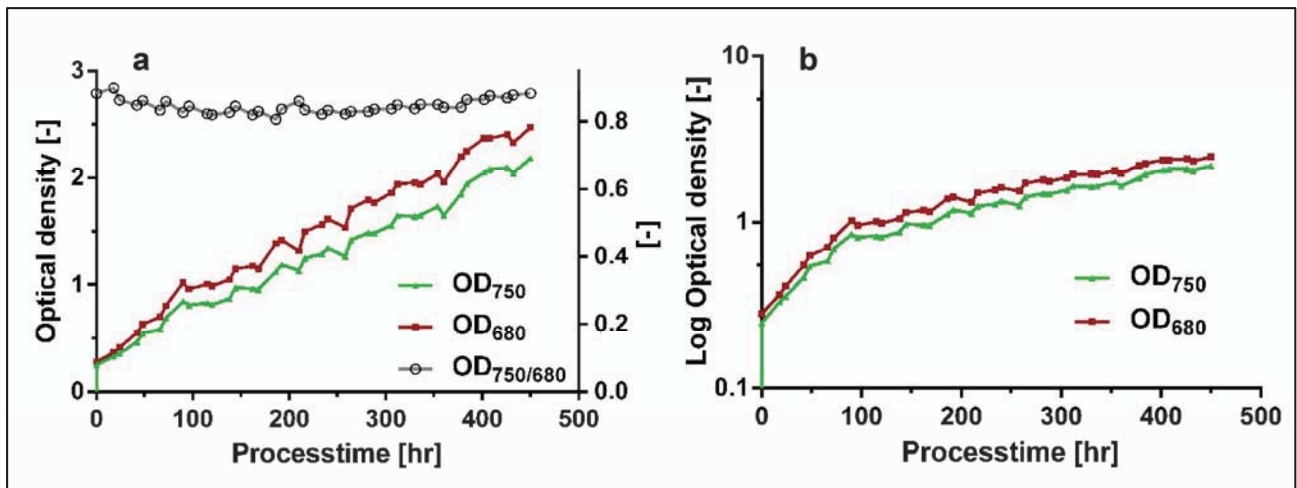

Figure 3.7 A: Culture performance and cultivation conditions of *Scenedesmus sp.* (Pinjarra 001): Display of the growth curve based on optical density a) linear and b) logarithmic.

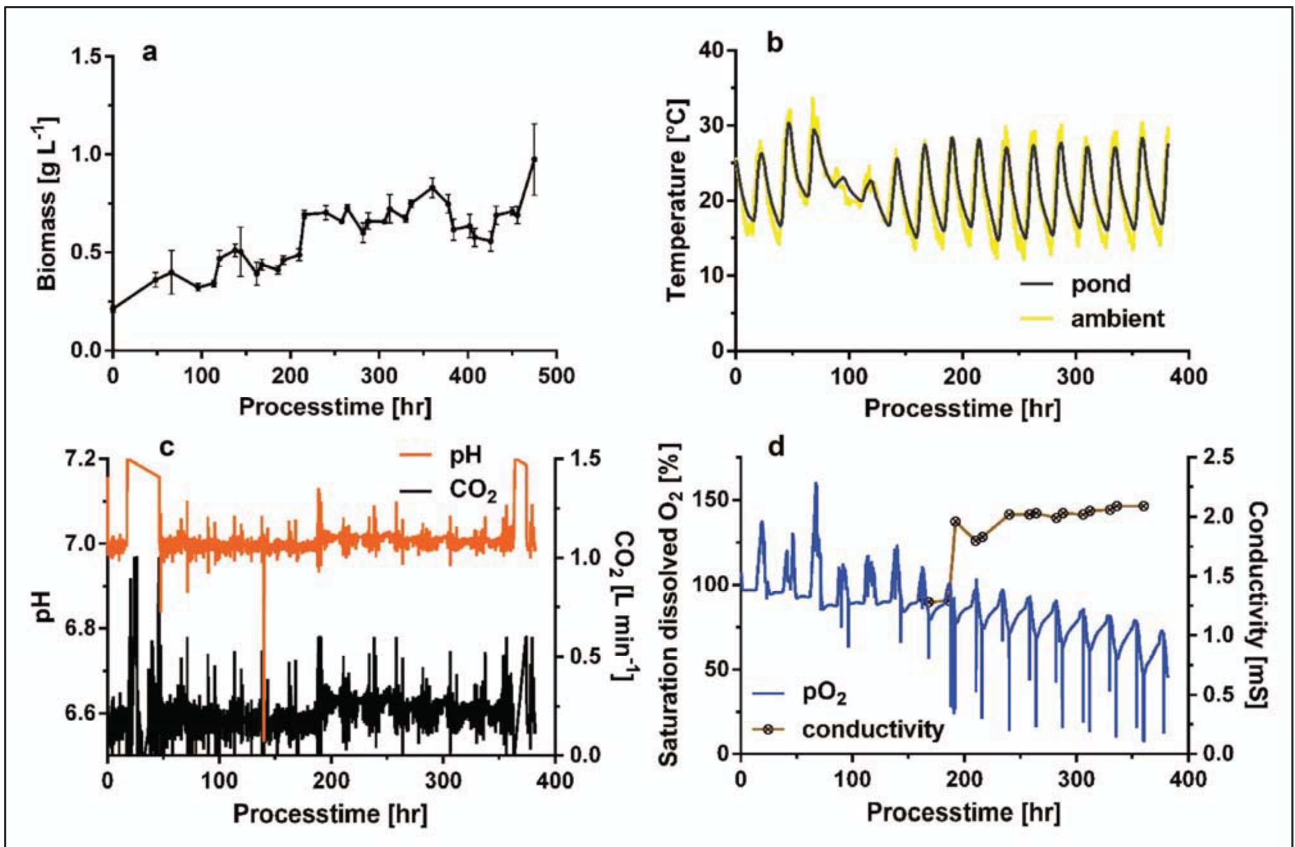

Figure 3.7 B: Culture performance and cultivation conditions of *Scenedesmus sp.* (Pinjarra 001): Display of the a) growth curve based on biomass density. b) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle. c) measured pH of the algal culture and dependent CO_2 inputand d) display of conductivity and dissolved oxygen.

Microscopic monitoring of the *Scenedesmus sp.* (Pinjarra001) culture provided additional insights into culture performance. Images are provided at 48, 240 and 360 hrs at low (x100) mid (x200) and high (x400) magnification.

Microscopic images on day 2 (48 hrs) showed a healthy culture containing cells which are oval shaped (some appear thinner, some thicker) with pointed ends and mostly clustered in stacks of 2 – 6 algae cells (Figure 3.8, a). Occasionally rotifer predators morphologically similar to *Brachionus* are seen swimming through the culture (Figure 3.8, b).

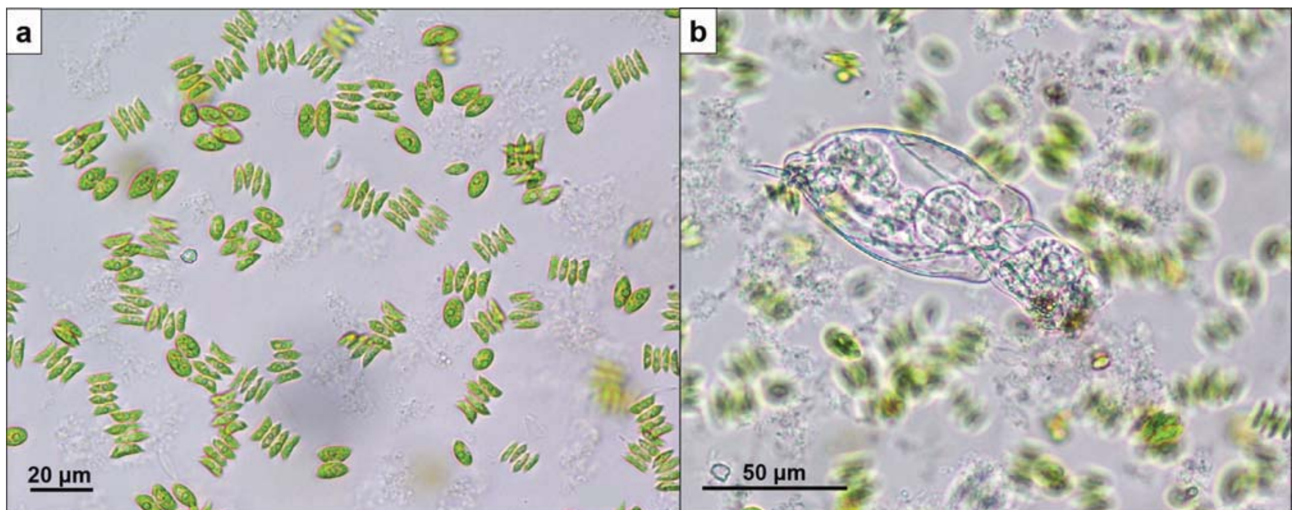

Figure 3.8: *Scenedesmus sp.* (Pinjarra001) pond culture 48 hours after inoculation (x400 magnification). a) cell morphology of *Scenedesmus sp.* (Pinjarra001). b) example of a rotifer organism detected.

Microscopic images on day 10 (240 hrs) showed a denser healthy green culture containing more single cell algae. Rotifers can be frequently observed in the culture (Figure 3.9, rotifer indicated by a red arrow). However, no algae cells are seen to be consumed by the predator.

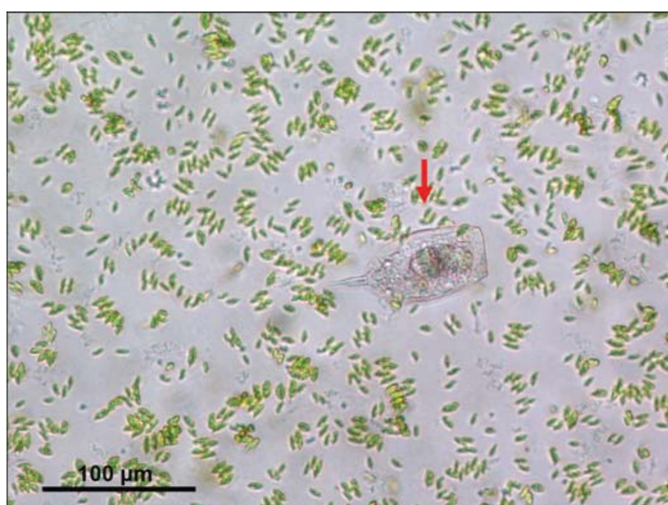

Figure 3.9: *Scenedesmus sp.* pond culture 240 hours after inoculation.

Microscopic images on day 12 (288 hrs) showed a similar picture as to that seen on day 10 (240 hours). The algae cell morphology shifted almost entirely towards single cells that had less pointed ends. (Figure 3.10, a, b).

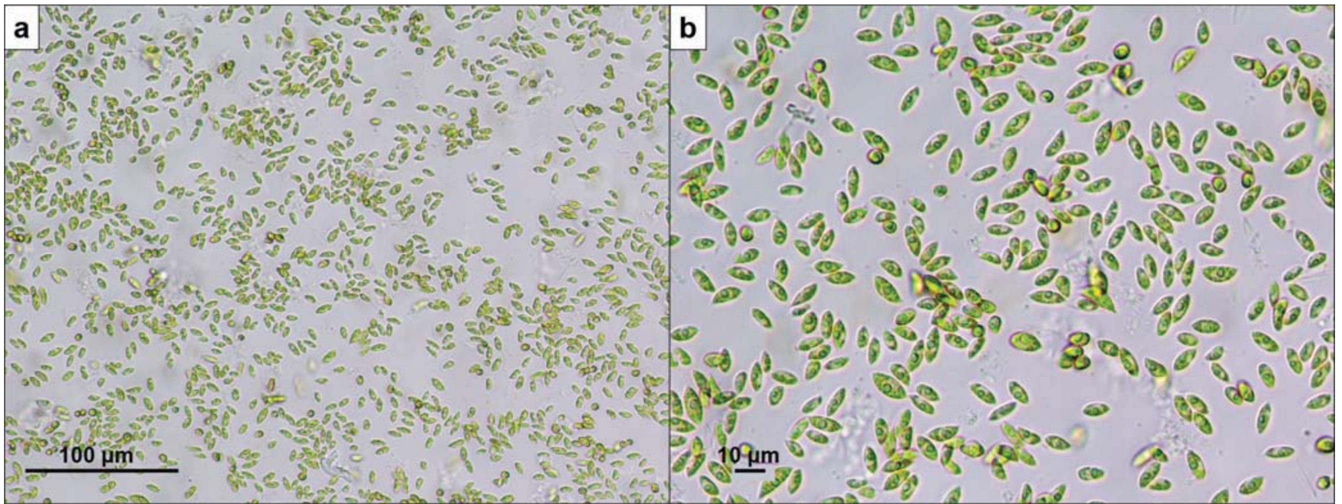

Figure 3.10: *Scenedesmus sp.* pond culture 288 hours after inoculation.

In summary: *Scenedesmus* sp. (Pinjarra001) appeared to be either thin longitudinal and thin with pointed ends or thicker and ovoid in appearance. The morphology of up to 6 clustered cell changed during the cultivation period. Rotifer predators (morphologically similar to *Brachionus*) were frequently detected but had no noticable influence on the algae culture or cell morphology. No further contaminants were detected within the culture despite some debris flocs.

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***Micractinium pusillum* (5_H4)** cultivation data and conditions are illustrated in figure 3.11 (A-B). The cultivation was inoculated at a starting OD_{750} of 0.1, and the resulting growth curves based upon optical density and dry weight measurements are presented in figure 3.11 A (a, b) and figure 3.11 B, (a). This species experienced a short lag phase to ~24 hours and then transitioned into a logarithmic growth phase up until ~96 hours. Similar to *Scenedesmus* sp. Pinjarra001 this strain tended to continue in growth at a slow rate and a true stationary phase was not observed during the cultivation period. At the cessation of the cultivation OD_{750} values of between 3-4 had already been achieved. It appeared to be a robust strain, and faster growing than the indigenous *Scenedesmus*.

There were 2 short pH disruptions between 200-240 hours due to an outage of the CO_2 regulation but the increase was minor. Due to a system fault no CO_2 data were recorded. The conductivity measured throughout the cultivation remained between 4.43 and 4.01 mS. *M. pusillum* (5_H4) which is considerably higher than the media used for the other strains in this project.

Rainfall levels were very low except for one higher rainfall event (4.5 mm h^{-1}) occurring after 283 hours (Figure 3.11 C, b). The pH of the system remained predominantly between 6.9 - 7.02 (Figure 3.11 C, a).

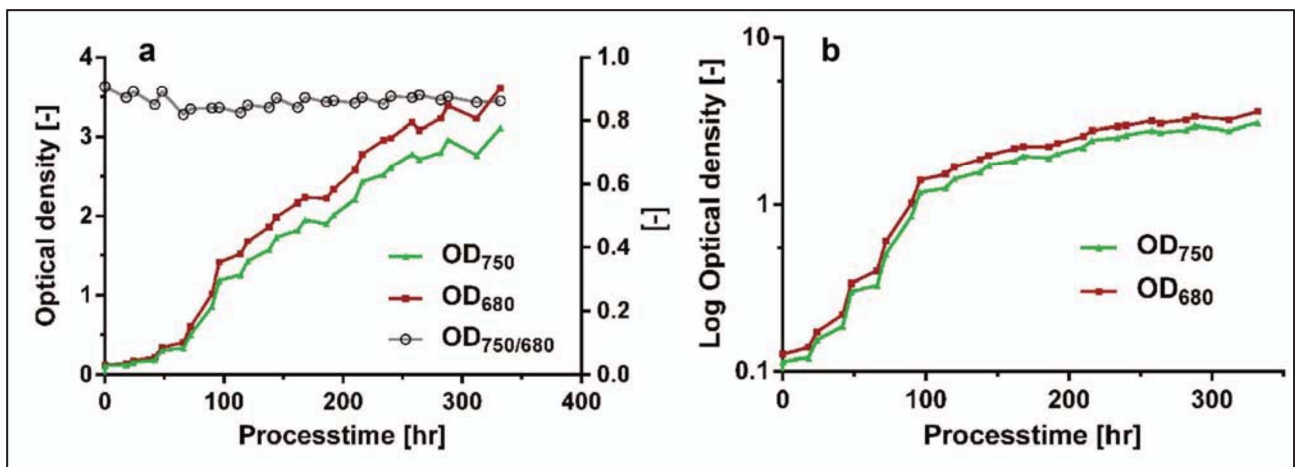

Figure 3.11 A: Culture performance and cultivation conditions of *M. pusillum* (5_H4): display of the growth curve based on optical density a) linear and b) logarithmic.

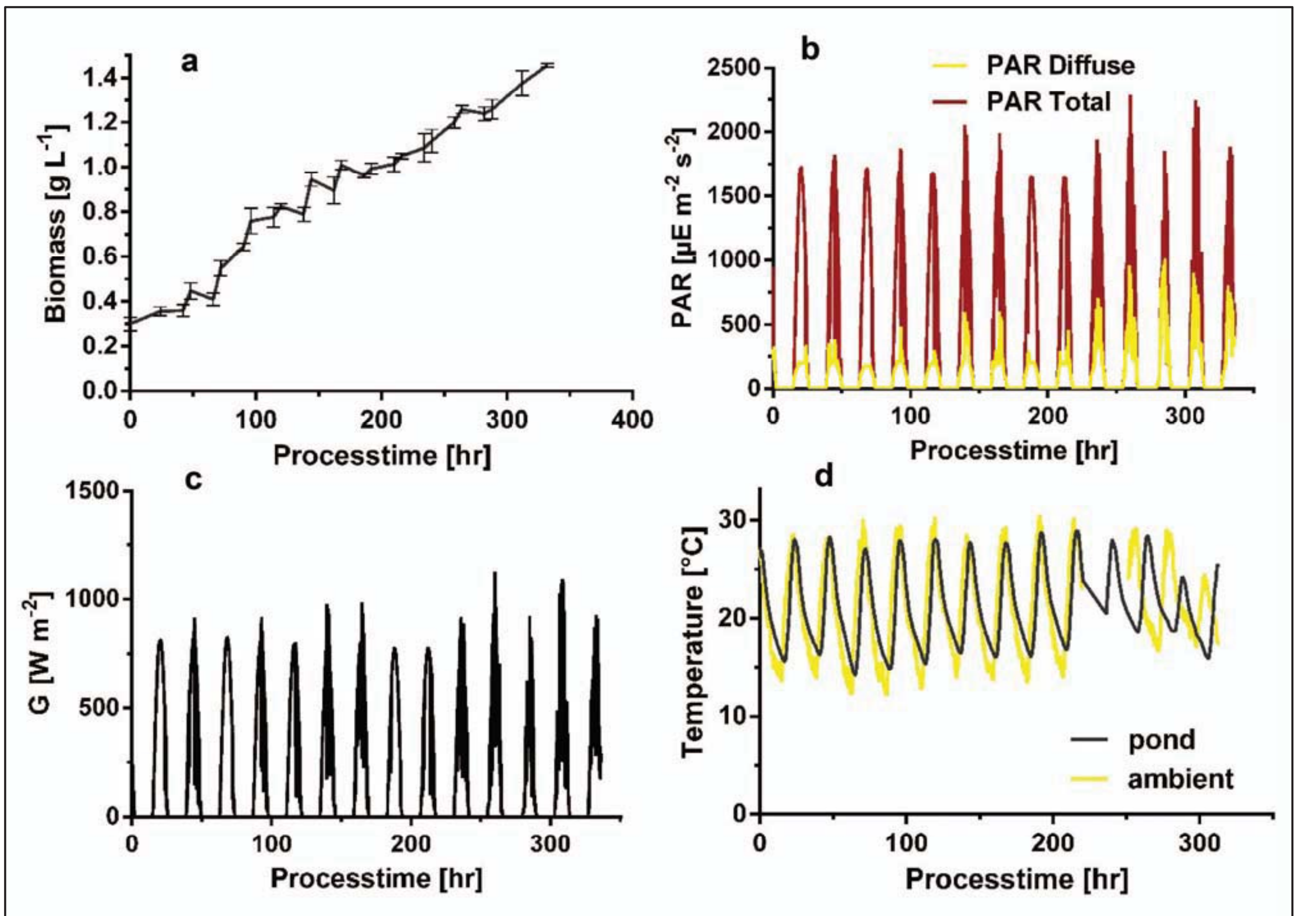

Figure 3.11 B: Culture performance and cultivation conditions of *M. pusillum* (5_H4): display of the a) growth curve based on biomass density. b) total and diffuse photosynthetically active radiation and c) global solar radiation. d) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle.

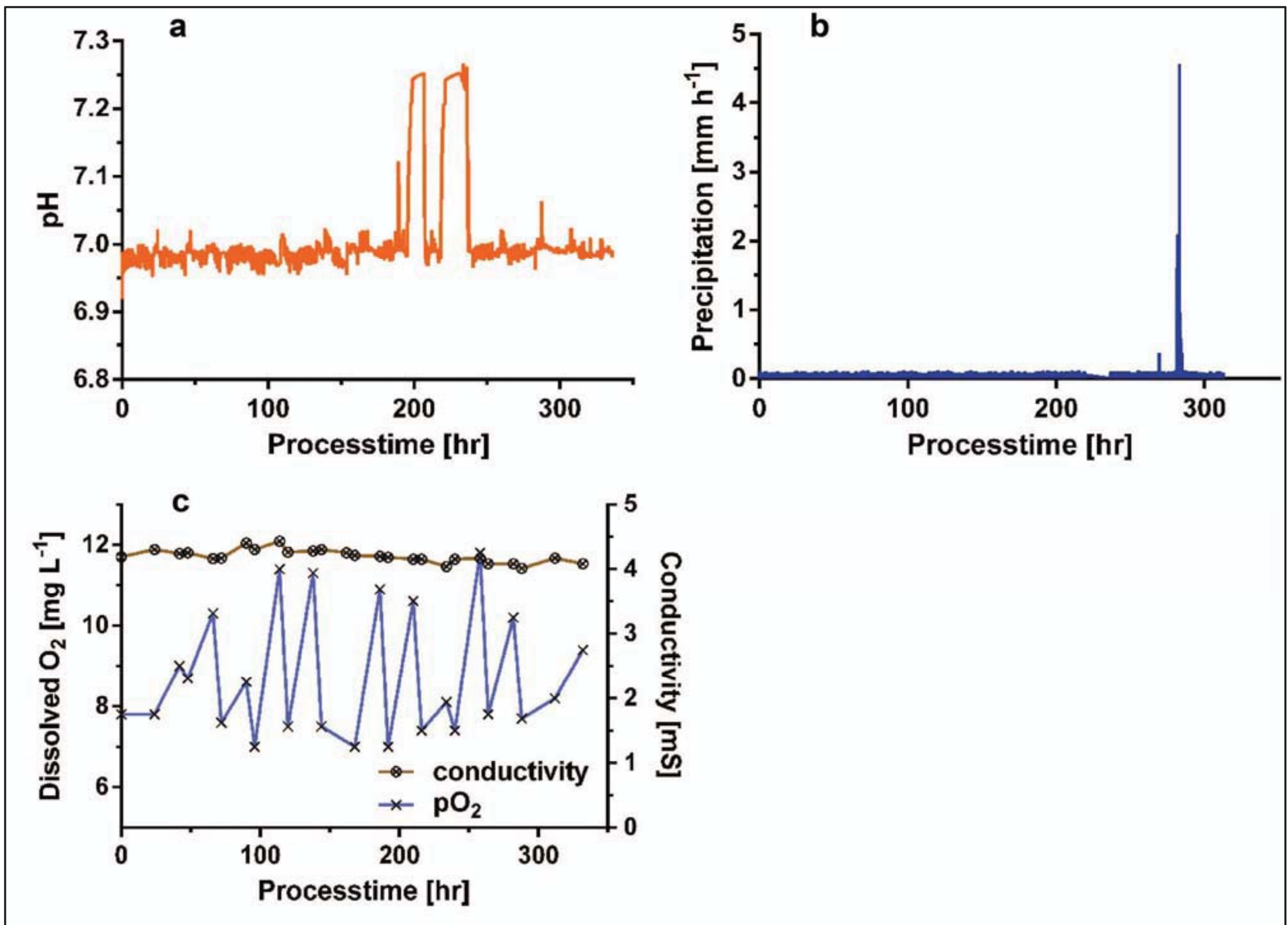

Figure 3.11 C: Culture performance and cultivation conditions of *M. pusillum* (5_H4): display of the a) measured pH of the algal culture and dependent CO_2 input. b) precipitation occurrence measured over the time course of the experiment. c) display of conductivity and dissolved oxygen.

Microscopic monitoring of the *M. pusillum* (5_H4) culture provided additional insights into culture performance. Images are provided at 72, 192 and 312 hrs at medium (x200) and high (x400) magnification.

Microscopic images on day 3 (72 hrs) showed mainly a single cell morphology containing low chlorophyll pigmentation and poor motility (Figure 3.12, a). Fungi contamination was observed in the pond (Figure 3.12, b) but with little evidence of significant negative impact on the algal population.

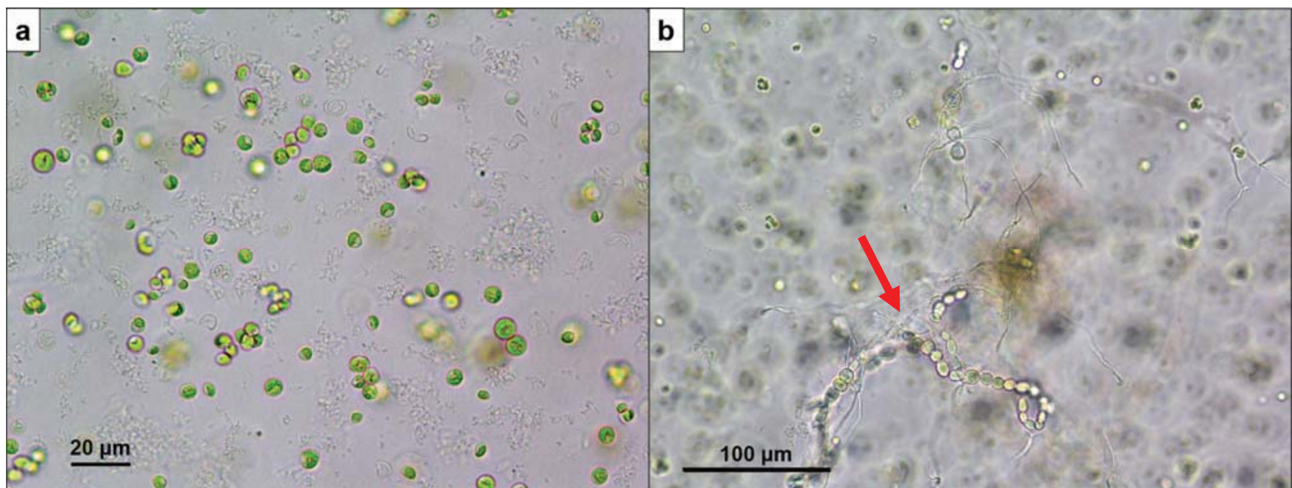

Figure 3.12: *M. pusillum* (5_H4) pond culture 72 hours after inoculation a) algal cells with dispersed debris b) visible fungi bodies/branches within the algal culture (example indicated by a red arrow).

Microscopic images on day 8 (192 hrs) showed a much denser green culture of algal cells which generally clustered in groups of 3-4 cells. Increased levels of bacterial contamination were visible. The fungi population initially observed was no longer detectable at this time point. Small numbers of green flagellates (similar in morphology to *Chlamydomonas*) were also evident. Notably these created empty cell circle around themselves presumably through their motility (Figure 3.13, a, b, examples are indicated by red arrows) (supplementary data video 3.3). Ciliates *Tetrahymena sp.* (morphological identified) were observed grazing through the culture.

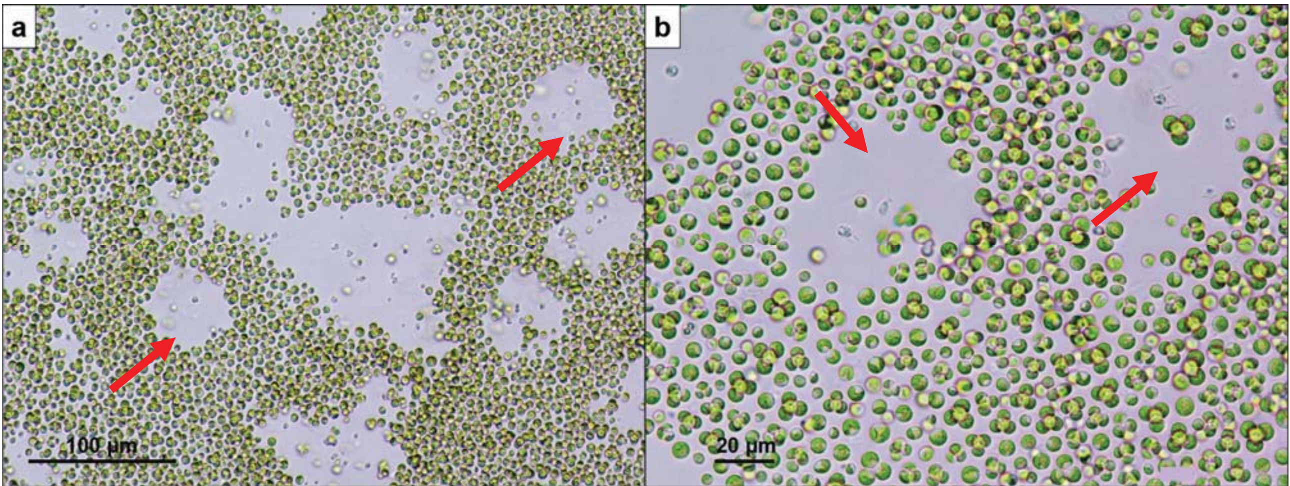

Figure 3.13: *M. pusillum* (5_H4) pond culture 192 hours after inoculation. Increased growth of algae culture on day eight including a small predator organism forming "holes" in the algae cell colonies. a) x200 and b) x400 magnification.

Microscopic images on day 13 (312 hrs) showed *M. pusillum* (5_H4) dominating the culture and a decreased appearance of contaminating microorganisms compared to previous time points (Figure. 3.14). Green flagellates were present but in significantly lower numbers. The ciliate population *Tetrahymena sp.* remains constant in the culture, and furthermore some small *Paramecium* were also occasionally seen.

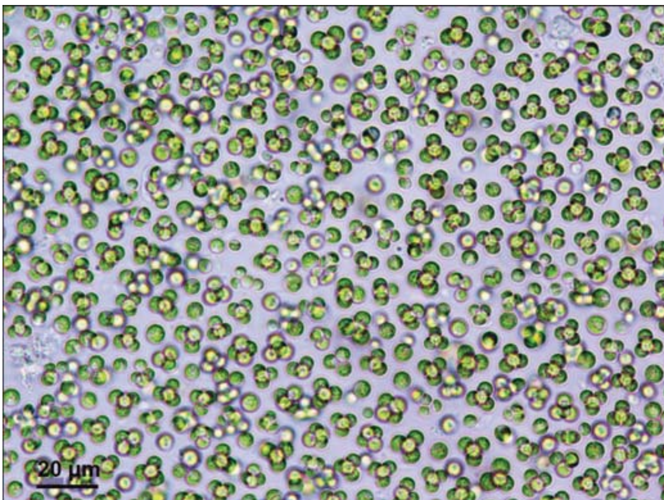

Figure 3.14: *M. pusillum* (5_H4) pond culture 312 hours after inoculation.

In summary: *M. pusillum* (5_H4) cells were round in appearance with spines during the growth and stationary phase (not readily apparent in the microscopic images shown). The spherical clusters of 3-4 cells may be indicative of cell division, and a healthy culture. At day 3, the culture contained fungi bodies which reduced in number as the algae culture density increased. This suggests that they were not seriously pathogenic to the algae. An increased number of bacterial cells on day 8 and fewer predatory flagellates and ciliates organism (possibly grazing mostly on bacteria) also had no detectable influence on the steady growth of the algae culture.

***Chlorella sp.* (11_H5)** cultivation data and conditions are illustrated in figure 3.15 (A-C). The cultivation was inoculated at a starting OD_{750} of 0.2, and the resulting growth curves based upon optical density and dry weight measurements are presented in figure 3.15 A (a, b) and figure 3.15 C (a). This species experienced a short lag phase to ~24 hours and then transitioned into a logarithmic growth phase up until ~114 hours. At this point there was a notable decrease of OD most likely due to the occurrence of high levels of foaming and biofouling. It is unknown as to if the logarithmic phase could have been further sustained if these issues could be addressed. This strain did enter a distinct stationary phase at OD_{750} of ~2.

There was a significant rainfall event during the first three days (2.4 – 5.4 mm h^{-1}) (Figure 3.15 C, b) however pH was well maintained. The pH of the system remained stable at around 7 with only a slight drop to 6.8 at round 77 hours as the rainfall ceased. The conductivity measured throughout the cultivation slightly fluctuated between 2.3 and 2.7 mS (Figure 3.15 C, c).

Chlorella sp. (11_H5) proved to be robust in that it exhibited low levels of bacterial and predator contamination and ranked no.8 of the nine strains in terms of maximum growth rate under the conditions tested.

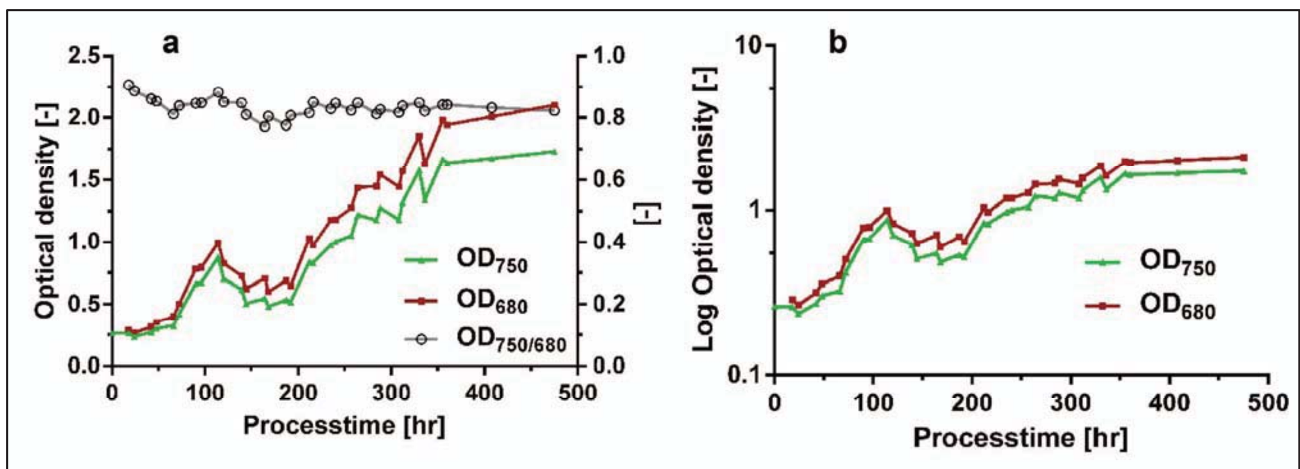

Figure 3.15 A: Culture performance and cultivation conditions of *Chlorella sp.* (11_H5): display of the growth curve based on optical density a) linear and b) logarithmic.

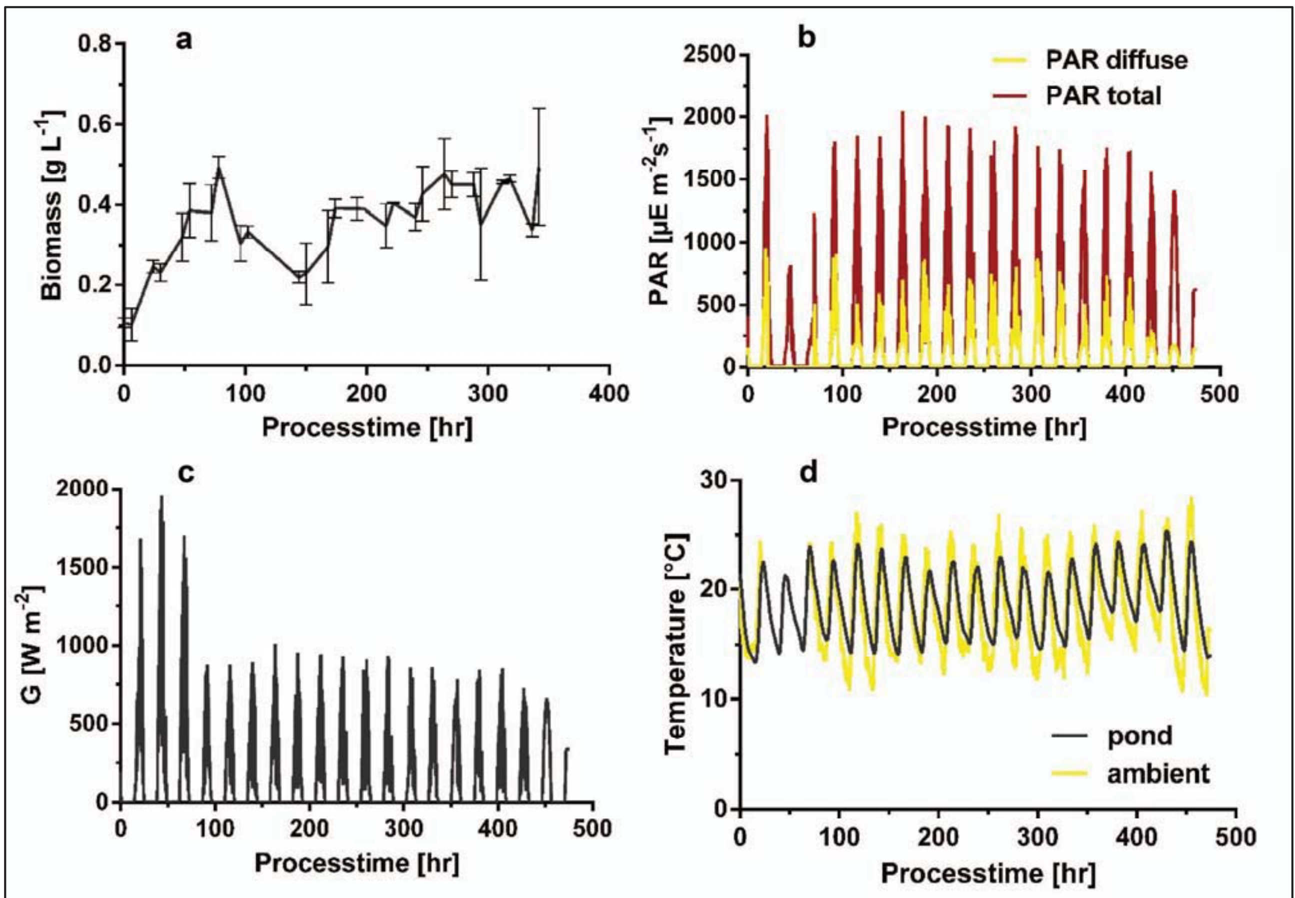

Figure 3.15 B: Culture performance and cultivation conditions of *Chlorella sp.* (11_H5): display of the a) growth curve based on biomass density. b) total and diffuse photosynthetically active radiation and c) global solar radiation. d) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle.

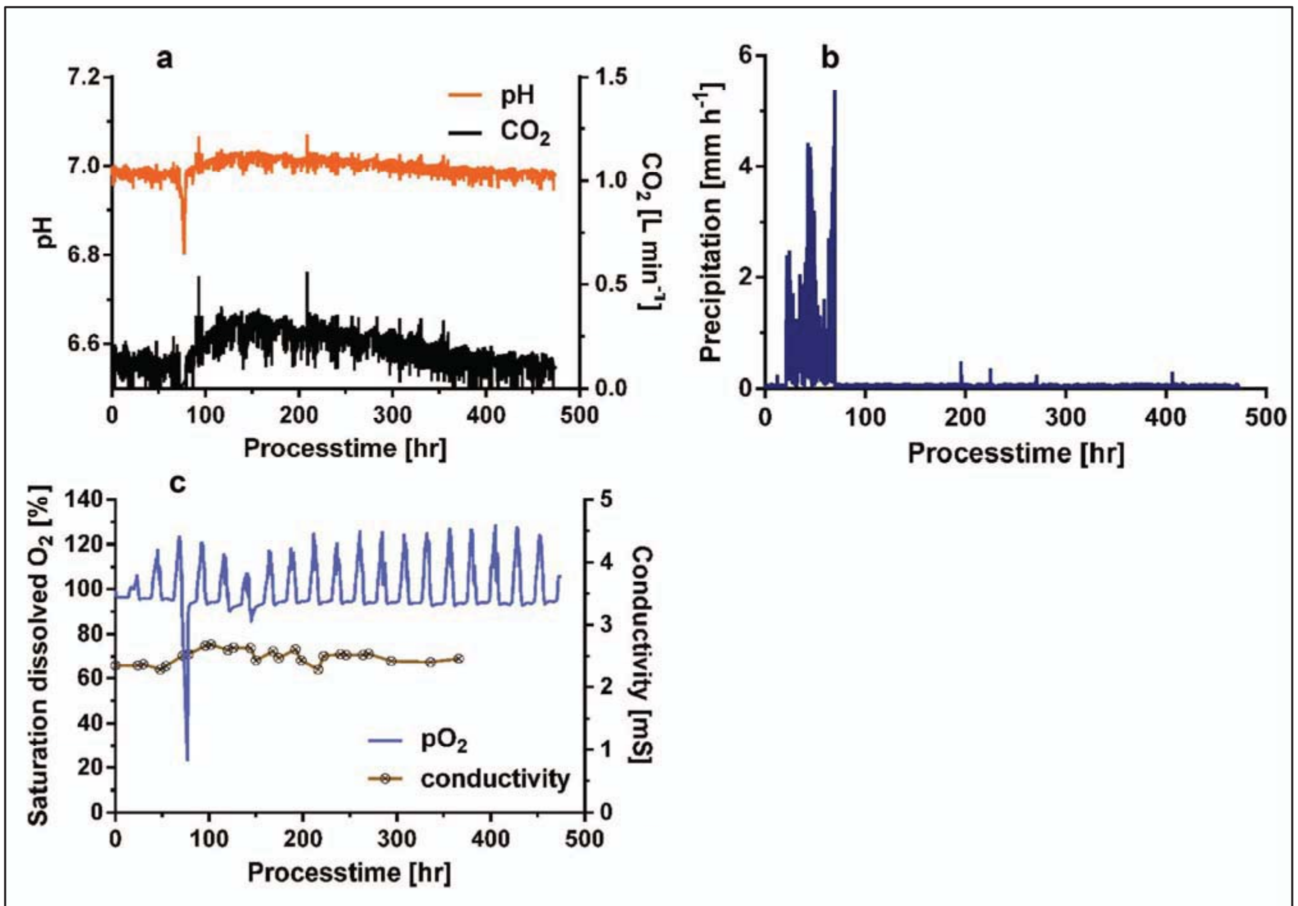

Figure 3.15 C: Culture performance and cultivation conditions of *Chlorella sp.* (11_H5): display of the a) measured pH of the algal culture and dependent CO_2 input. b) precipitation occurrence measured over the time course of the experiment. c) display of conductivity and dissolved oxygen.

Microscopic monitoring of the *Chlorella sp.* (11_H5) culture provided additional insights into culture performance. Images are provided at 168, 192, 360 and 475 hrs at low (x100) mid (x200) and high (x400) magnification.

Microscopic images on day 7 (168 hrs) showed single cell algae interspersed with many flocs of debris, high amount of bacteria as well as fungi contamination (Figure. 3.16).

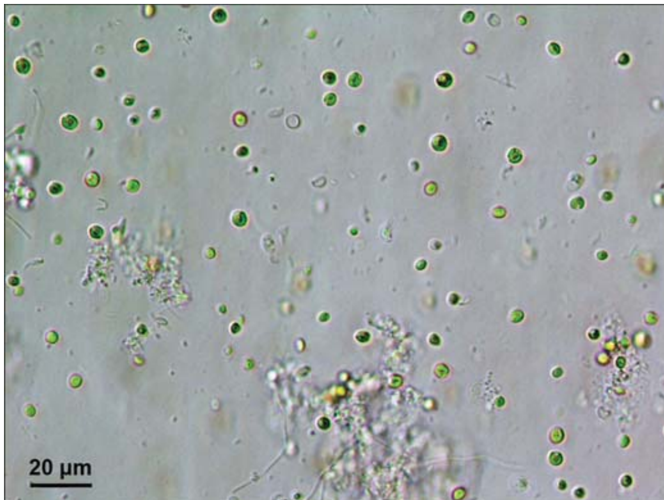

Figure 3.16: *Chlorella sp.* (11_H5) pond culture 168 hours after inoculation showing algal cells with dispersed debris, bacteria and fugi.

Microscopic images on day 8 (192 hrs) showed a denser and more healthy green culture containing a high amount of bacteria, debris and fungi contamination which was trapping algae. (Figure. 3.17, a, b). Small motile ciliates could be detected as well as a small number of elongated fast moving ciliates (morphological similar to *Amphileptus* [5]) grazing around the flocks.

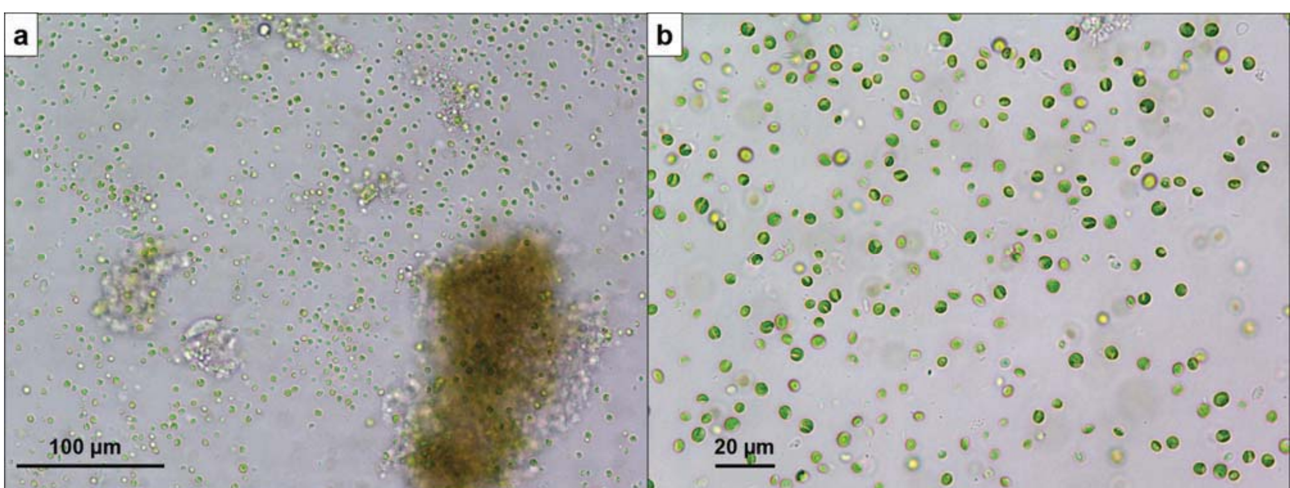

Figure 3.17: *Chlorella sp.* (11_H5) pond culture 192 hours after inoculation. a) showing algae cells and flocs of debris (x200 magnification). b) single cell morphology of dispersed algal cells (x400 magnification).

Microscopic images on day 15 (360 hrs) showed besides many single cell algae an increased flocculation of algae. The predator load was increasing in both their number and type's e.g. morphological identified ciliates similar to *Amphileptus* and flagellates (Figure 3.18, c, d) similar to *Chlamydomonas*. The ingestion of cells by the flagellates was clearly visible (Figure 3.18, c, d) (supplementary data video 3.4).

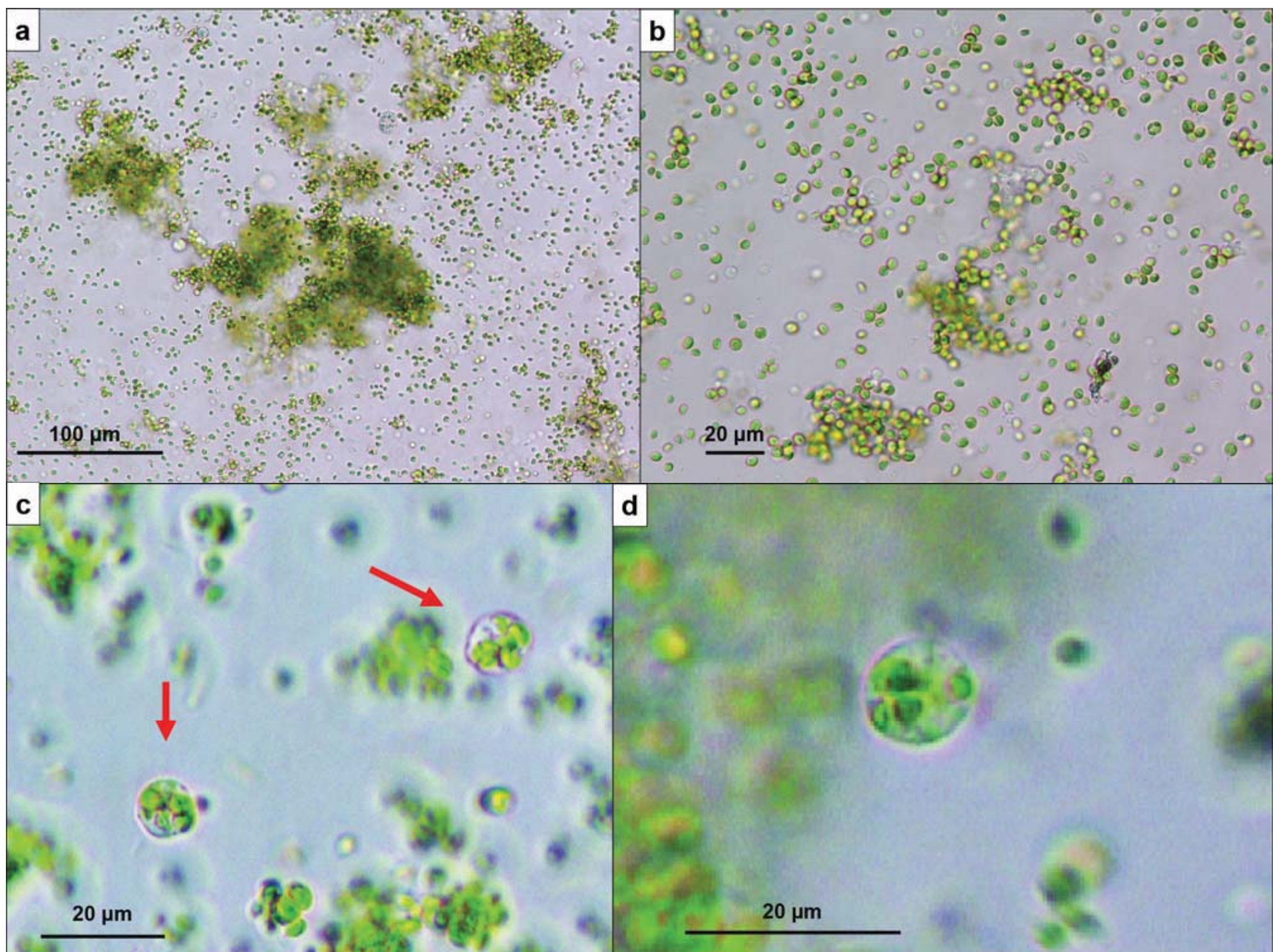

Figure 3.18: *Chlorella sp.* (11_H5) pond culture 360 hours after inoculation showing increased flocculation of the algal cells a) and b). Flagellates similar morphology to *Chlamydomonas* with ingested algae cells c) and d).

Microscopic images on day 20 (475 hrs) show a similar picture to day 15, however the flocculation amount and size of *Chlorella sp.* (11_H5) increased drastically whereas single cell numbers decreased. Many different predators could be observed which were described on day 15. Of note was the high number of a particular ciliate type not detected on previous days, which established itself in the culture and is morphologically and behaviourally very similar to the ciliate *Cyclidium* (locomotion by "jumping") (Figure. 3.19, d) (supplementary data video 3.5). This ciliate type and the ciliate described as *Amphileptus* (Figure 3.19, c) were the two dominating protozoa detected grazing around

the algae flocs (supplementary video 3.6 and 3.7). A one off observation of the ciliate morphological similar to the genus *Didinium* (blue arrow) and pseudopodia similar to *Actinosphaerium* (red arrow) was observed grazing on an algal floc (Figure 3.19, b) (supplementary data video 3.8).

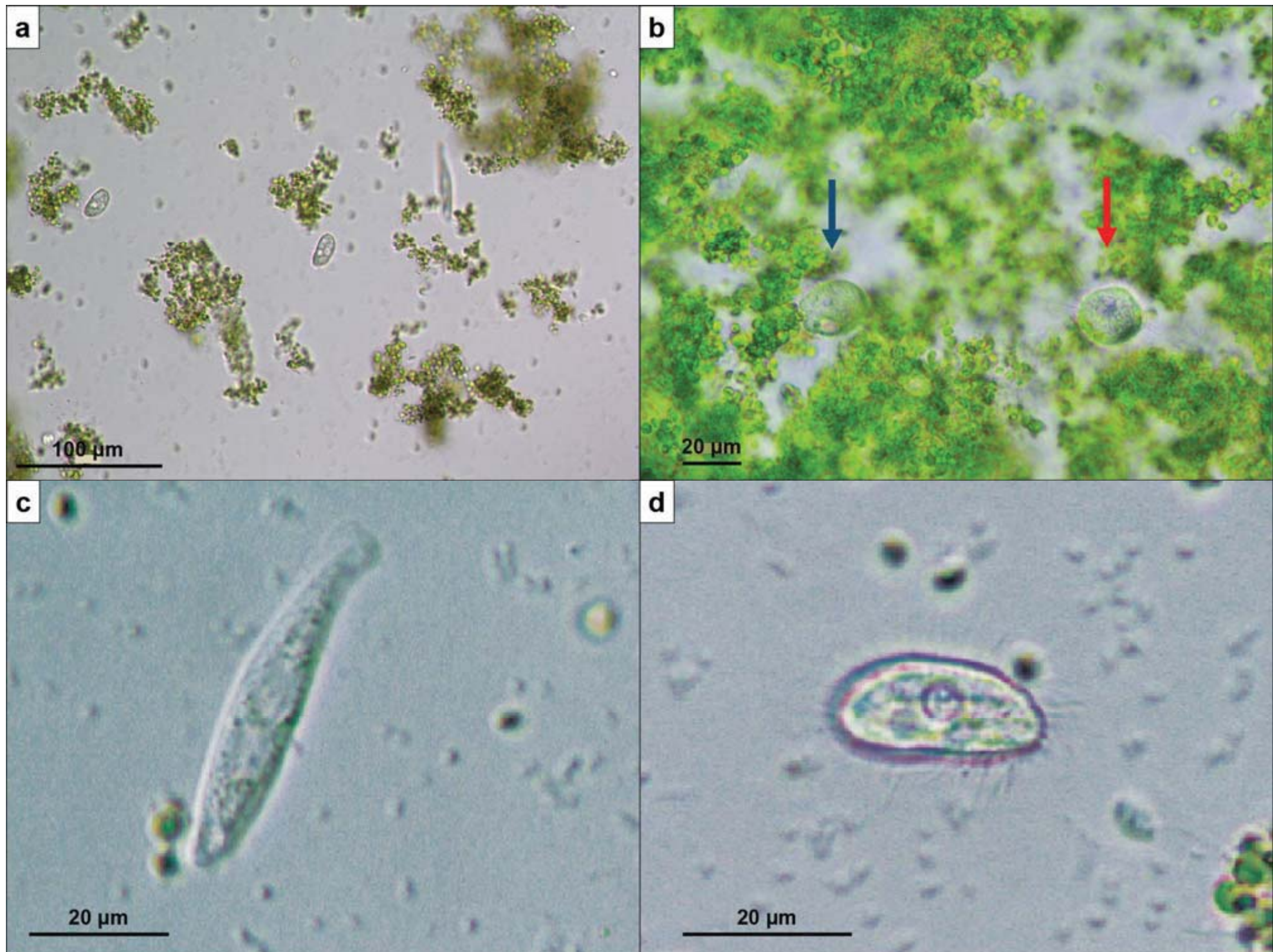

Figure 3.19: *Chlorella sp.* (11_H5) pond culture 475 hours after inoculation showing flocculation of algae cells a). b) Grazing predators similar to *Didinium* (blue arrow) and *Actinosphaerium* (red arrow). c) close up image of a ciliate similar to *Amphileptus*. d) close up image of a ciliate similar to *Cyclidium.*

In summary: *Chlorella sp.* (11_H5) appeared to be highly sensitive to the impact of predators. The highest diversity of protozoa was detected within this cultivation, however no predator species were directly detected ingesting the algae as was shown during the cultivation of *C. sorokiniana* (8_C4). The single cell morphology of *Chlorella sp.* (11_H5) shifted to an aggregated state with increased predator load. This leads to the assumption of a high impact from the protozoa on the behaviour and growth of the algae.

***Desmodesmus intermedius* (1_C4)** cultivation data and conditions are illustrated in figure 3.20 (A-C). The cultivation was inoculated at a starting OD_{750} of 0.1, and the resulting growth curves based upon optical density and dry weight measurements are presented in figure 3.20 A (a, b) and figure 3.20 B (a). This species exhibited a short lag phase to ~18 hours and then transitioned into a logarithmic growth phase up until ~92 hours. At this point there was a slowing of growth but growth did continue until ~250 hours.

Besides the steady increase in optical density, *D. intermedius* (1_C4) did not correspond in an increase of dry weight. The disparity between both growth curves may be explained by changes in cell morphology (clusters of 2-5 cells to ovoid single cells), increase of cell size and volume but not necessarily an accumulation of biomass, as well as increasing amount of cell debris along the cultivation period. These factors mentioned change light scattering parameters, governing optical density.

There were no perturbing rainfall events during the cultivation period and the pH was well maintained. The conductivity and dissolved oxygen measured throughout the cultivation remained between 2.32 and 2.81 mS and 7.1 and 10.3 mg L^{-1} respectively. *D. intermedius* (1_C4) proved to be robust in that it exhibited low levels of bacterial and predator contamination.

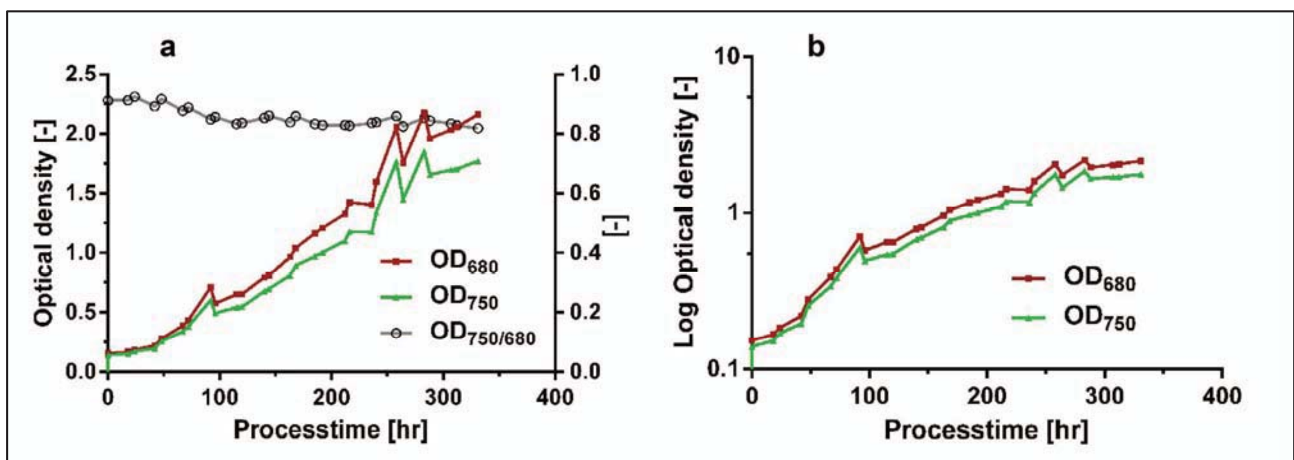

Figure 3.20 A: Culture performance and cultivation conditions of *D. intermedius* (1_C4): display of the growth curve based on optical density a) linear and b) logarithmic.

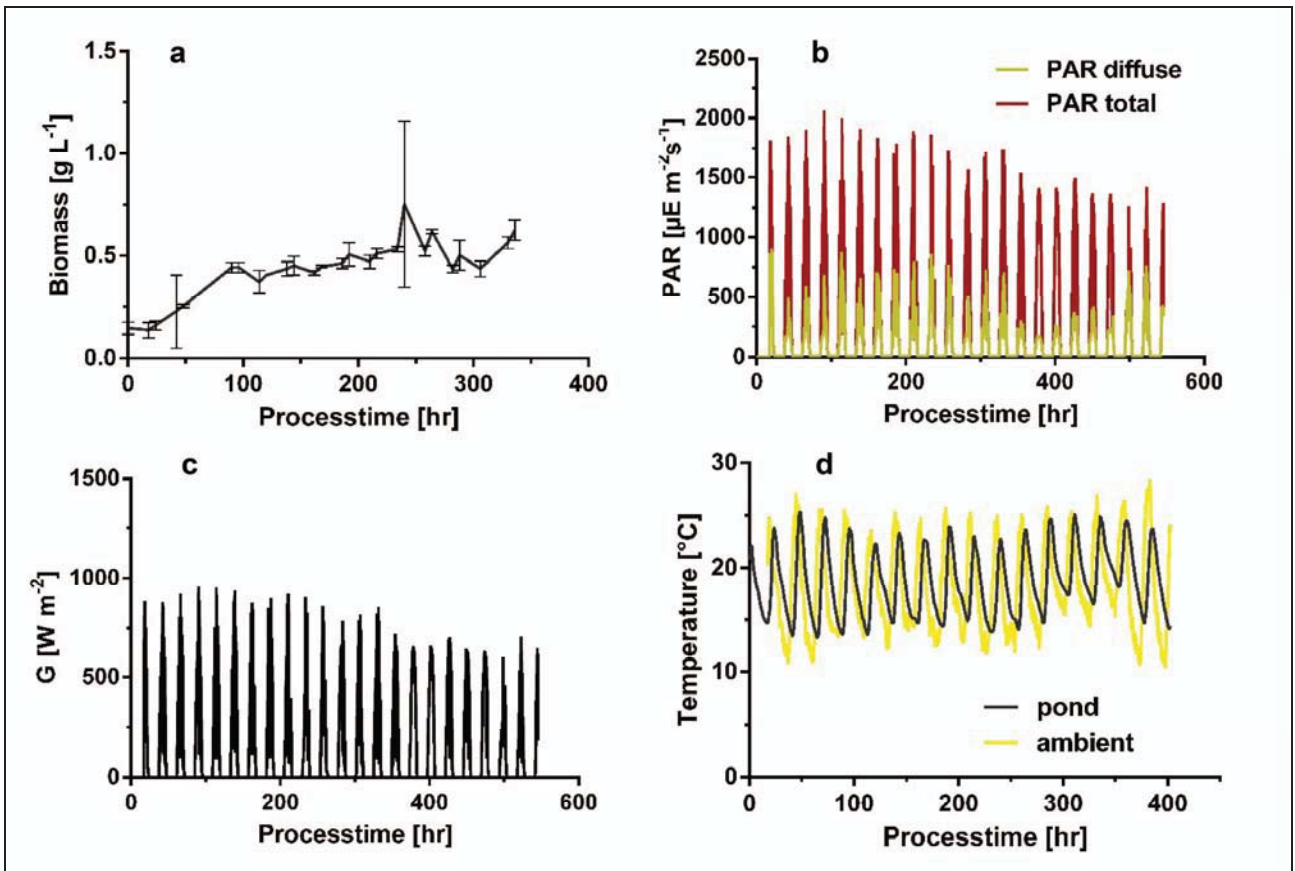

Figure 3.20 B: Culture performance and cultivation conditions of *D. intermedius* (1_C4): display of the a) growth curve based on biomass density. b) total and diffuse photosynthetically active radiation and c) global solar radiation. d) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle.

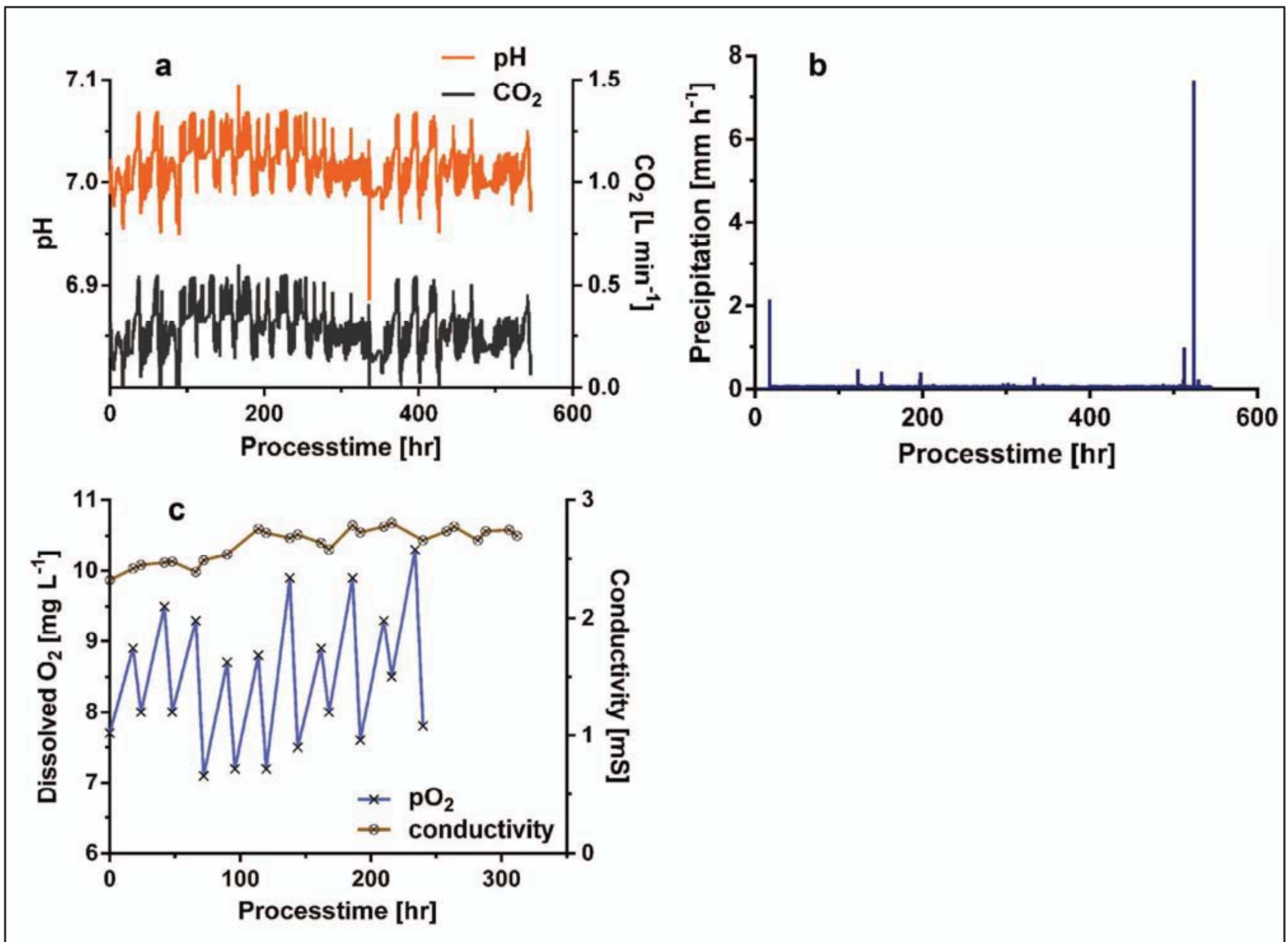

Figure 3.20 C: Culture performance and cultivation conditions of *D. intermedius* (1_C4): display of the a) measured pH of the algal culture and dependent CO_2 input. b) precipitation occurrence measured over the time course of the experiment. c) display of conductivity and dissolved oxygen.

Microscopic monitoring of the *D. intermedius* (1_C4) culture provided additional insights into culture performance. Images are provided at 96, 144 and 288 hrs at low (x100) mid (x200) and high (x400) magnification.

Microscopic images on day 4 (96 hrs) showed single cell algae interspersed with algae flocs. Rod shaped bacteria were clearly visible in the culture (indicated by red arrow (Figure 3.21, a, b).

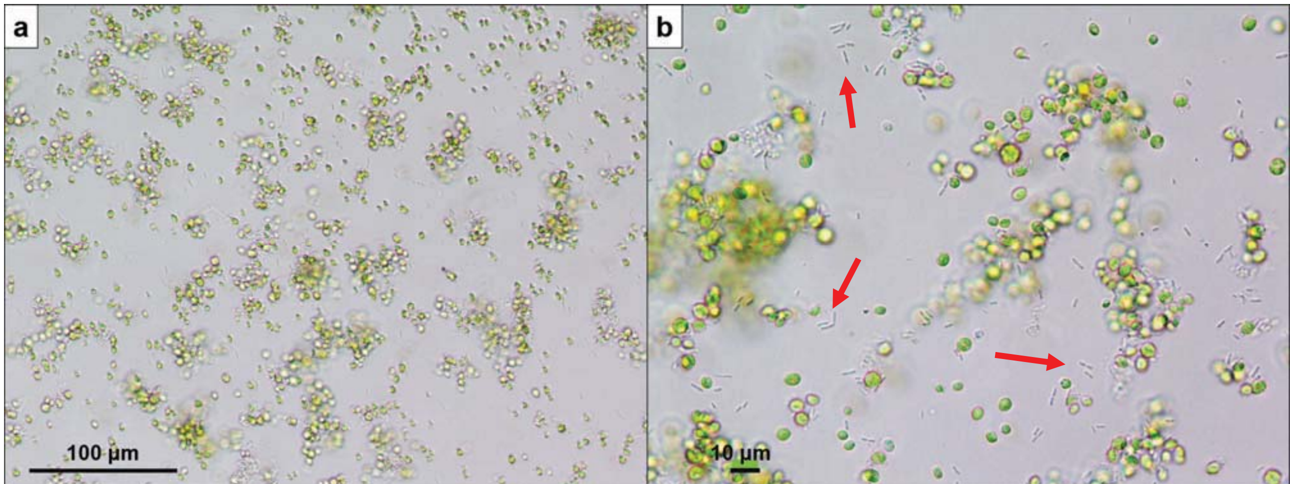

Figure 3.21: *D. intermedius* (1_C4) pond culture 96 hours after inoculation. Bacteria cells are indicated by a red arrow.

Microscopic images on day 6 (144 hrs) showed a denser and visibly healthier culture containing less single cell algae interspersed mostly with clusters/stacks of 2-5 algae which may be indicative of cell division. Spines typical of healthy *D. intermedius* (1_C4) cells were clearly visible as was cell debris which may be due to cell death or cell division. Rod shaped bacteria and other predatory organisms were not visible in the culture at this stage (Figure 3.22, a, b).

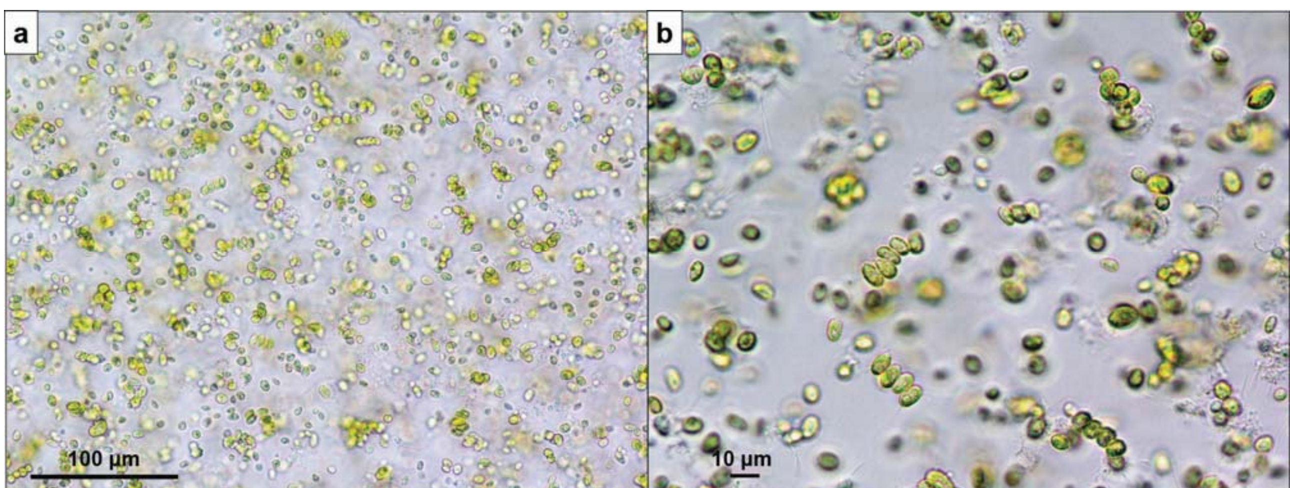

Figure 3.22: *D. intermedius* (1_C4) pond culture 144 hours after inoculation

Microscopic images on day 12 (288 hrs) showed single ovoid algal cells without clusters or aggregation. No spines could be seen on the cells. High amounts of cell debris were visible in the culture (Figure 3.23, a, b). Negligible numbers of predatory organisms were detected except for the occasional small and fast swimming *Paramecium.*

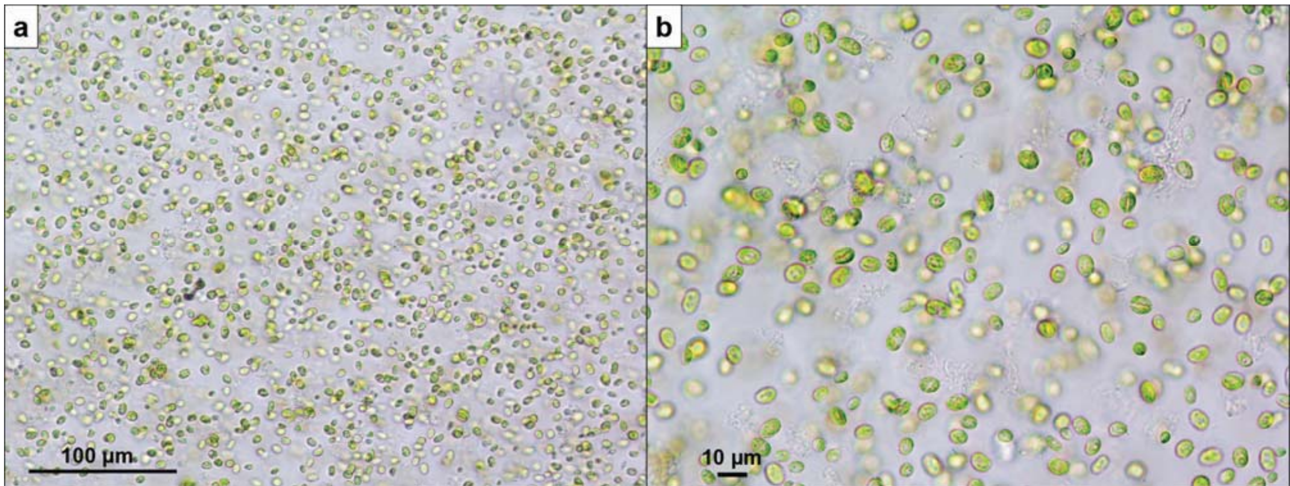

Figure 3.23: *D. intermedius* (1_C4) pond culture 288 hours after inoculation

In summary: *D. intermedius* (1_C4) appeared to be either ovoid or spiny in appearance during the growth phase and clusters of 2-5 cells may be indicative of cell division, though stress responses cannot be excluded. At day 4, the culture contained rod shaped bacteria which reduced in number as the algae culture density increased. This suggest that they were not seriously pathogenic to the algae. Foam was observed on the culture surface at the end of the cultivation period (Day 13 - 312 hrs) and coincided with the stationary phase.

***Chlorella sorokiniana* (8_C4)** cultivation data and conditions are illustrated in figure 3.24 (A and B). The cultivation was inoculated at a starting OD_{750} of 0.16, and the resulting growth curves based upon optical density and dry weight measurements are presented in figure 3.24 A (a, b) and figure 3.24 B (a). This species experienced a longer lag phase up until ~42 hours and then transitioned into a logarithmic growth phase up until ~144 hours. There was a continuance of slower growth until ~250 hours (OD750 = ~2.0).

The pH of the system mainly remained between 6.8 - 7.16 (Figure 3.24 B, c). This was supported by the fact that the CO_2 input stayed stable at a maximum of 0.3 to 0.5 L min^{-1} (Figure 3.24 B, c). The conductivity measured throughout the cultivation remained between 1.35 and 1.71 mS. The dissolved oxygen was measured at the beginning of the cultivation between 8 – 9 mg L^{-1} increasing constantly after 114 hours. A noticeable drop of dissolved oxygen to 6.7 mg L^{-1} was visible after 90 hours (Figure 3.24 B, d).
C. sorokiniana (8_C4) did exhibit extreme flocculation after 144 hours (supplementary data video 3.9).

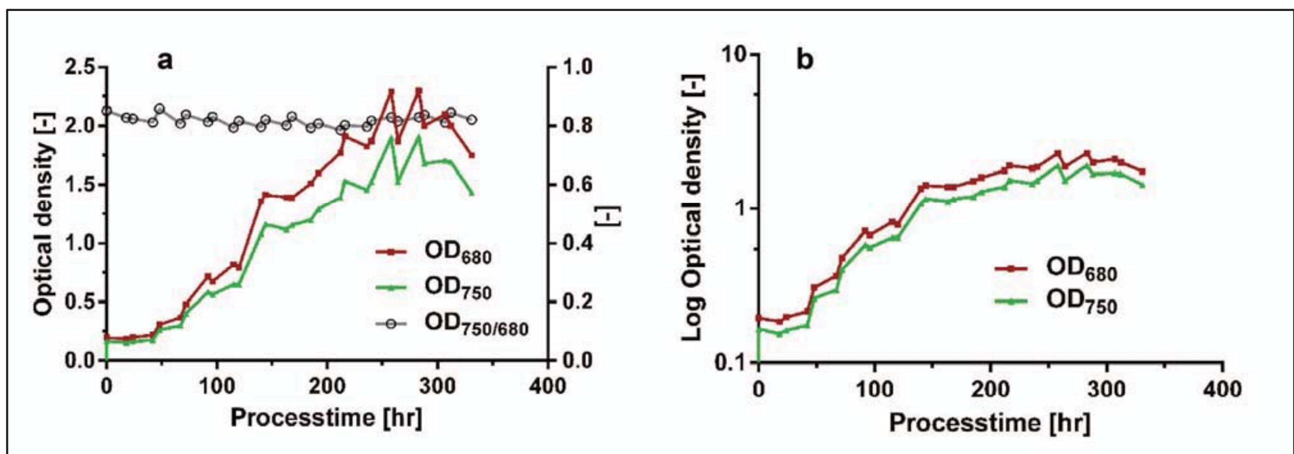

Figure 3.24 B: Culture performance and cultivation conditions of *C. sorokiniana* (8_C4): display of the growth curve based on optical density a) linear and b) logarithmic.

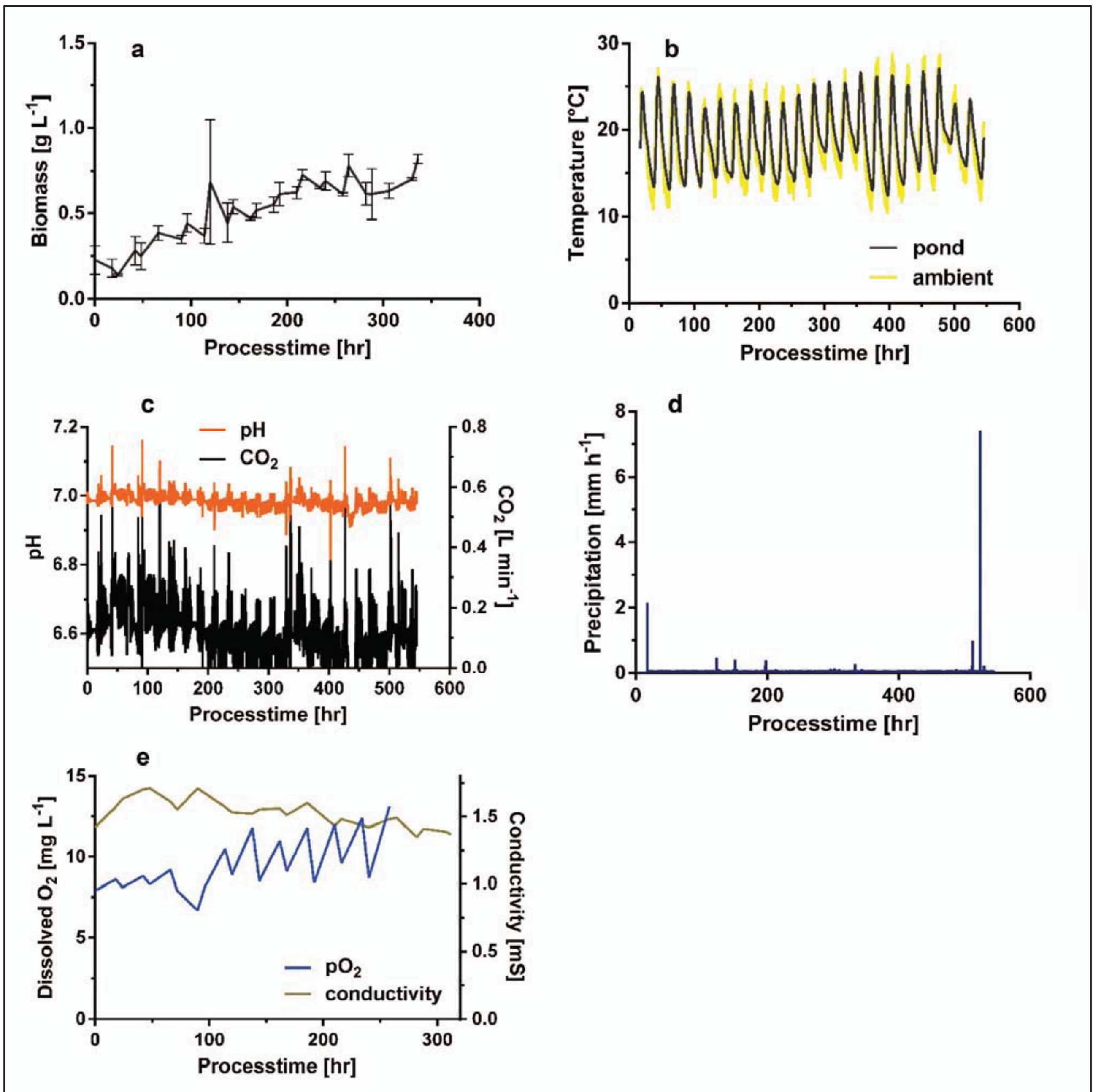

Figure 3.24 B: Culture performance and cultivation conditions of *C. sorokiniana* (8_C4): display of the a) growth curve based on biomass density. b) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle. c) measured pH of the algal culture and dependent CO_2 input. d) precipitation occurrence measured over the time course of the experiment. e) display of conductivity and dissolved oxygen.

Microscopic monitoring of the *C. sorokiniana* (8_C4) culture provided additional insights into culture performance. Images are provided at 96, 144 and 288, 408 and 432 hrs at low (x100) mid (x200) and high (x400) magnification.

Microscopic images on day 4 (96 hrs) showed healthy, low in numbers single cell algae cells. (Figure 3.25). Amoeba organisms were visible in the culture

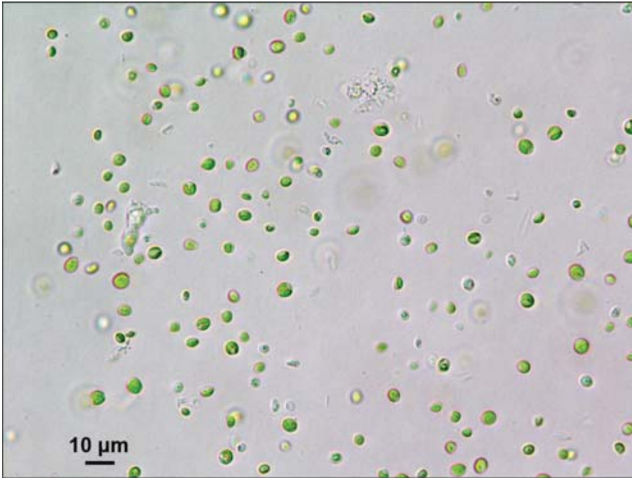

Figure 3.25: *C. sorokiniana* (8_C4) pond culture 96 hours after inoculation.

Microscopic images on day 6 (144 hrs) showed a much denser culture containing single cell algae interspersed with thick algae flocs. Coccoid shaped bacteria, mostly together in clusters were visible. Within the algae flocs a transparent substance (e.g. debris) was present between the cells. Predatory amoeba organisms (supplementary data video 3.10) and a very high number of the ciliate type *Tetrahymena sp.* moved between the algae cells. (Figure 3.26, c). *Tetrahymena sp.* movement was very fast and occasionally they were seen connected to pairs (Figure 3.26, d indicated by a red arrow) (supplementary data video 3.11).

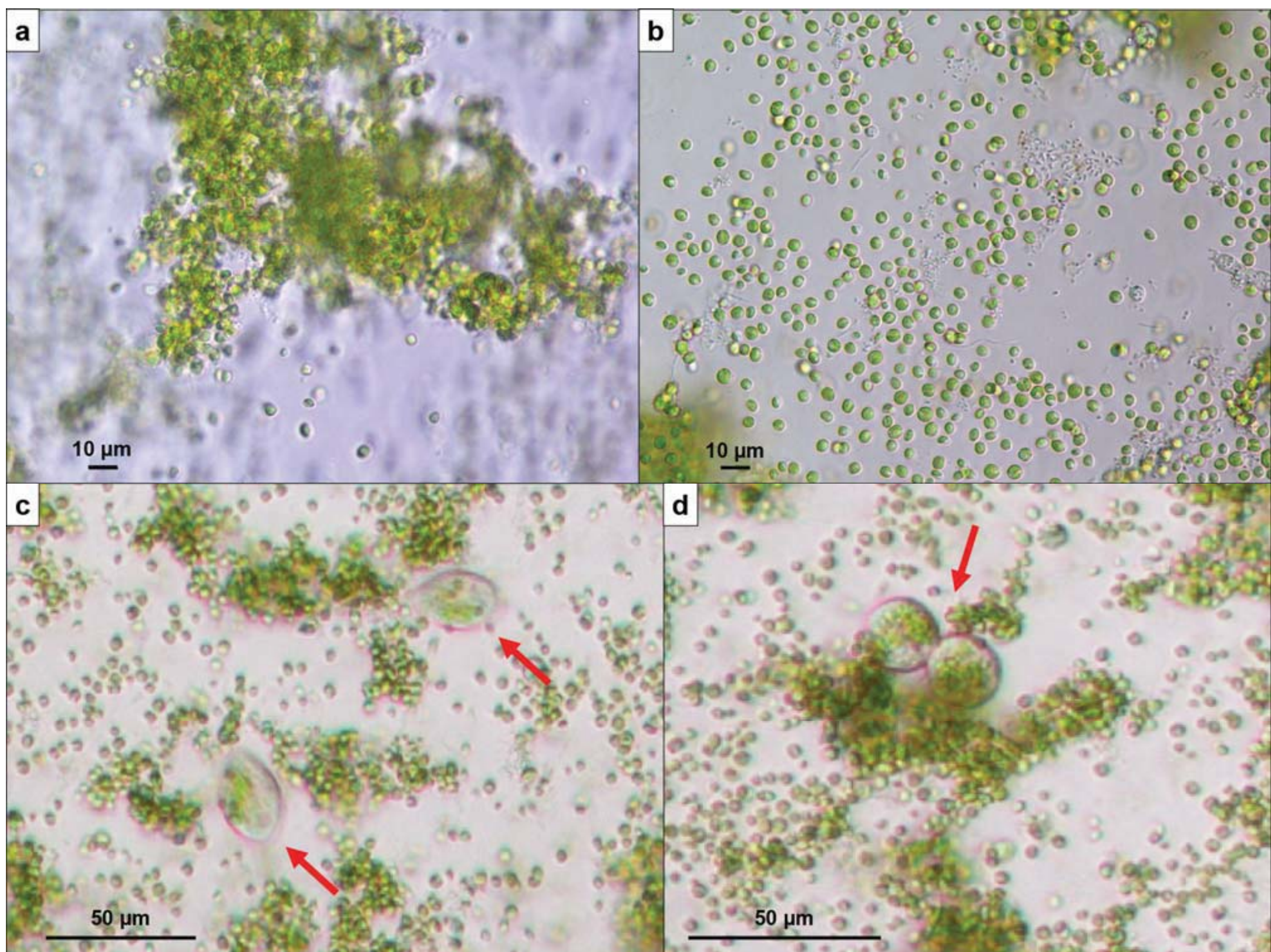

Figure 3.26: *C. sorokiniana* (8_C4) pond culture 144 hours after inoculation a) dense algae floc of *C. sorokiniana* (8_C4). b) dispersed algal cells including bacteria contamination. c) fast moving ciliates presumably *Tetrahymena* (indicated by a red arrow), d) connection occurring between ciliate cells (indicated by a red arrow).

Microscopic images on day 12 (288 hrs) showed an increased aggregation of algal cells forming big flocs. No previous detected ciliate could be seen in the culture. Occasionally small cells with flagella were visible (Figure 3.27).

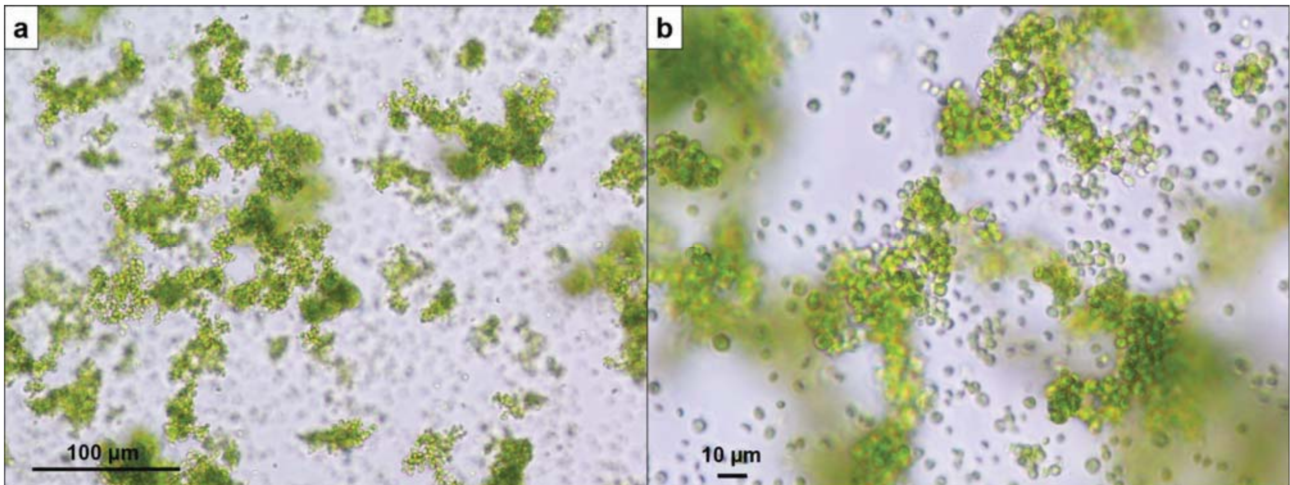

Figure 3.27: *C. sorokiniana* (8_C4) pond culture 288 hours after inoculation.

Microscopic images on day 17 (408 hrs) showed a decrease of the aggregation. More algae flocs but smaller in size were present including many single cell algae interspersed. (Figure. 3.28, a, b).

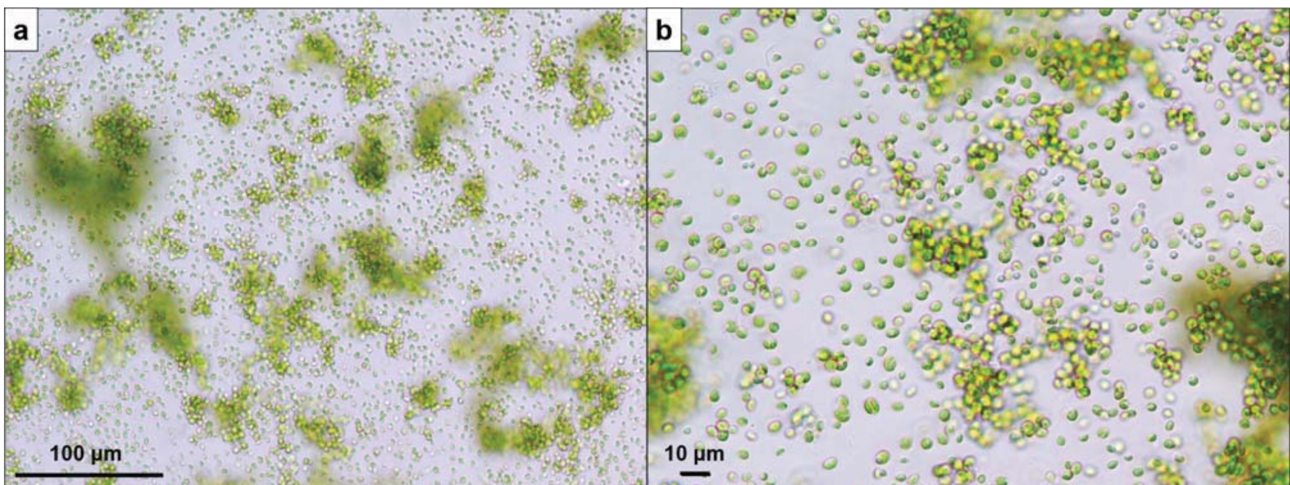

Figure 3.28: *C. sorokiniana* (8_C4) pond culture 408 hours after inoculation.

Microscopic images on day 18 (432 hrs) at the end of the experiment the culture contained mostly of thick but healthy looking algae aggregations, increased in density and size with clearly less single cells in between (Figure. 3.29, a, b). Occasionally *Chlamydomonas* like flagellates and *Tetrahymena* ciliates were visible without notable impact on the algae culture.

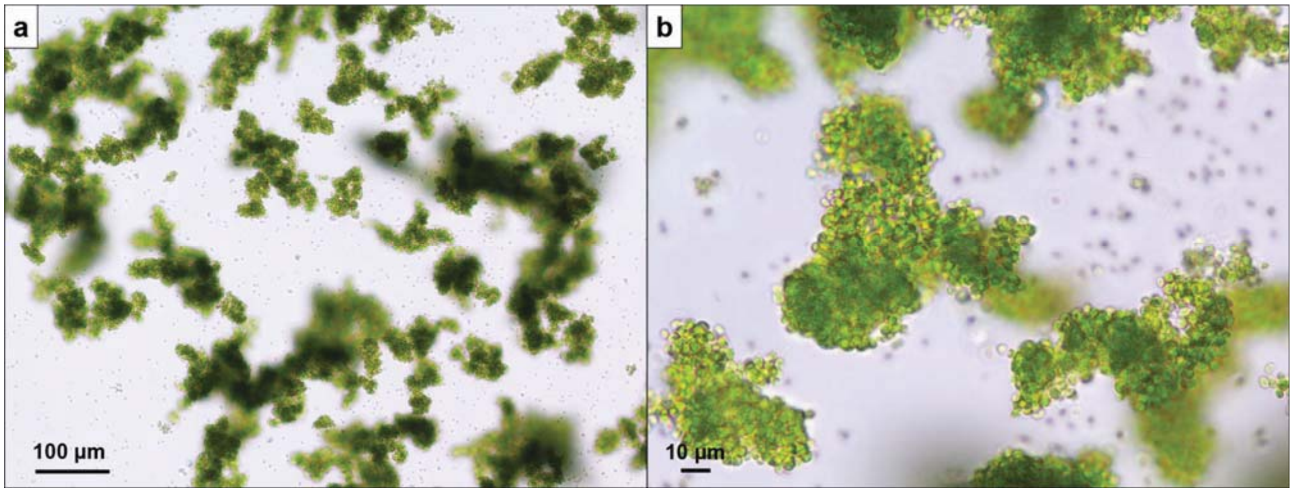

Figure 3.29: *C. sorokiniana* (8_C4) pond culture 432 hours after inoculation.

In summary: *C. sorokiniana* (8_C4) appeared to be susceptible to a range of bacteria and predators (amoebae, flagellates and ciliates). However a healthy culture developed throughout the cultivation with no significant grazing impact of the predators detected. A high amount of the ciliate *Tetrahymena* (morphological identified) was observed after 144 hours and was no longer detected after 288 hours. Starting from day 6 (144 hrs) the algal cells showed increased flocculation which intensified and persisted until the end of the cultivation.

Winter Cultivations

***Chlorella pyrenoidosa* (10_B9)** cultivation data and conditions are illustrated in figure 3.30 (A and B). The cultivation was inoculated at a starting OD_{750} of 0.1, and the resulting growth curves based upon optical density measurements are presented in figure 3.30 A (a-b). This species experienced a short lag phase up until ~18 hours and then transitioned into a logarithmic growth phase up until ~114 hours. There was a continuance of slower growth until ~310 hours (OD_{750} = ~1.8).

Rainfall levels were low overall with the highest rainfall event (31.4 mm h^{-1}) occurring after 364 hours (Figure 3.30 B, e). The pH of the system remained between 6.9 - 7.1 (Figure 3.30 B, d) except for a pH spike of 7.4 at 326 hours. The CO_2 input remained stable at a maximum around 0.3 L min^{-1} except for several spikes (0.6 L min^{-1}) between 60-100 hours to regulate pH during the growth phase and at 325 hours which was corresponding to the pH peak due to a set up agent fault (Figure 3.30 B, d). No dissolved oxygen and conductivity data are available for the cultivation.

C. pyrenoidosa (10_B9) was observed to start dying after 300 hours indicated by the drop of OD_{750} and OD_{680}. No biomass dry weight data is available for this cultivation.

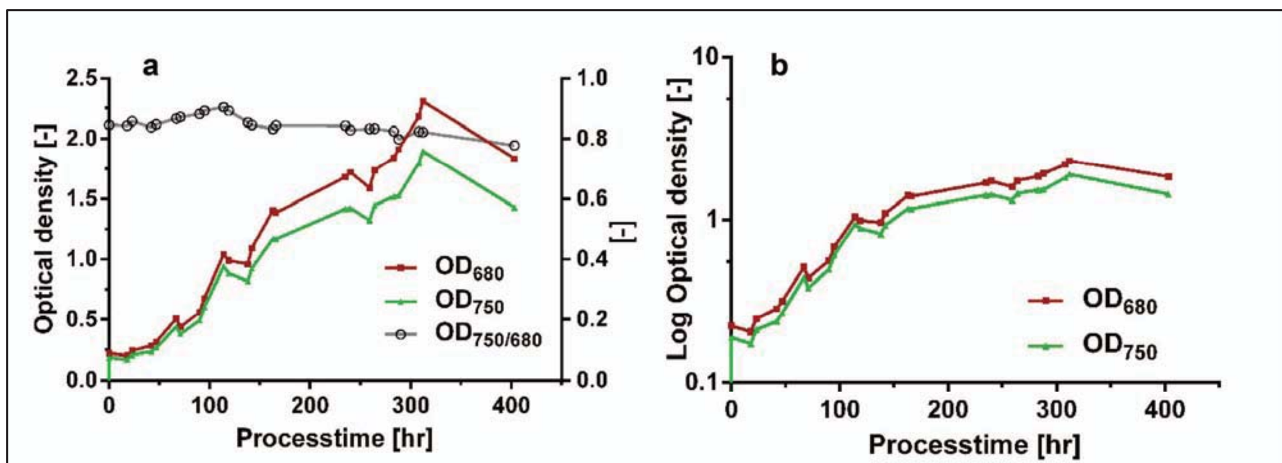

Figure 3.30 A: Culture performance and cultivation conditions of *C. pyrenoidosa* (10_B9): display of the growth curve based on optical density a) linear and b) logarithmic.

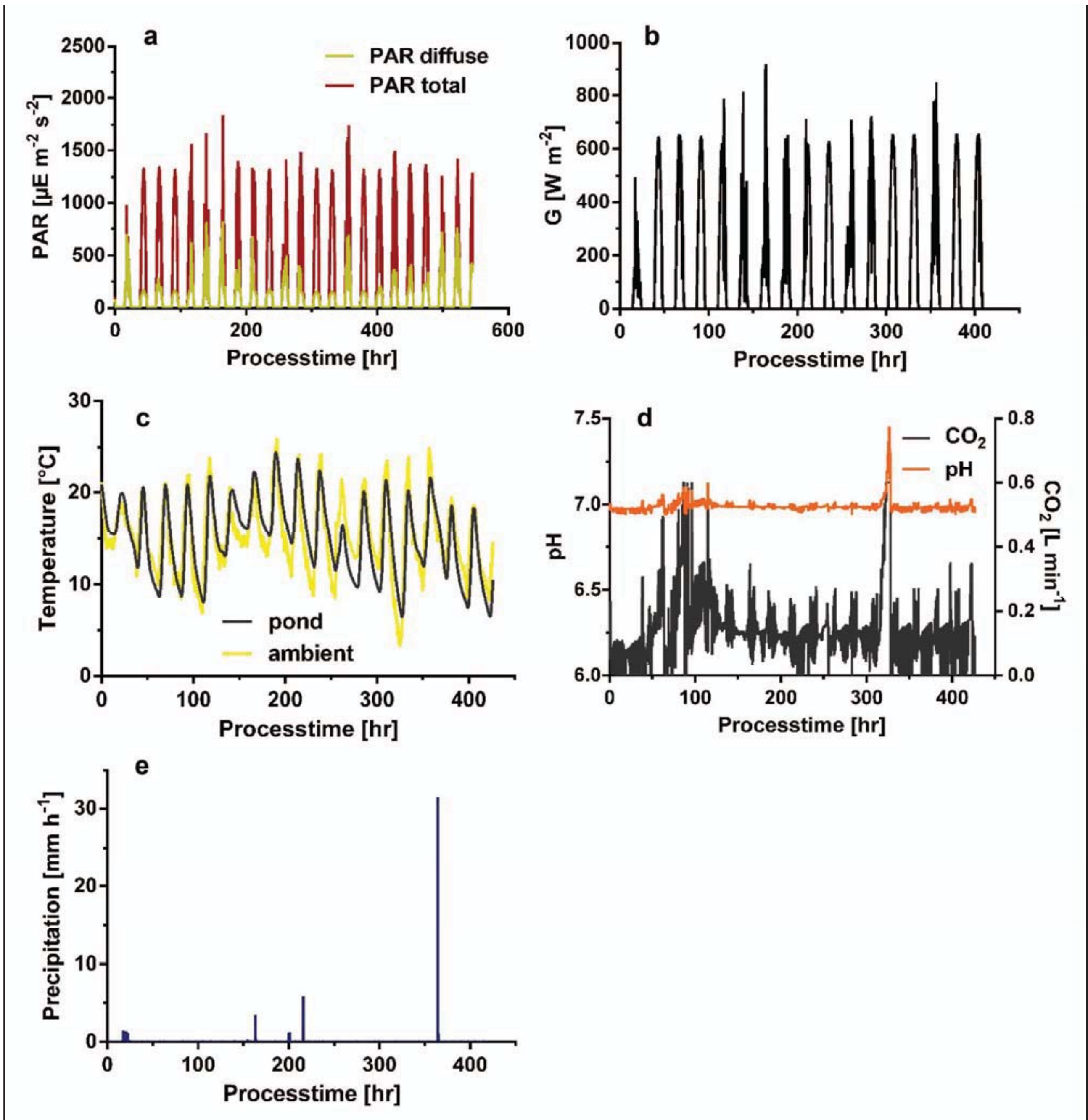

Figure 3.30 B: Culture performance and cultivation conditions of *C. pyrenoidosa* (10_B9): display of the a) total and diffuse photosynthetically active radiation and b) global solar radiation. c) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle. d) measured pH of the algal culture and dependent CO_2 input. e) precipitation occurrence measured over the time course of the experiment.

Microscopic monitoring of the *C. pyrenoidosa* (10_B9) culture provided additional insights into culture performance. Images are provided at 119 and 403 hrs at low (x200) and high (x400) magnification.

Microscopic images on day 5 (119 hrs) showed healthy single cell algae species with no predatory organisms detected. Larger oval shaped non-target algae cells were visible (examples indicated by a red arrow), however not dominating the culture (Figure. 3.31, a, b). Visible foam development on the water surface on day 6 (142 hrs) correlated to an increased growth in algal cells.

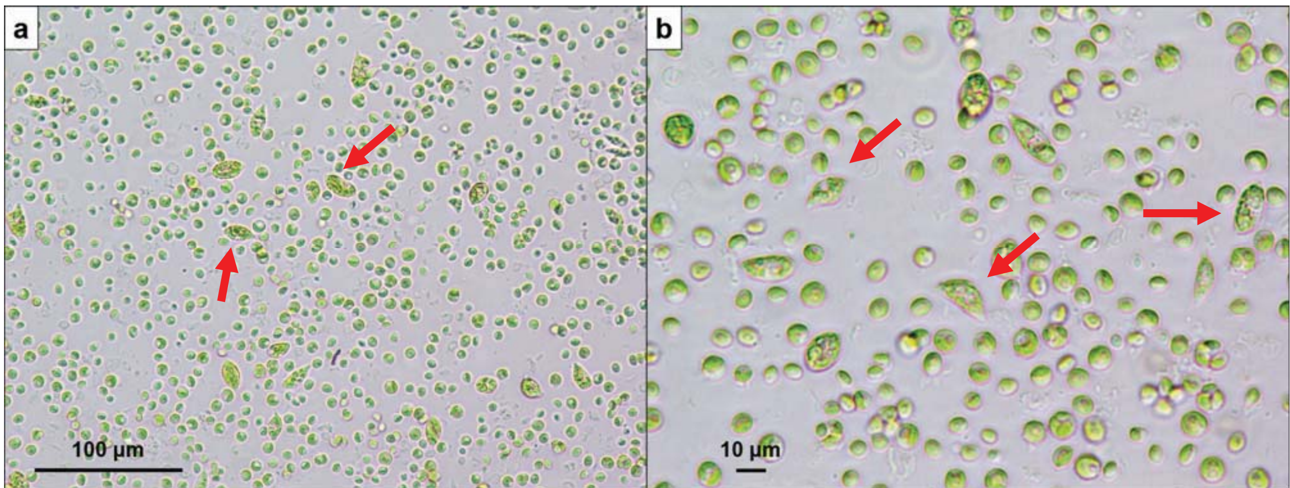

Figure 3.31: *C. pyrenoidosa* (10_B9) pond culture 119 hours after inoculation.

Microscopic images on day 12 (403 hrs) showed a very dense and healthy green culture. Besides a minor number of oval to sickle shaped algal cells, no other contaminating organisms were detectable. (Figure. 3.32, a, b).

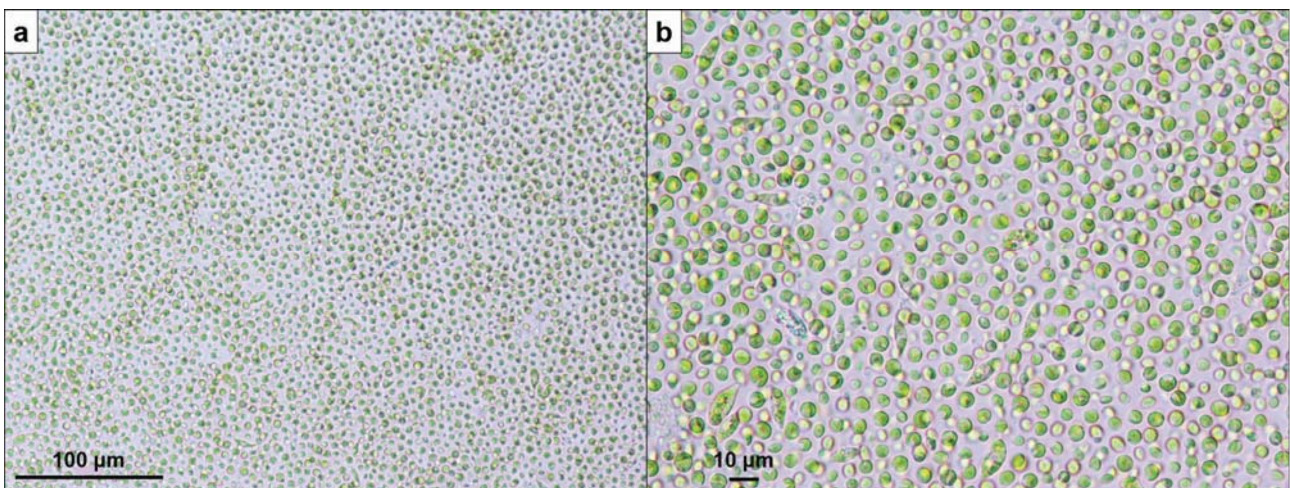

Figure 3.32: *C. pyrenoidosa* (10_B9) pond culture 403 hours after inoculation.

In summary: *C. pyrenoidosa* (10_B9) appeared to be robust in that it exhibited low levels of bacterial or predator contamination. Non-target algal cells established themselves from day 5 of the cultivation, however no major impact on the growth of *C. pyrenoidosa* (10_B9) was detected and no outcompeting of the target algae occurred. Foam was observed on the culture surface at the end of the logarithmic phase of the cultivation (day 6, 142 hours) assuming from algae protein released during cell division. Furthermore no cross contamination from the culture *C. sorokiniana* (15_E4) with *Tetrahymena* like ciliates was detected despite the proximity to other ponds where *Tetrahymena* populations had established.

***Chlorella sorokiniana* (15_E4)** cultivation data and conditions are illustrated in figure 3.33 (a-d). The cultivation was inoculated at a starting OD_{750} of 0.1, and the resulting growth curves based upon optical density measurements are presented in figure 3.33 (a-b). This species experienced a short lag phase up until ~18 hours and then transitioned into a logarithmic growth phase up until ~71 hours. There was a continuance of slower growth until ~260 hours (OD_{750} > 2.0). At ~312 hours the cultivation went into gradual decline. No dissolved oxygen and conductivity data are available for the cultivation.

During the cultivation the pH experienced several peaks of up to pH = 8 (Figure 3.33, c). This is due to a system fault of the set up agent system (breaking of the ammonia dispenser increased set up agent flow into the culture).

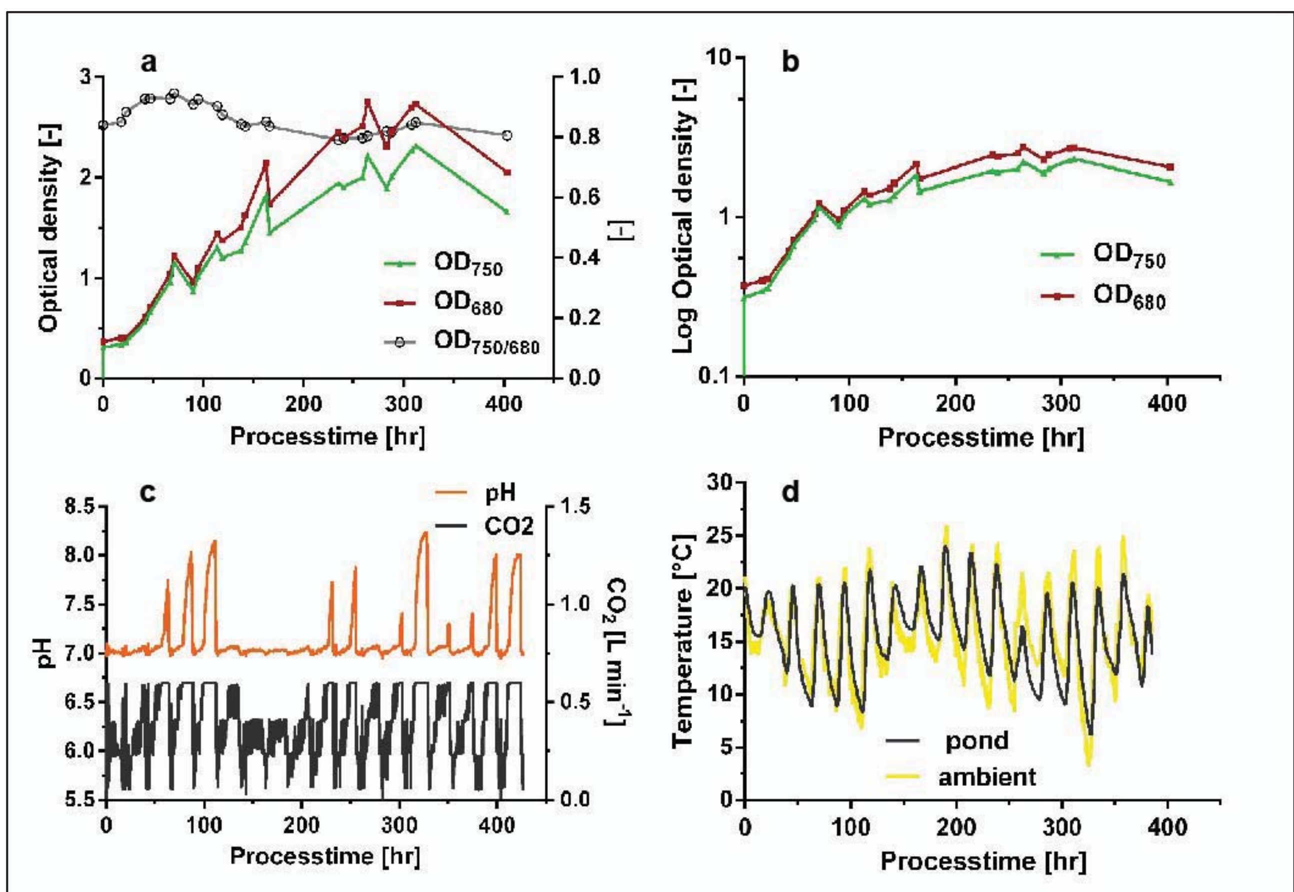

Figure 3.33: Culture performance and cultivation parameters of *C. sorokiniana* (15_E4): display of the growth curve based on optical density a) linear and b) logarithmic. c) measured pH of the algal culture and dependent CO_2 input. d) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle.

Microscopic monitoring of the *C. sorokiniana* (15_E4) culture provided additional insights into culture performance. Images are provided at 119 and 403 hrs at mid (x200) and high (x400) magnification.

Microscopic images on day 5 (119 hrs) showed round single cell algae interspersed with some debris (Figure 3.34, a, b). No contamination or predatory organisms were visible.

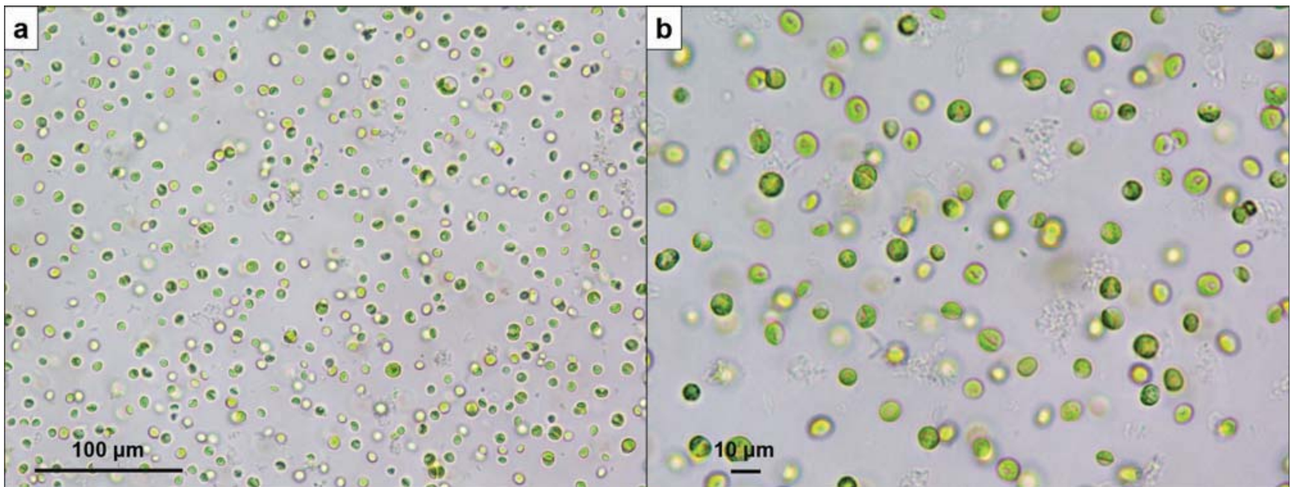

Figure 3.34: *C. sorokiniana* (15_E4) pond culture 119 hours after inoculation.

Microscopic images at the end of the experiment on day 14 (403 hrs) showed besides a denser algae culture a high amount of debris flocs and possible fungi contamination (Figure 3.35, a, b). Some algae were trapped within the debris flocks. A high amount of the ciliate protozoa *Tetrahymena* (morphological identified, indicated by a red arrow) was visible grazing throughout the culture (Figure 3.35, c) (supplementary data video 3.12). Occasionally tiny fast swimming *Paramecium* were observed.

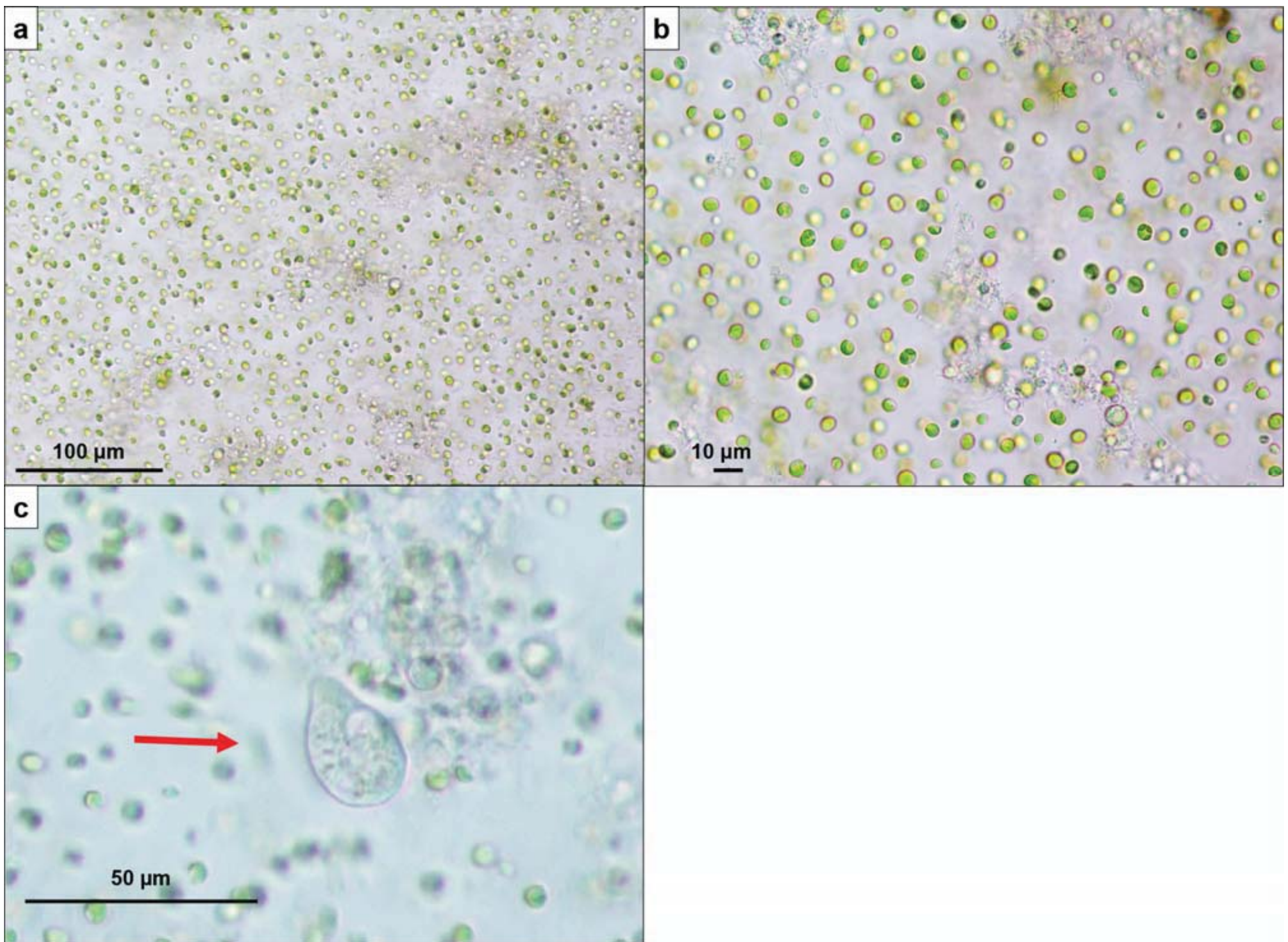

Figure 3.35: *C. sorokiniana* (15_E4) pond culture 403 hours after inoculation.

In summary: *C. sorokiniana* (15_E4) appeared to be round single cell algae in appearance with a relatively constant amount of debris within the culture. During the cultivation a high amount of ciliates established themselves in the algae broth similar to predator organisms found during the cultivation of 8_C4. However no flocculation occurred and no significant evidence of negative impact on the algal population from the contaminants was found.

Chlorella sorokiniana **(12_A9)** cultivation data and conditions are illustrated in figure 3.36 (A and B). The cultivation was inoculated at a starting OD_{750} of 0.1, and the resulting growth curves based upon optical density measurements are presented in figure 3.36 A (a-b). This species experienced a lag phase up until ~23 hours and then transitioned into a logarithmic growth phase up until ~ 71 hours. There was a continuance of slower growth until ~260 hours (OD_{750} > 2.0). At ~312 hours the cultivation went into gradual decline.

The pH of the system remained stable between 6.9 - 7.1 with an insignificant single peak of 7.2 at around 90 hours. This is supported by the fact that the CO_2 input remained stable (Figure 3.36 B, b, maximum of 0.3 – 0.6 L min^{-1}). No conductivity data are available for the cultivation. The dissolved oxygen measured throughout the cultivation showed a strong circadian rhythm mainly between 95 - 120 % saturation, with a maximum of 136% at around 164 hours. A noticeable drop of dissolved O_2 can be seen at around 157 hours down to 64% saturation.

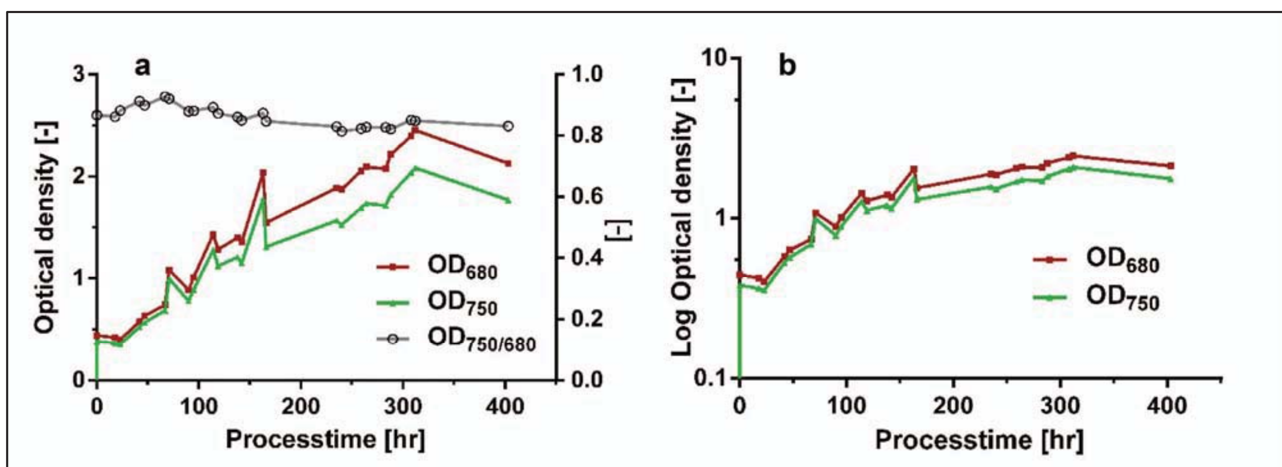

Figure 3.36 A: Culture performance and cultivation conditions of *C. sorokiniana* (12_A9): display of the growth curve based on optical density a) linear and b) logarithmic.

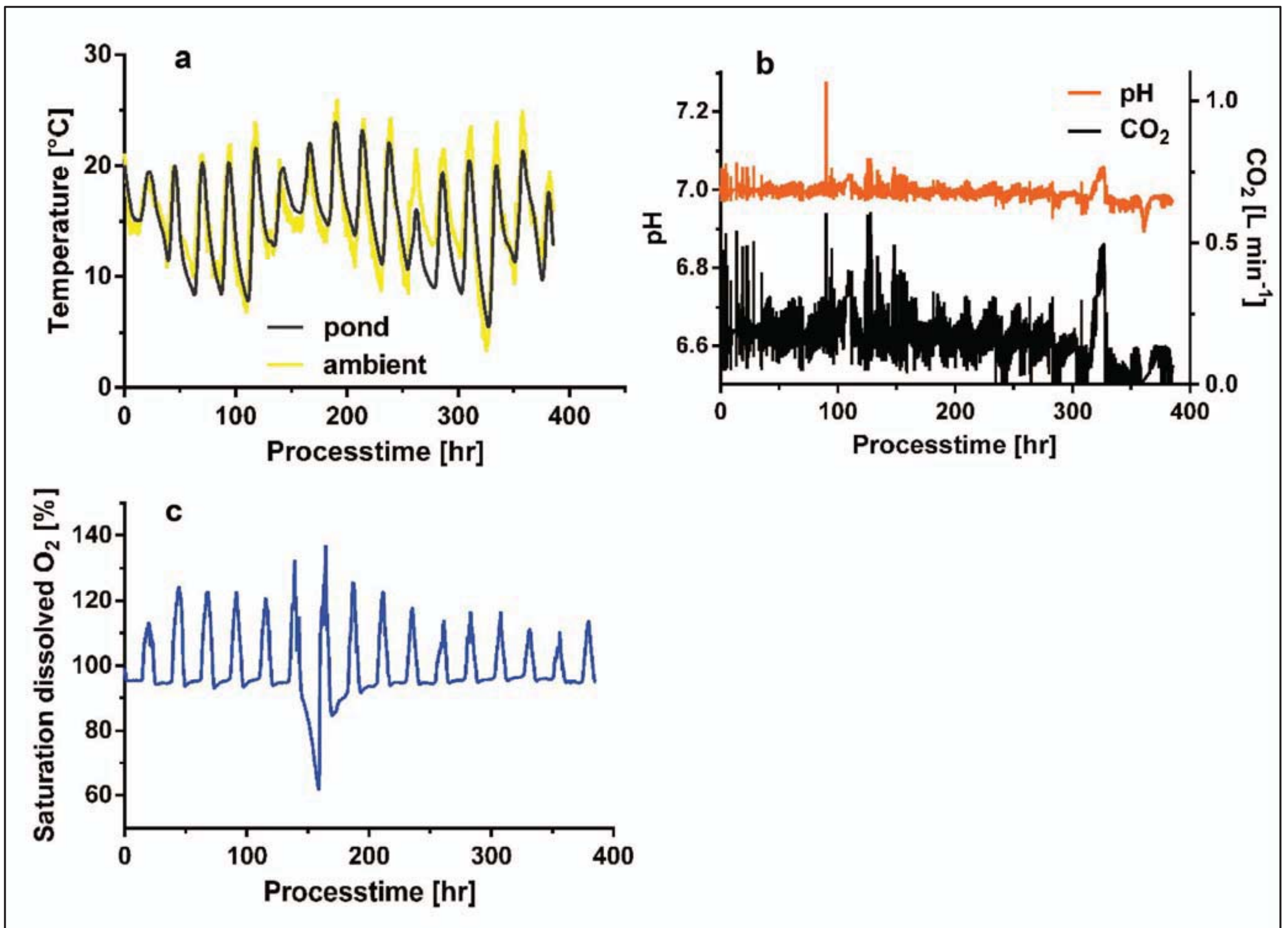

Figure 3.36 B: Culture performance and cultivation conditions of *C. sorokiniana* (12_A9): display of the a) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle. b) measured pH of the algal culture and dependent CO_2 input. c) display dissolved oxygen.

Microscopic monitoring of the *Chlorella sorokiniana* (12_A9) culture provided additional insights into culture performance. Images are provided at 119 and 403 hrs at low (x200) and high (x400) magnification.

Microscopic images on day 5 (119 hrs) showed a dense culture of variably sized single oval shaped algal cells. No predator or other contaminants were visible in the culture (Figure 3.37, a, b). A small amount of debris was present between the cells.

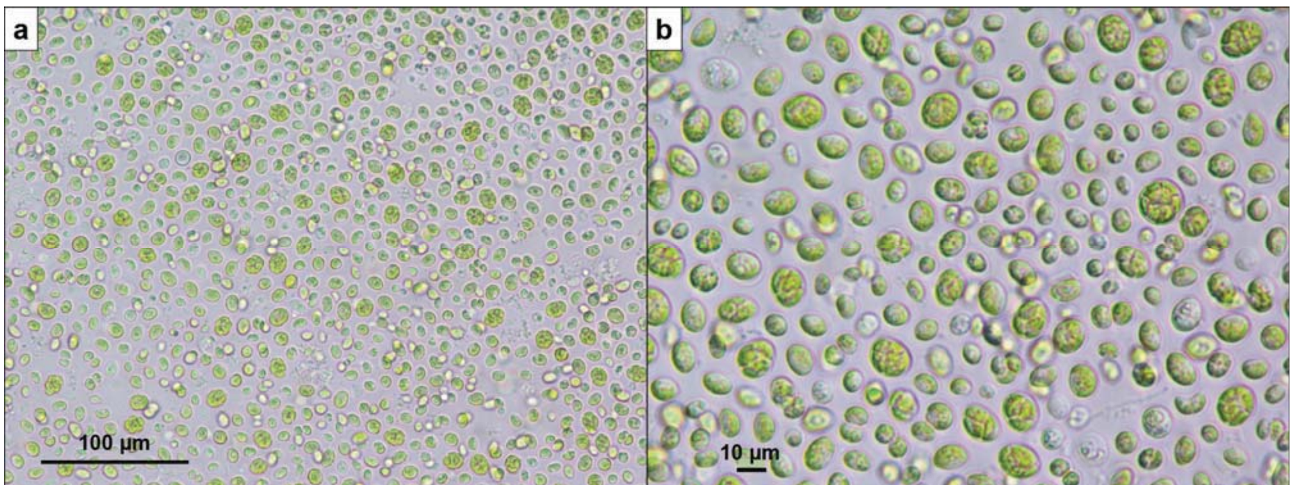

Figure 3.37: *C. sorokiniana* (12_A9) pond culture 119 hours after inoculation.

Microscopic images at the end of the experiment on day 14 (403 hrs) showed a denser and healthy green culture containing same size single cell algae. More debris was dispersed in the culture. Single cell contaminants with flagella were detected in the culture, noticeable by creating an empty cell circle around themselves (Figure 3.38, a, b).

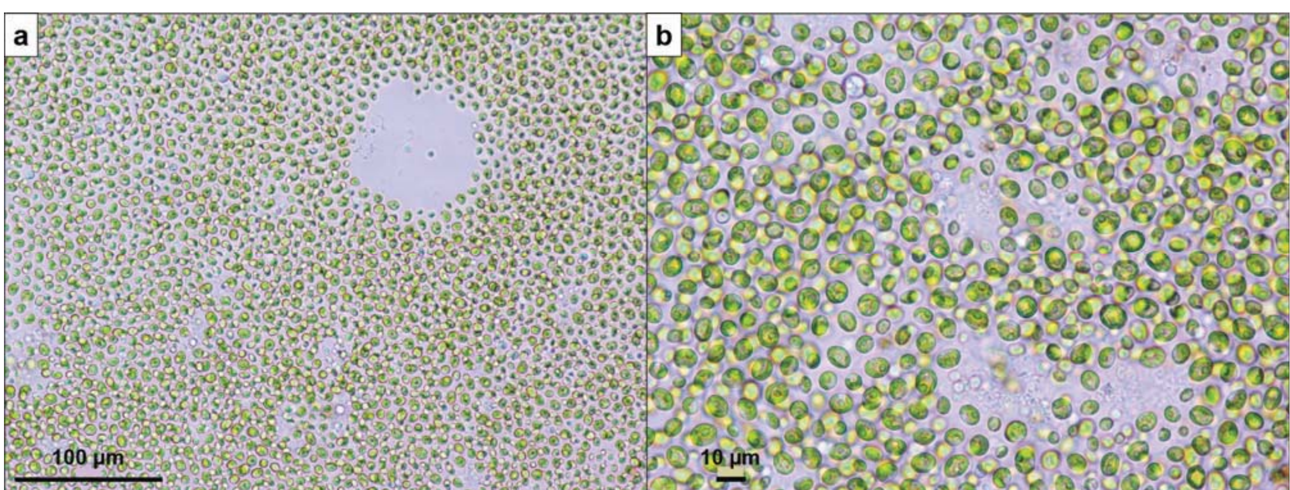

Figure 3.38: *C. sorokiniana* (12_A9) pond culture 403 hours after inoculation.

In summary: *C. sorokiniana* (12_A9) appeared to be an ovoid cell type with slightly different sizes during the growth phase and uniform size in the stationary phase. The culture showed only minor predator contamination of flagellates which seemed to be of no harm or influence on the culture growth. Furthermore no cross contamination from the culture *C. sorokiniana* (15_E4) with *Tetrahymena* like ciliates was detected despite their side by side arrangement of the ponds.

Spring Cultivations

3.3.4 Multiple species (polyculture) experiments

Following the monoculture experiments, two parallel polycultures were established. These contained equal cell density of *Desmodesmus intermedius* (1_C4), *Micractinium pusillum* (5_H4), *Chlorococcum sp.* (12_02), *Chlorella sorokiniana* (12_A9) and *Chlorella sp.* (11_H5). Cultivation data for both ponds and conditions are illustrated in figure 3.39 (A and B). The cultivation was inoculated at a starting OD_{750} of 0.1, and the resulting growth curves based upon optical density and biomass dry weight measurements are presented in figure 3.39 A, (a-d) and figure 3.39 B, (a). These cultures both rapidly transitioned into a logarithmic growth phase up until ~ 94 hours. There was a continuance of slower growth until ~312 hours and both cultures achieved high optical density (OD_{750} = ~3-4).

The weather conditions during the time of cultivation stayed mostly sunny and hot and heavy rainfall occurred only during the last 3 days (~ 281 – 335 hours). The pH of the two ponds remained mainly between 6.9 – 8, except for two pH peaks of 9.5 at ~ 160 hours and 229 hours which was due to a control system fault (Figure 3.39 B, c). This is supported by the fact that the CO_2 input remained stable except for the outage at 136 and 215 hours and an increased input of CO_2 at 162 and 232 hours to stabilise the pH (Figure 3.39, c) CO_2 average min ~ 0.029-0.169 and max ~ 0.3 L min^{-1}). Towards the end of the cultivation the average pH maximum slightly increased resulting in a simultaneous increase of CO_2 input to the culture. The conductivity of both ponds was measured throughout the cultivation period and started between 1.18 (pond 1) and 1.0 mS (pond 2) and as expected declined throughout the experiment to 0.92 (pond 1) and 0.86 mS (pond 2), as the nutrients were depleted. The dissolved oxygen values measured were overall higher in pond 1 and reached its maximum of 277 % saturation on day 4 and was declining towards the end of the cultivation. In pond 2 dissolved oxygen measurements remained lower at a maximum of 170 % saturation during the day and remained stable over the time period over cultivation. The minimum pO_2 saturation dropped in pond 1 to 76 % and in pond 2 to 66 % during the night time.

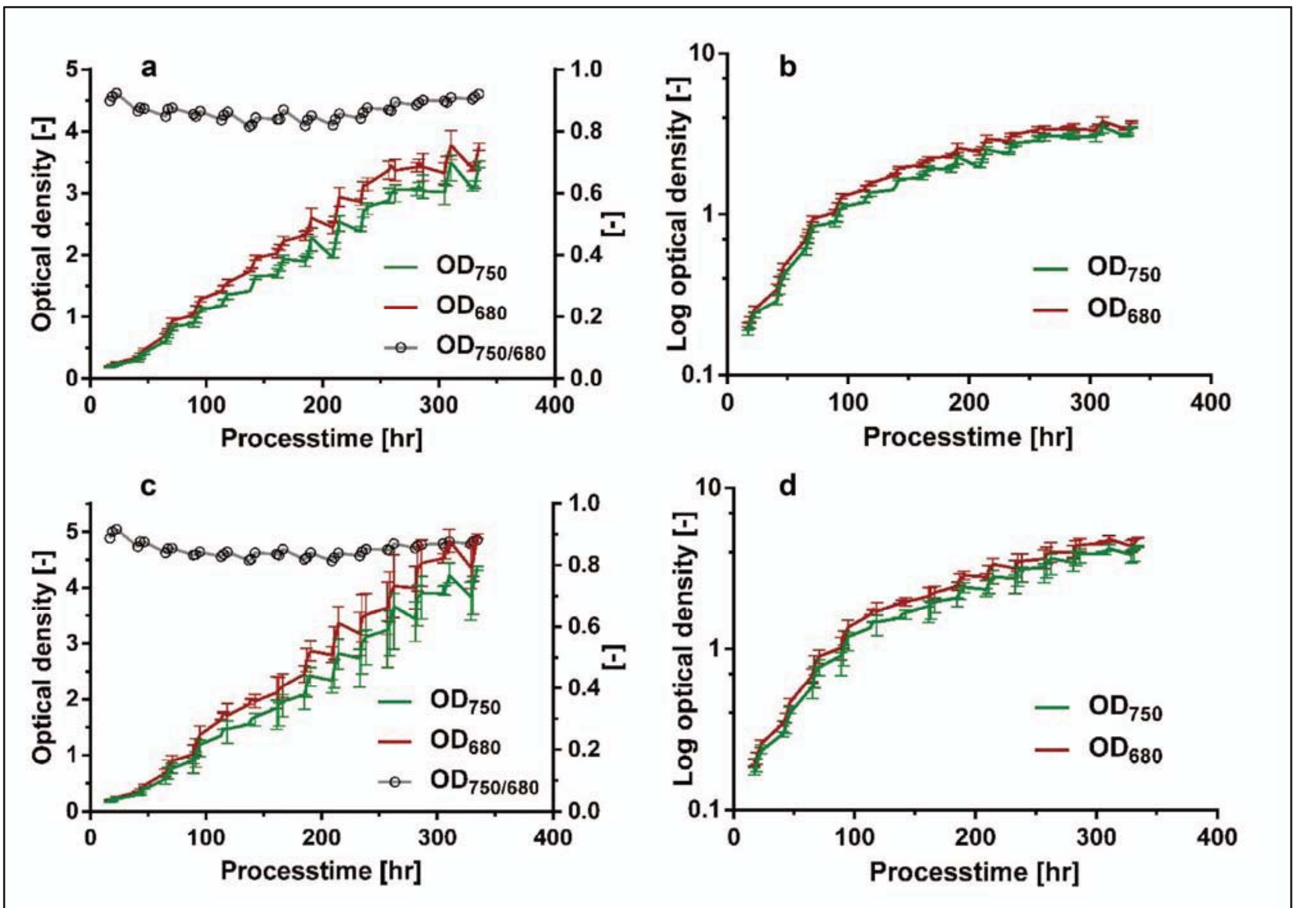

Figure 3.39 A: Culture performance and cultivation conditions of the polyculture pond duplicates: display of the growth curve based on optical density in pond 1 (a) linear, b) logarithmic) and pond 2 (c) linear, d) logarithmic).

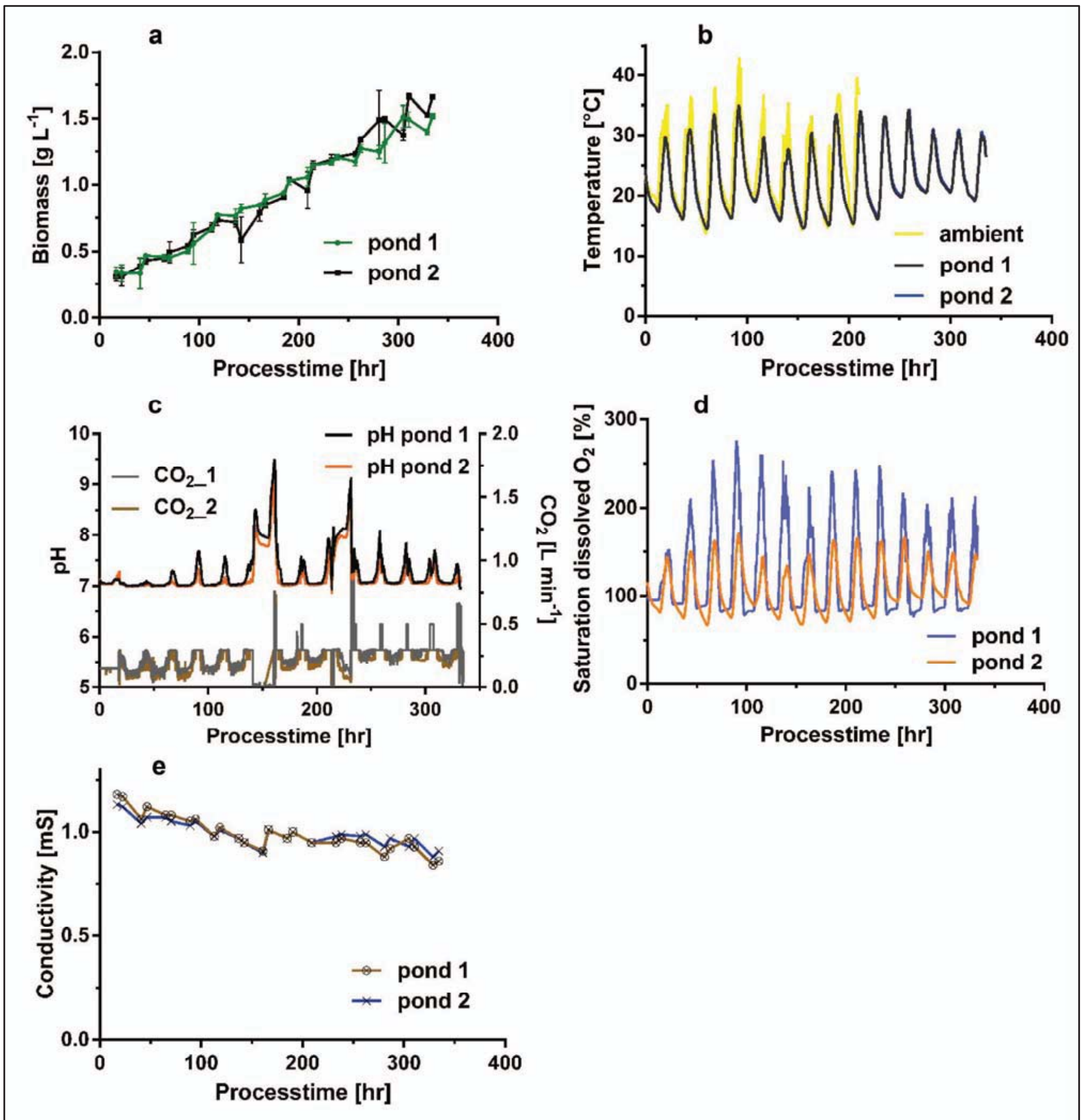

Figure 3.39 B: Culture performance and cultivation conditions of the polyculture pond duplicates: display of the a) growth curves of pond duplicates based on biomass density. b) pond culture and ambient temperature over the time course of cultivation, fluctuation during day and night cycle. c) measured pH of the algal culture and dependent CO_2 input. d) dissolved oxygen and e) display of conductivity of both systems.

Cultivation Data from parallel Flasks experiment

Duplicate polyculture flasks (5 litre Erlenmeyer flasks, culture volume 2 litre, indoor cultivation, air permeable sealed, shaken, no additional CO_2 input) established from the outdoor pond cultivations had the same starting OD_{750} of 0.1. Cultivation data and conditions for both flasks are illustrated in figure 3.40 (A and B). The resulting growth curves based upon optical density and biomass dry weight measurements are presented in figure 3.40 A (a, d) and figure 3.40 B (a). These cultures experienced a logarithmic growth phase up until ~46 hours. Although they did exhibit higher than expected maximum growth rates, they did not achieve high optical density before entering the stationary phase.

The culture temperatures of the two flasks ranged between 25 to 28.5 °C (Figure 3.40, b). Illumination was at 24 hours per day and light intensity remained constant at 400 μE m^{-2} s^{-1}. The pH of the two flasks cultures remained between 6.9 to 7.8 (Figure 3.40, c). The conductivity of both flasks was measured throughout the cultivation period and stayed constant between 1.08 and 0.93 mS.

A high amount of debris and flocculation of the morphologically distinct *Chlorococcum sp.* (12_02) strain as well as many kinds of predators were observed which influenced growth of the culture.

The polyculture in the flask experiment proved to be somewhat robust against the high levels of predator contamination in that no culture crash occurred during the time of cultivation. However growth and productivity was significantly reduced by the lower levels of CO_2 in the flasks and by the increased activity of the predatory microorganisms present.

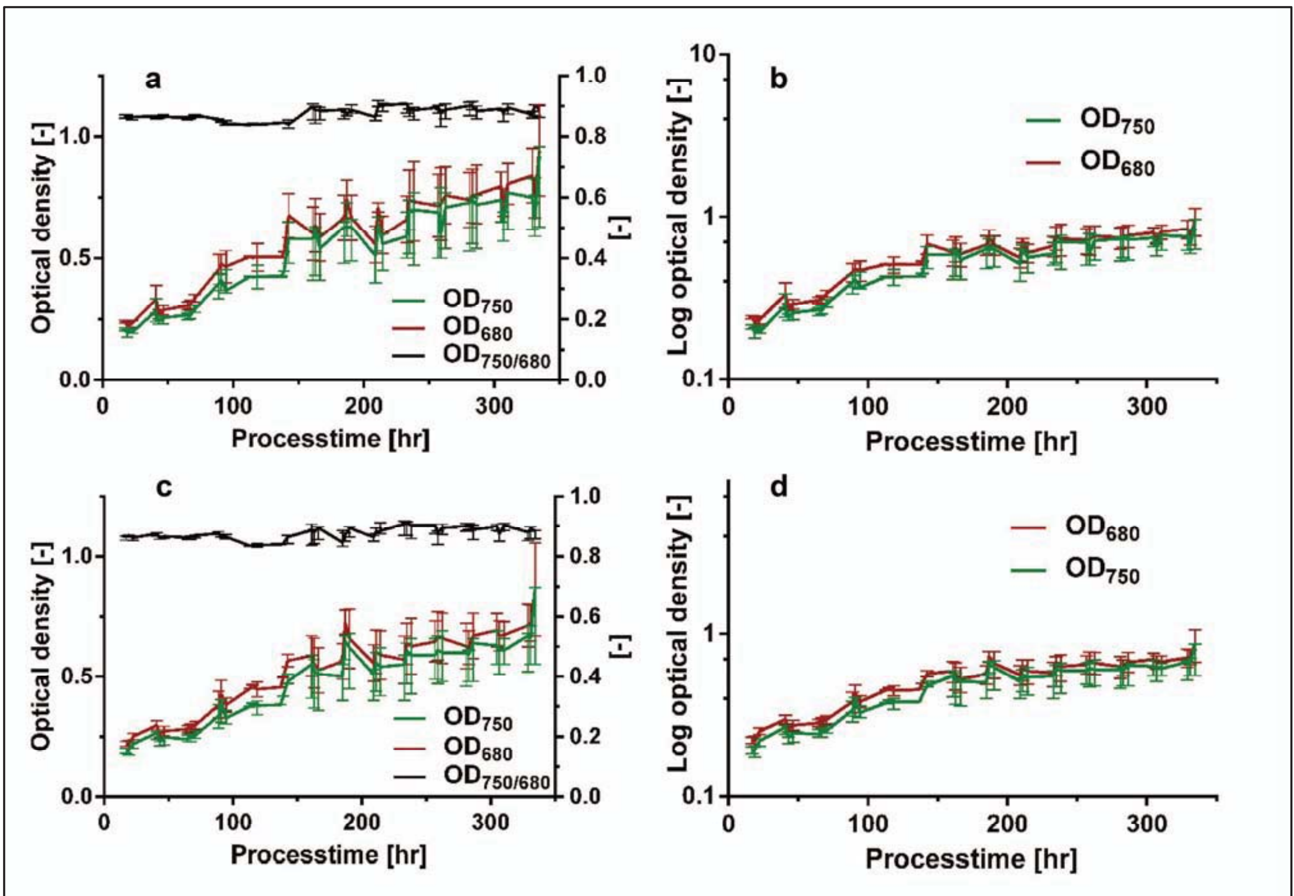

Figure 3.40 A: Culture performance and cultivation conditions of the polyculture flask duplicates: display of the growth curve based on optical density in flask 1 (a) linear, b) logarithmic) and flask 2 (c) linear, d) logarithmic).

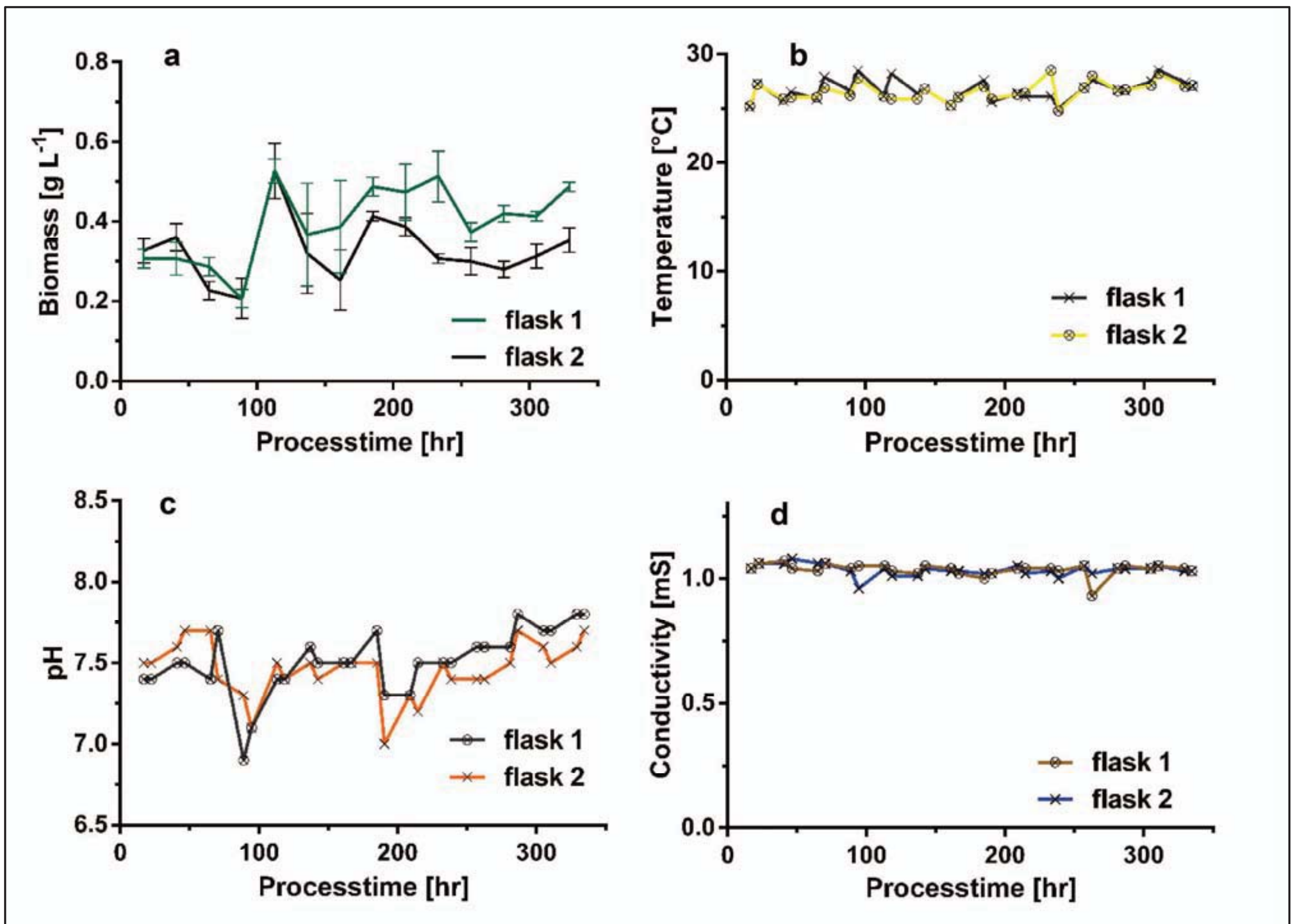

Figure 3.40 B: Culture performance and cultivation conditions of the polyculture flask duplicates: display of the a) growth curves of flask duplicates based on biomass density. b) flask culture temperature over the time course of cultivation. c) measured pH of the flask culture. d) display of conductivity.

Strain distribution and shifts in populations

Microscopy open pond culture

Monitoring via microscopic observation of the communal populations in open pond polycultures provided additional insights into culture performance and was conducted at 10 am and 3:30 am each day. Images in figure 3.40-45 are provided at the time points 70.5, 142.5, 190.5, 238.5, 262.5 and 334.5 hours at low (x200) and high (x400) magnification.

Microscopic images on day 3 (70.5 hrs) allowed the detection of all algae species in both ponds due to their morphological diversity. Furthermore no major culture shifts and strain dominance was observed (Fig 3.41, a, b).

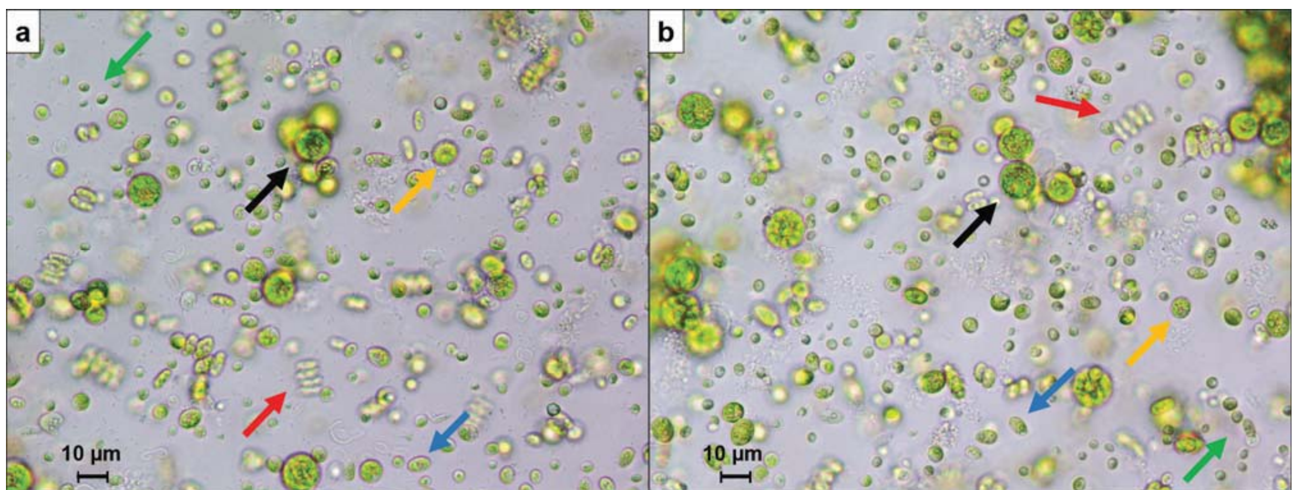

Figure 3.41: Pond polyculture 70 hours after inoculation. a) pond 1, b) pond 2. Algae cells of different species are indicated by coloured arrows: 1_C4 red, 12_A9 blue, 12_02 black, 5_H4 orange, 11_H5 green.

Microscopic images on day 6 (142.5 hrs) showed a more dense and healthy culture in both ponds, which contained a high amount of small coccoid algal cells indicating *Chlorella sp.* (11_H5) as one of the dominating strains, consistent with its previously reported fast growth rate. *Chlorococcum sp.* (12_02) showed the anticipated flocculation, as well as big algal cells which disaggregated and released small motile cells. A slightly higher amount of *Chlorococcum sp.* (12_02) was observed in pond 1 compared to pond 2. *M. pusillum* (5_H4) and *C. sorokiniana* (12_A9) were present in moderate numbers. *D. intermedius* (1_C4) cells were identified based upon their characteristic 2-5 algae cell cluster morphology and the presence of spines on the cells, appeared to be on the decline. Apart from a few small ciliates, no major predatory organisms were present in the culture. (Figure. 3.42).

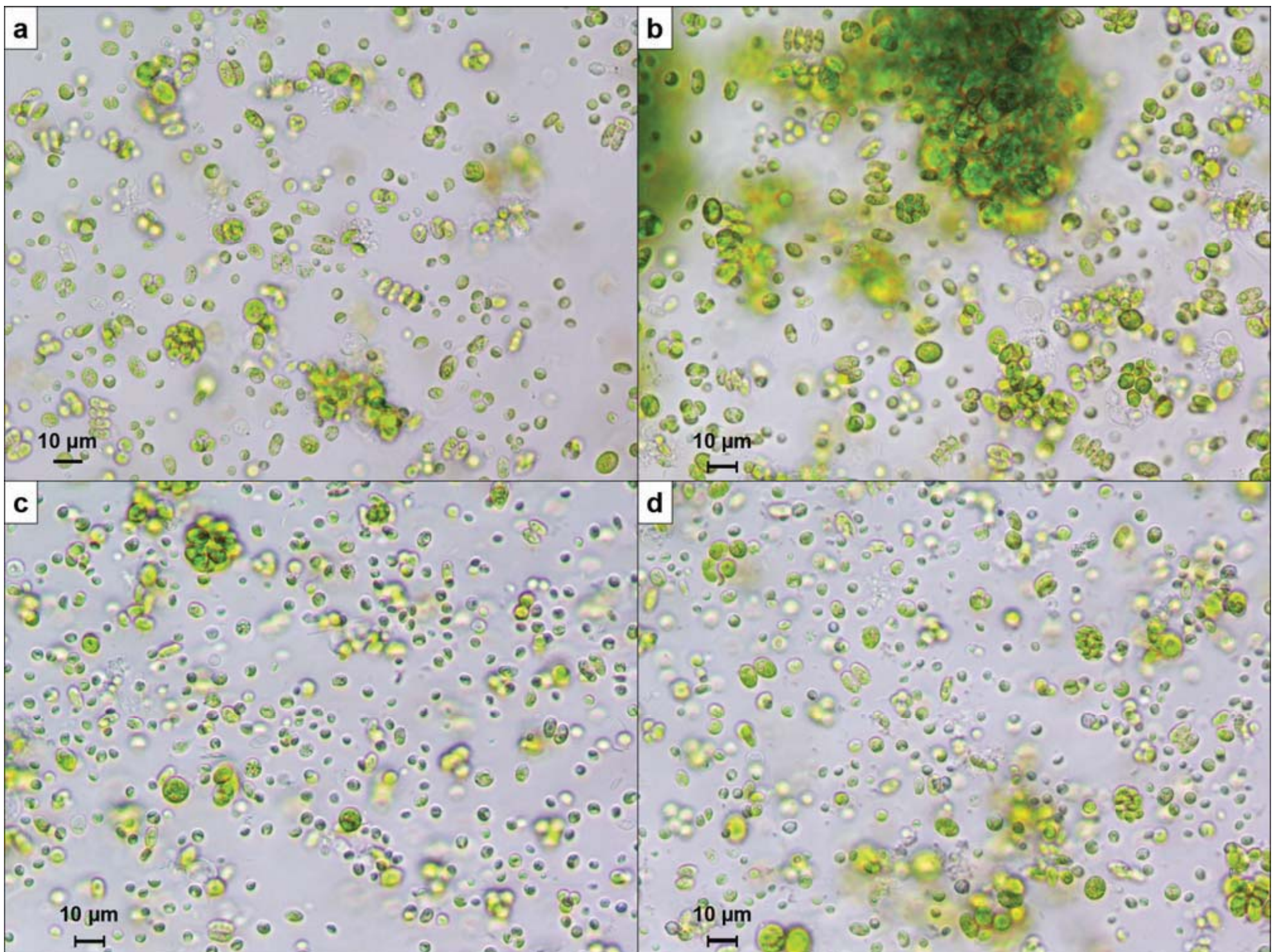

Figure 3.42: Pond polyculture 142 hours after inoculation. a, b) pond 1, c, d) pond 2.

Microscopic images on day 8 (190.5 hrs) showed a divergence of the population in the two ponds with fast motile algal cells including *Chlorococcum sp.* (12_02) more prevalent in pond 1. In contrast in pond 2 less motile cells were observed. Both ponds contained *Chlorella sp.* (11_H5) and *M. pusillum* (5_H4) (distinctly growing in 3-4 cell spherical clusters) which appeared to be increasing in numbers since the previous time point. *C. sorokiniana* (12_A9) and *D. intermedius* (1_C4) were present but appeared to be reducing in number compared to the previous time point. Predatory organisms were almost undetectable except for small numbers of *Paramecium*. The pattern observed appears to correspond with the relative growth rate of the species, and due to the low number of predators may have been relatively unaffected by them (Figure. 3.43).

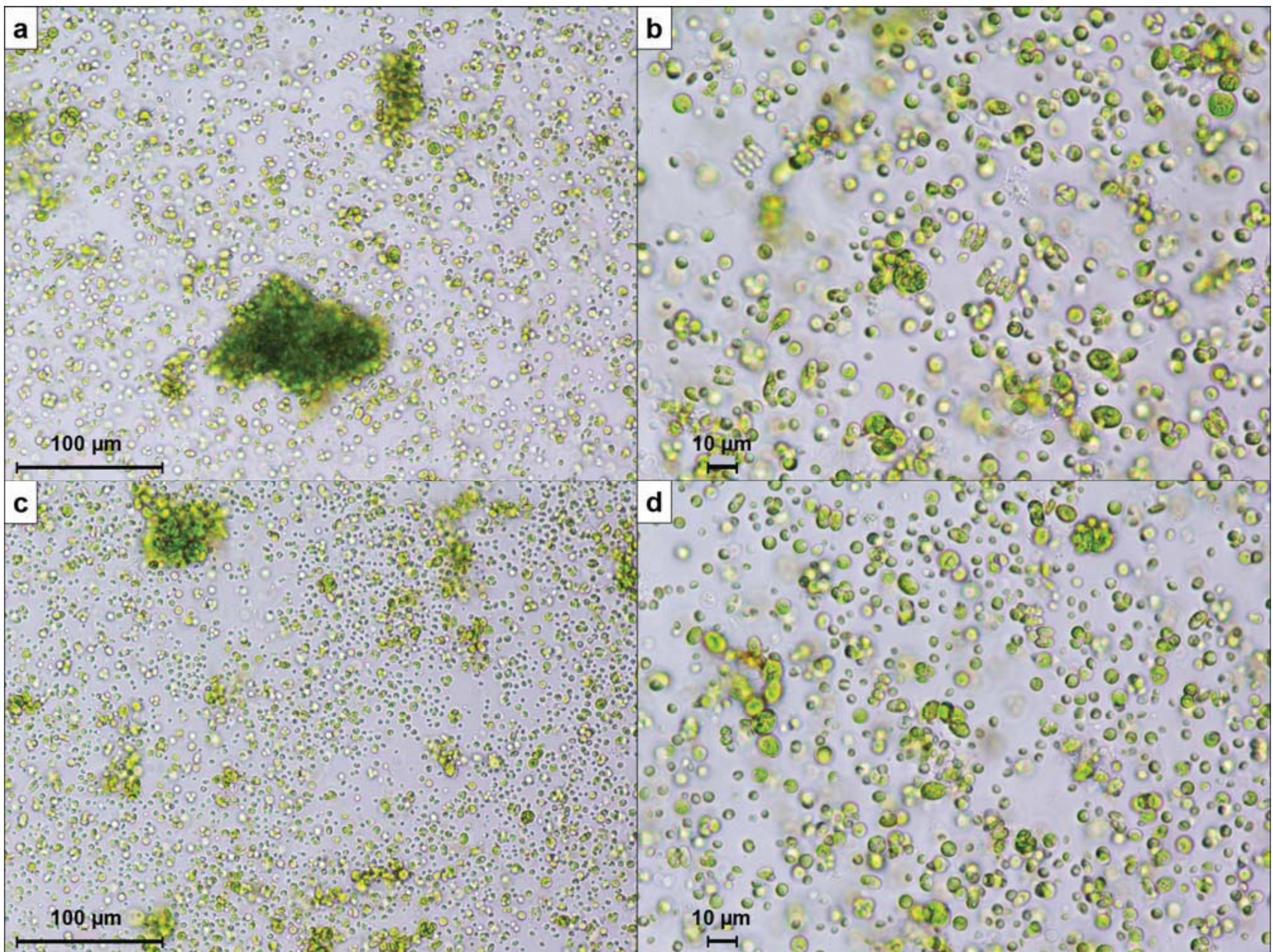

Figure 3.43: Pond polyculture 190 hours after inoculation. a, b) pond 1, c, d) pond 2.

Microscopic images on day 10 (238.5 hrs) showed an increasing pattern of the dominant *Chlorella sp.* (11_H5) and 5_H5 cells in pond 1. The motile *Chlorococcum sp.* (12_02) cells were also common but decreased slightly compared to the previous time point that remained mostly in a self-aggregating state. *D. intermedius* (1_C4) was detectable but low in numbers and *C. sorokiniana* (12_A9) could only be seen sporadically. Pond 2 showed an approximately similar cultural behaviour except for the fact that *D. intermedius* (1_C4) was clearly less present than in pond 1 (Figure. 3.44).

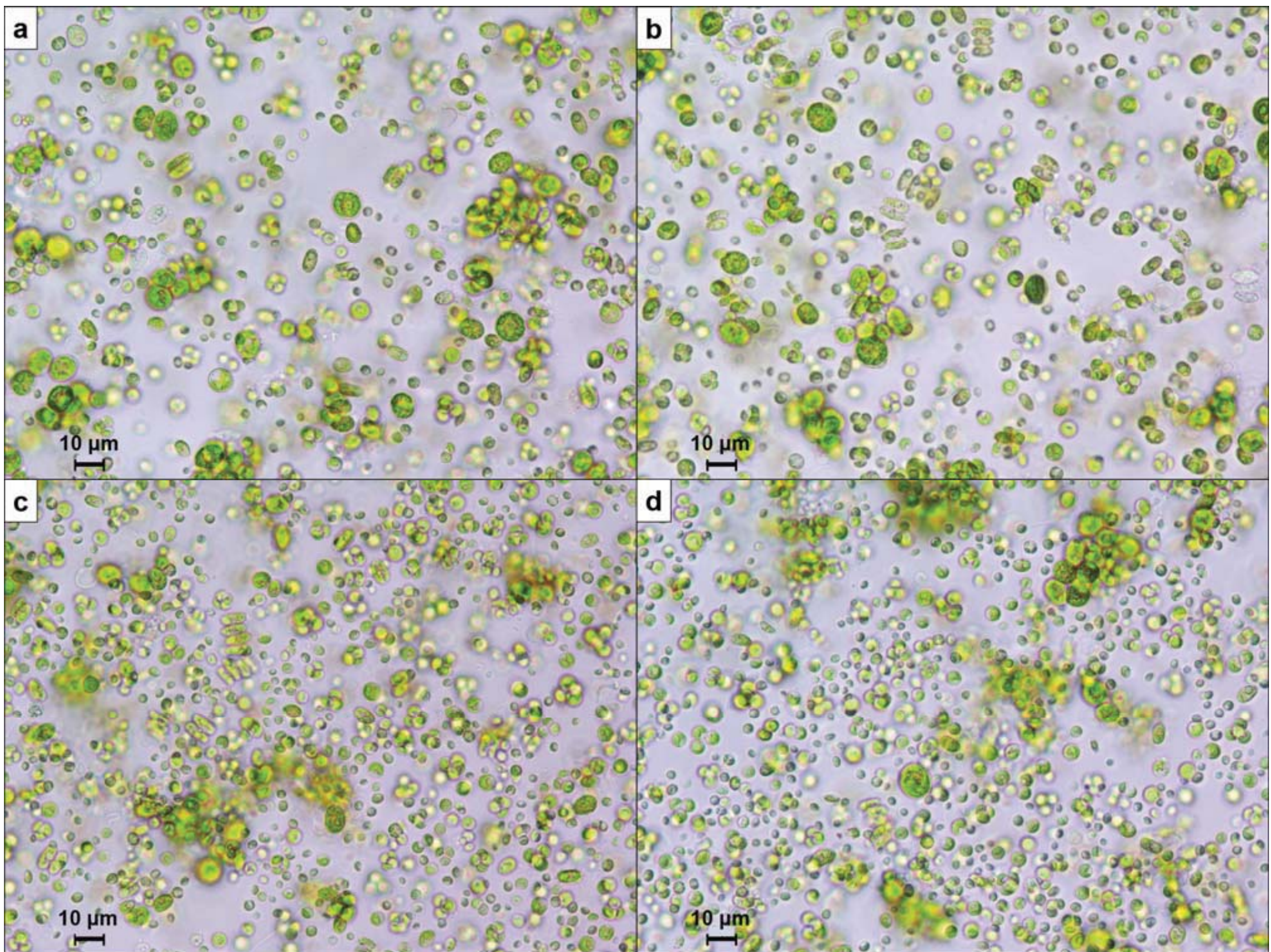

Figure 3.44: Pond polyculture 238 hours after inoculation. a, b) pond 1, c, d) pond 2.

Microscopic images on day 11 (262.5 hrs) showed the presence of *Chlorella sp.* (11_H5), *Chlorococcum sp.* (12_02) and *M. pusillum* (5_H4) in both pond cultures. *C. sorokiniana* (12_A9) and *D. intermedius* (1_C4) could hardly be detected anymore. Many motile *Chlorococcum sp.* (12_02) cells were observed in pond 1 but these had decreased dramatically in pond 2 compared to the previous time point. Consequently *M. pusillum* (5_H4) seems to dominate the pond 1 culture, while *Chlorella sp.* (11_H5) was the most common species in pond 2. Overall no predators were detected except for infrequently grazing rotifers in pond 2, likely due to the high amount of *Chlorella sp.* (11_H5) in the culture (Figure. 3.45).

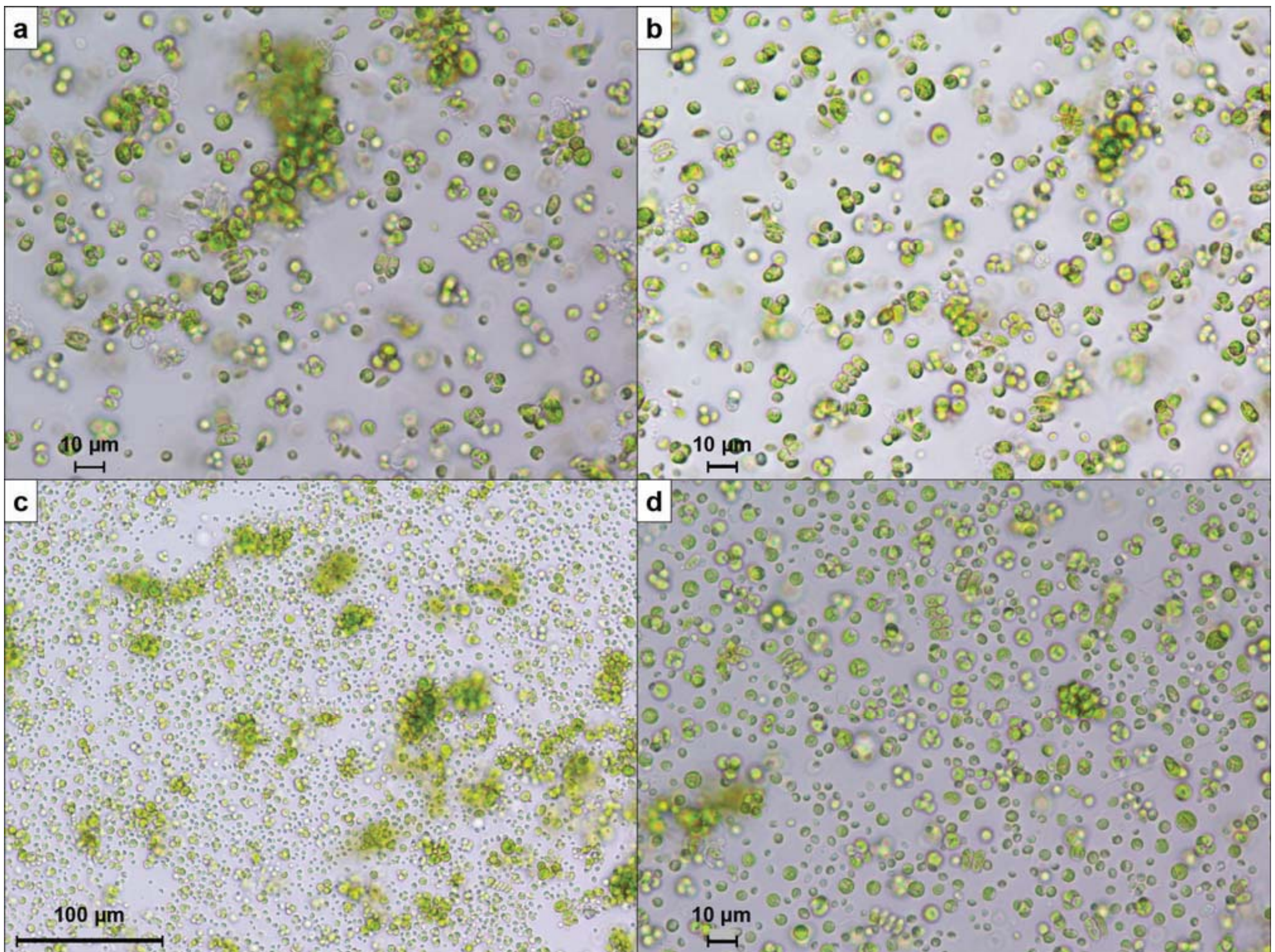

Figure 3.45: Pond polyculture 262 hours after inoculation. a, b) pond 1, c, d) pond 2.

Microscopic images on day 12 (286.5 hours), day 13 (310.5 hours) and day 14 (334.5 hours) show the same pattern of strain dominance in both ponds with *Chlorella sp.* (11_H5), *M. pusillum* (5_H4) and *Chlorococcum sp.* (12_02) being the most prevalent. No further predators capable of influencing the culture were detected (Figure. 3.46).

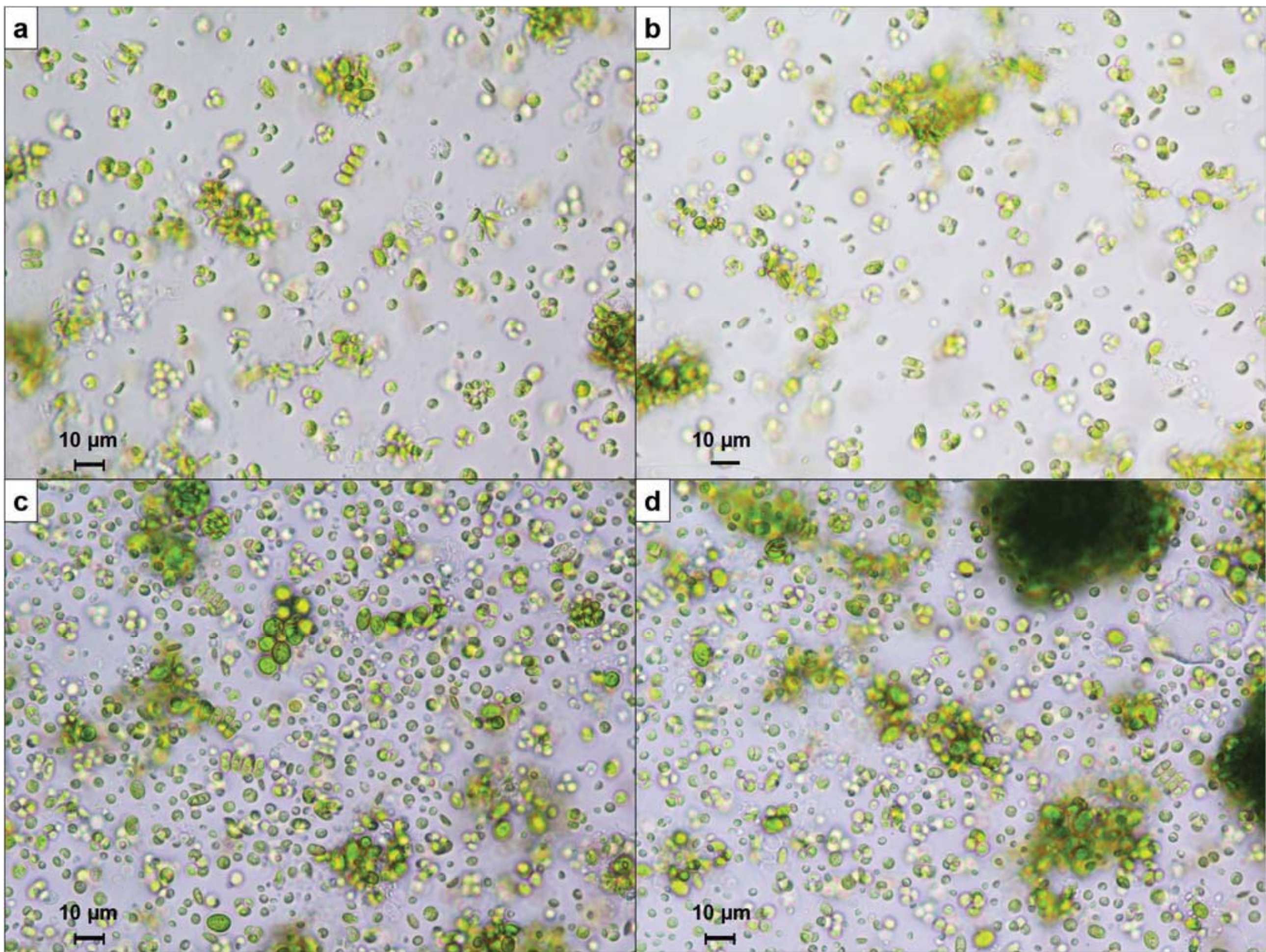

Figure 3.46: Pond polyculture 286 hours after inoculation. a, b) pond 1. c, d) pond 2.

In summary: during the open pond trials the initial relative cell density of the inoculated species were maintained for approximately 2 to 3 days. After this *Chlorella sp.* (11_H5), *Chlorococcum sp.* (12_02) and *M. pusillum* (5_H4) quickly emerged as the most dominant species presumably due to their rapid growth rate rather than a negative effect due to predation. Although the ratio between these 3 strains differed slightly between the two ponds, a similar pattern emerged for both ponds with ~80% of the culture biomass composed of the top three strains. At the end of the experiment the final ratio between the algal cell numbers was estimated for 11-H5 and 5-H4 with 35%, *Chlorococcum sp.* (12_02) with 20 %, followed by *D. intermedius* (1_C4) and *C. sorokiniana* (12_A9) with only ~ 5%.Very few predators were detected during the cultivation time which could have influenced growth or culture crash.

Microscopy flask culture

Microscopy was also performed on the flask trials to evaluate the effect of the different parameters on the productivity of the culture which was run in parallel with the pond experiments. Conditions in the flask cultures differed from the pond cultures in that the temperature range was more tightly controlled and the flasks were not supplied with additional CO_2, so CO_2 was only available via passive diffusion from ambient levels. Overall as a result, biomass productivity in the flasks was much lower due to CO_2 limitation and predator grazing. Furthermore this strategy allowed us to try to identify predators in the culture as part of an ongoing program to protect against them. Microscopic monitoring was conducted at 10 am and 3:30 am each day and images are provided at 70.5, 142.5, 190.5, 238.5 and 262.5 hour time points at low (x200) and high (x400) magnification.

Microscopic images on day 3 (70.5 hrs) showed an approximately equal mixture of all five algal strains. Many motile algal cells were present indicating *Chlorococcum sp.* (12_02) growth. A high amount of debris was also visible which may be due to cell death, or cell division of *Chlorococcum sp*. (12_02). At this time point no predatory organisms were observed (Figure 3.47).

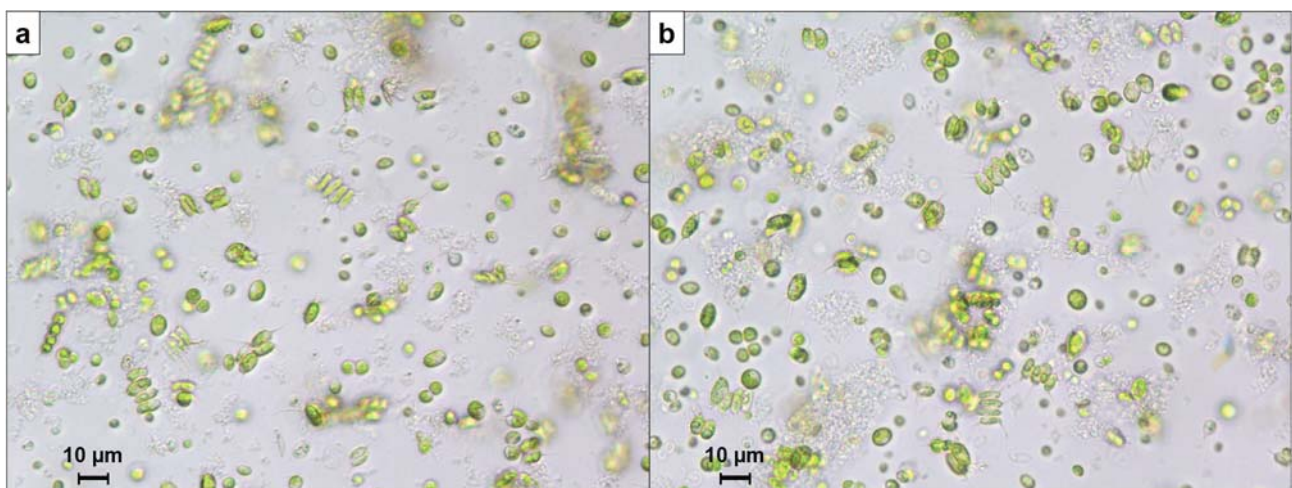

Figure 3.47: Flask polyculture duplicates 70 hours after inoculation: flask 1 (a) and flask 2 (b)

Microscopic images on day 6 (142.5 hrs)

Many algae aggregation flocs deriving from *Chlorococcum sp.* (12_02) and cell debris were observed. Other algae species were found to be trapped in flocs of debris. Single algal cells of *Chlorella sp.* (11_H5) were seen to be interspersed between larger cells of *M. pusillum* (5_H4) and *D. intermedius* (1_C4). *C. sorokiniana* (12_A9) was almost undetectable. Grazing predators were attached onto flocs via their tails (ciliate morphologically similar to *Vorticella*) (Figure 3.48, b indicated by a red arrow). Many other small motile ciliates were also observed within the culture. Long spines deriving from *D. intermedius* (1_C4) cells are visible (Figure 3.48, d)

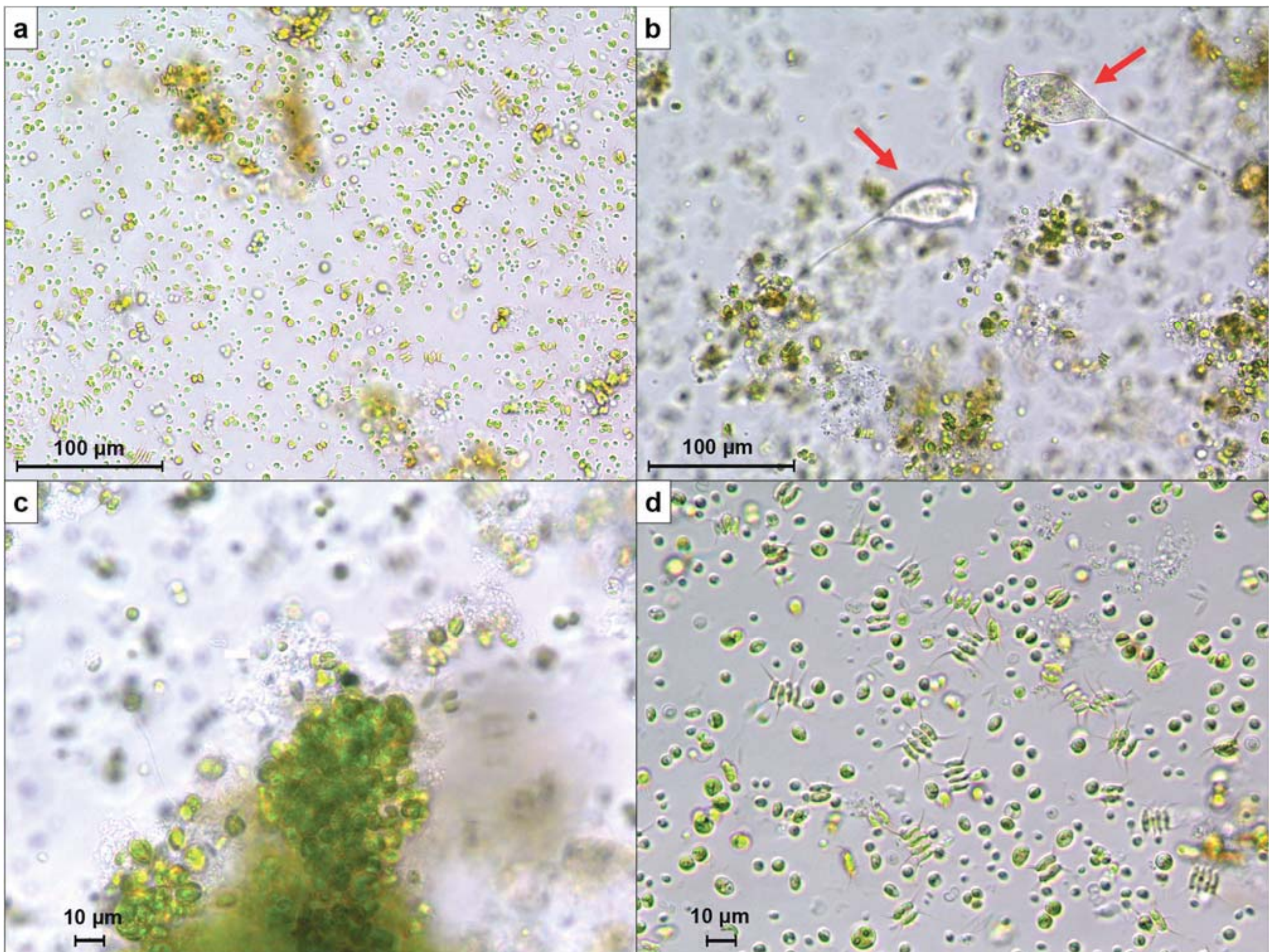

Figure 3.48: Flask polyculture duplicates 142 hours after inoculation. Both cultures show very similar culture development therefore different pictures are used to illustrate all main findings. Flask 1 (a) culture at x200 magnification and (b) culture at x400 magnification with grazing predators marked by a red arrow. Flask 2 (c) close up picture of an algae cell cluster, (d) x400 magnification illustrating the algal cell diversity, long spines deriving deriving from *D. intermedius* (1_C4) can be seen.

Microscopic images on day 8 (190.5 hrs)

Small motile cells of *Chlorococcum sp.* (12_02) decreased as did *Chlorella sp.* (11_H5) cells. Larger cell types of *D. intermedius* (1_C4), and *C. sorokiniana* (12_A9) as well as *M. pusillum* (5_H4) (clustered in groups of 3-4 cells) were more prevalent. Interestingly the decrease in populations of the small *Chlorococcum sp.* (12_02) and *Chlorella sp.* (11_H5) cells may have been caused both by CO_2 limitation in the flasks and predation by the ciliate. This clearly suggests that predation can have a significant effect on a polyculture consisting of these species but that this was masked under the outdoor conditions in which algae growth rates were high (due to higher light and CO_2 availability) and predator numbers were lower, potentially influenced by lower night time temperatures (Figure 3.49).

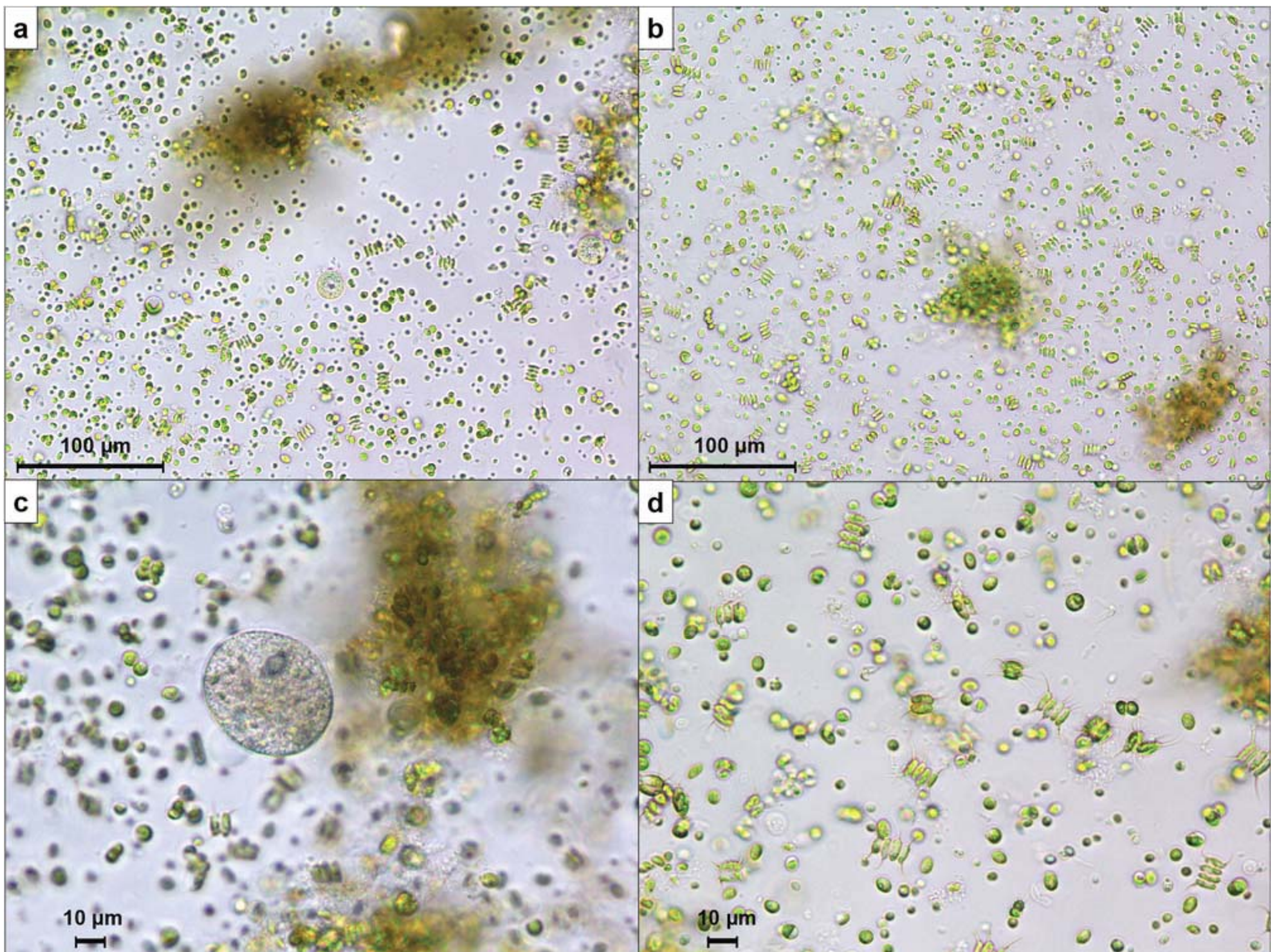

Figure 3.49: Flask polyculture duplicates 190 hours after inoculation. Flask 1 (a) and (c), flask 2 (b) and (d). Both flasks cultures developed very similar to each other. Panel (c) represents a onetime observed round ciliate.

Microscopic images on day 10 (238.5 hrs) clearly shows a decrease of *Chlorella sp.* (11_H5) cells throughout the culture. A higher amount of single cells of *M. pusillum* (5_H4) was observed and these exhibited protective spines. Many *D. intermedius* (1_C4) cells were present in stacks of 2-4 cells which also had spines (Figure 3.50, a). Furthermore many flocs containing *Chlorococcum sp.* (12_02) and other debris were observed. Many ciliate predators (morphologically similar to *Tetrahymena*, Figure 3.50 b, and *Vorticella*, Figure 3.50, c, indicated with a red arrow) were seen to be attached on the flocs as well as grazing throughout the liquid sample. They contained small round algae cells (most likely *Chlorella sp.* (11_H5) but also presumably small motile cells of 12_02. These observations suggest that 11_H5 was susceptible to predation and that the presence of predators may have induced the production of protective spines for some species.

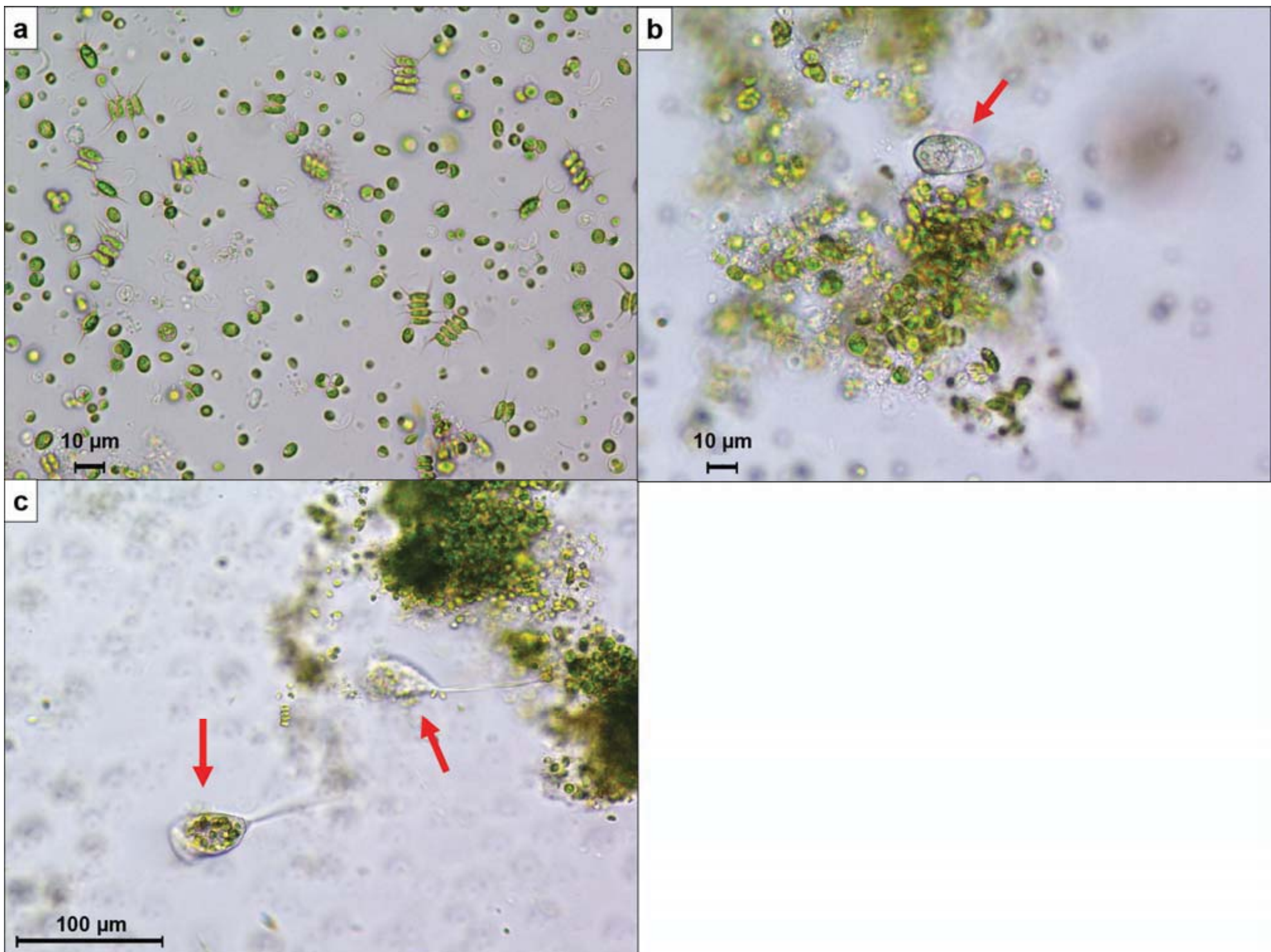

Figure 3.50: Flask polyculture 238 hours after inoculation.

Microscopic images on day 11 (262.5 hrs) and day 14 (335 hrs) (Figure 3.51 a, b) showed a similar pattern to the previous time point.

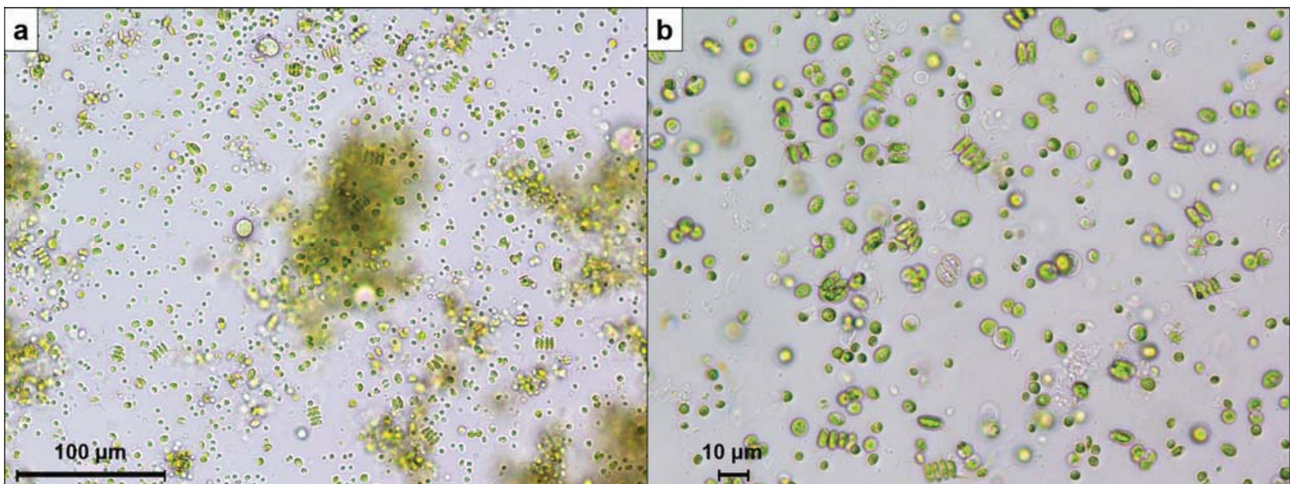

Figure 3.51: Flask polyculture 335 hours after inoculation.

Summary: Over the first 2 to 3 days of the culture approximately similar cell concentrations were observed for the inoculated species. Although the small motile *Chlorococcum sp.* (12_02) species

were initially present their occurrence decreased as the cultivation period progressed. However the *Chlorocoocum sp*. (12_02) which was present as flocs was able to sustain itself. *Chlorella* sp. (11_ H5) which grew well at the beginning, decreased quite rapidly most likely due to a limitation on growth rate because of the lack of CO_2 and simultaneous predation by a diverse range of microorganism. Interestingly *Chlorella sp.* (11_H5) was not eradicated completely and continued to be present in the culture in moderate numbers. Overall *D. intermedius* (1_C4) proved to be the dominating strain under the flask trial conditions. It grew healthily and its 2-4 cell clusters presented spines which appeared to protect it from predation as these species were not detected inside the predators themselves. *M. pusillum* (5_H4) was present mostly as single cells but in lower numbers and appeared to develop protective bristles. *C. sorokiniana* (12_A9) cells were observed but in low numbers.

Predatory organisms were grazing throughout the culture, influencing culture growth by keeping the density low. It was seen that they mostly consumed the small round cells of *Chlorella sp.* (11_H5) and presumably the small motile cells of *Chlorococcum sp.* (12_02). Dense flocs derived from natural flocculation of *Chlorococcum sp.* (12_02) cells as well as debris accumulation. Furthermore the flocs showed to trap other non-flocculating algae species within them which made clear cell distinguishing difficult. At the end of the experiment the final ratio between algal cells was estimated based on their morphology to be 35 % *D. intermedius* (1_C4) , with around 20% *M. pusillum* (5_H4) and *Chlorella sp.* (11_H5,) 15% *Chlorococcum sp.* (12_02) and 10% *C. sorokiniana* (12_A9) .

3.4 Discussion

It has long been known that maximum achievable growth rates of algae in controlled laboratory conditions are higher than those achievable in field trials where both environmental and biological factors impede production, despite the often higher levels solar energy input. Biomass productivity has been identified as one of the primary drivers for commercial feasibility in techno-economic analysis [6]. Consequently the identification of strains which could serve as suitable production candidates [2] and strategies to maximise their final productivity in field conditions remains one of the foremost challenges for this emerging industry. Dependent on species, climate and operation, open pond cultivation productivities can range from 5 – 50 g m^{-2} d^{-1} [7-10]. Schenk *et al* (2008) reported possible productivities of 10-25 g m^{-2} d-[1] in raceway ponds operated with a water depths of 15-20 cm [11]. Furthermore an average of 19-25 g m^{-2} d^{-1} can be reportedly achieved in well managed ponds, with an achieved peak up to 40 g m^{-2} d^{-1} [12]. Maximum achieved growth rates of the pond trials in this study ranging from 11 g m^{-2} d^{-1} to 24 g m^{-2} d^{-1}, which were within the range of the generalised standards reported in the literature. Nevertheless longer term studies need to be performed to obtain annual mean values for true comparison.

In this study maximum specific growth rates of ~30-50% of those achieved in previous laboratory screens under controlled conditions [1] were achieved. Productivity (volumetric and areal) and specific growth rate maxima are illustrated in figure 3.52.

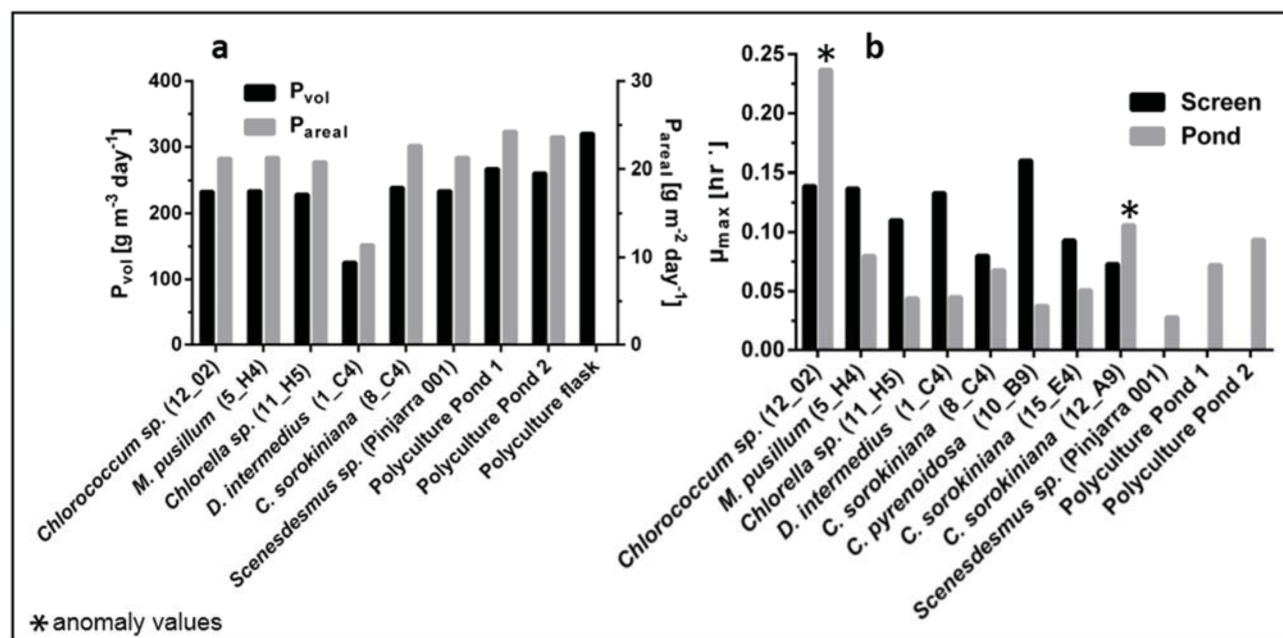

Figure 3.52: Comparison of (a) the maximum volumetric and areal productivities of tested strains; and (b) maximum specific growth rates achieved in open pond experiment trials versus the laboratory

screen conditions presented in Wolf *et al* [1]. Clearly for all algae species tested, a much higher growth rate was achieved in closed sterile systems with constant conditions of light and temperature.

Scaling up of production compared to small scale laboratory trials inevitably increases costs which incurs limitations at the pilot plant scale which may be more easily circumvented at laboratory scale. Due to this, both equipment limitations and time constraints occur as a function of the sharing of facilities with other staff and experimentation needs. Thus not all experiments could be replicated as widely as desired and certain anomalies in the data must be identified at the outset. Most notably these are the μ_{max} values for *Chlorococcum sp.* (12_02), and the μ_{max} [hr^{-1}] (day only) values for *C. sorokiniana* (12_A9) and indoor polyculture flasks. For *Chlorococcum sp*. (12_02) this is largely due to the effect of its frequent auto-flocculation upon optical density measurements, and while dry weight measurements in this case were ineffective due to high error levels, this strain is still considered as one of the high performance strains in terms of potential growth. In contrast the auto-flocculation can have positive effects in terms of reducing energy and cost of harvesting and attributes some degree of protection and resilience against predation which is also an important trait for commercial production. Thus this remains an interesting production candidate for further investigation. Conditions in the flask trials varied from those in open pond systems by lower levels of available CO_2, shorter light path, optimal light levels that do not lead to photoinhibition, higher levels of energy for mixing, and more optimal temperature levels and lower temperature flux.

While the cultivations conducted in this project were performed in a batch production regime it is known that semi-continuous production is a more promising strategy for commercial systems. Thus the output data is interpreted in terms of maximum potential in a semi-continuous production regime where frequent harvesting is performed to maintain cultures in the logarithmic growth phase. In this regard, the suitability of strains for further investigation is considered on the basis of maximum potential growth performance (e.g. doubling times quicker than once per 2 days), continued growth to high density culture (e.g. cultures that maintain reasonable productivity above an OD_{750} of 2), and stability/robustness of cultures with regard to environmental (e.g. seasonal variability) and biological challenges (e.g. predation). Strains that achieved highest growth rates were *M. pusillum* (5_H4), *Chlorella* sp. (11_H5), and *Chlorococcum* sp. (12_02), and the cultivations which achieved continued growth at higher optical densities were *M. pusillum* (5_H4), *Scenedesmus* sp. (Pinjarra001), and the polycultures.

In each cultivation non-target microorganisms were detected but diversity, load and impact varied significantly. Earlier incidence or a greater degree of bacterial contamination will establish itself in

each system regardless of whether open or closed bioreactors are used. In open pond systems, different types of bacteria are always present and were detected in higher numbers for example during the cultivation of *D. intermedius* (1_C4) and *M. pusillum* (5_H4) but these do not necessarily have a high negative impact on the culture growth, despite competing for nutrition.

The invasion of protozoan organisms can occur when favourable environmental factors are present. Besides the right growth conditions (pH, temperature, nutrition), protozoa are attracted by either the bacterial load or the algae itself as potential food sources in the culture solution. As an example, *Tetrahymena* ciliates established themselves in the pond cultivation of *C. sorokiniana* (15_E4) but none of these species were detected in cultivations of *C. pyrenoidosa.* (10_B9) and *C. sorokiniana* (12_A9), leading to the assumption that a certain condition must have been preferred. Debris flocs to which bacteria adhere could have been one of the differences which was more prevalent within the *C. sorokiniana* (15_E4) culture. But despite many bacteria being observed in the cultivation of *D. intermedius* (1_C4) no notable other protozoa were detected during this cultivation. It may be that the growth condition, type of bacteria or the algae itself (with its pointed morphology and spines on the cells) displayed a less preferable environment for protozoa.

Protozoa can have a negative impact on the cultivated algae species as was observed during the cultivation of *Chlorella sp.* (11_H5). Overall the bacterial load was very high throughout the time of this cultivation resulting in contamination of many different types of protozoan. Furthermore the morphology of *Chlorella sp.* (11_H5) is a small and coccoid cell, which is presumably more sensitive to predator attack (easy to ingest). Thus while *Chlorella* sp. (11_H5) was considered one of the top production candidates from a growth performance perspective, it’s sensitivity to predation and potential for rapid culture decline somewhat attenuate it’s commercial suitability, at least in monoculture cultivations. Shifting from single cell morphology into a colonial (e.g. *Scenedesmus* sp.) or flocculation (e.g. *Chlorococcum* sp.) state might be a possible defence strategy against the grazing pressure.

The single species cultivation trial of the nine top growing strains showed a diverse behaviour of the target species and non-target microorganism establishment, resulting from almost none to a very high impact on the whole system. The knowledge of the sensitivity and robustness of each algae strain towards a fluctuating multi-microorganism environment is an essential basis for further improvement in open mass cultivation.

Polyculture

It has previously been reported that algae polycultures can achieve increased growth rates and dry-biomass yields compared to cultures of single-strains and can exhibit increased robustness against adverse environmental factors [13]. This research project aimed to investigate this phenomena by using a mixture of five morphologically distinct strains. The use of a defined polyculture with fixed proportions of inoculum instead of random natural colonisation facilitates explanations of the resultant culture trajectory in terms of the specific characteristics of each algal subtype and is thus a more tractable system to investigate than a complex natural ecosystem.

The primary hypothesis tested was whether the fastest growing species would dominate the culture under outdoor conditions and secondarily to identify other factors which favoured any dominance by slower growing strains. Furthermore we aimed to establish whether the combined productivity of all species lead to higher yield overall compared to productivity of pure cultures of the fastest growing strains.

Overall between all open cultivations tested, both pond polycultures ranked highest in terms of their maximum areal and volumetric productivity and are considered to be a potential alternative to single species cultivation, though the cultivation timing in the spring season resulted in higher mean solar energy as well as higher absolute temperatures and greater temperature flux. Thus while further investigation is certainly warranted, it is suggested that long term (i.e. over 1 year) parallel cultivations should be conducted in order to discern the real benefits.

Despite the overall high performance of the polycultures, the monoculture of the strain *M. pusillum* remained the top candidate in terms of specific growth rate. This may indicate that rapid exponential growth is restrained by slower growing strains in the algae mixture due to shared access to light, CO_2, and nutrients. The relatively good performance of the polycultures can be explained in two ways. First that the culture is dominated by the fastest growing organisms lending to high productivity values; and secondly that all species in the culture contribute to the total growth rate and that collectively biological challenge from predation is constrained by the complex ecosystem of predation resistant strains which provides smaller fast growing strains like *Chlorella* sp. (11_H5) which are susceptible to predation in monoculture, a somewhat protected environment in relation to biological challenge.

Microscopy Polyculture:

Due to their morphological differences, the abundance of each of the strains of the polycultures could be tracked via microscopic imaging making it possible to determine whether the fastest growing strains dominated, or whether the overall culture performance was due to significant biomass contributions of many species.

A similar cell distribution trend in both polycultures was revealed. At the start point approximately equal numbers of all strains were present and this was maintained within the first 2-3 days. However as the culture proceeded it was noted that the fastest growers *M. pusillum* (5_H4). *Chlorococcum sp*. (12_02) and *Chlorella sp.* (11_H5) dominated the culture. This is in line with the top three maximum growth rate strains in monoculture as noted above.

Within the flask cultivation *D. intermedius* (1_C4) and *Chlorococcum sp*. (12_02) and *C. sorokiniana* (12_A9) were the leading species in the culture which appeared to be the result of selection pressure from predators. With no additional CO_2 input in the flask polycultures, growth of the algae was limited. Furthermore the constant culture temperature (25.0 – 28.5 °C) which was generally warmer and more stable than the outdoor pond cultivations, potentially enhanced growth of other microorganisms and bacteria which were competing for nutrition and made the algae subject to predator attack.

This was experienced in previous outdoor cultivation trials. When night time temperature dropped it was observed that predator levels declined. Similarly, predatory organisms were more prevalent in the algal culture at temperatures constantly above 16 °C (e.g. during summer season, data not shown).

Consequently the lower temperature flux experienced indoors for the flask experiments allowed us to assess the effect on predator survival and its corresponding influence on algae attack and dominance. While two variables were adjusted simultaneously in this experiment the flask trials represented a low productivity regime in which the effects of predators might be considered to be at the high end of that observed in the field trials.

Predator impact

It is known that the selection pressure of predators on microalgae populations is at least partially dependent upon the cell size of the algae. This can be confirmed by several observations. For example *Chlorella sp.* (11_H5) showed a high sensitivity for predation as a monoculture. During the flask polyculture experiment *Chlorella sp.* (11_H5) as well as the small motile cells of *Chlorococcum sp.*

(12_02) were decreasing in numbers with increased predator presence. Evidence is demonstrated by protozoa which readily ingest algae e.g. *Vorticella* like organism in the polyculture or, *Tetrahymena* like ciliates during the cultivation of *C. sorokiniana* (8_C4). Furthermore in-house trials of isolated rotifers, obtained from pond cultivations were fed and successfully maintained with the algal strains of *Chlorella sp.* (11_H5), *C. sorokiniana* (8_C4) and *C. pyrenoidosa.* (10_B9) (Supplementary data video 3.13 and 3.14).

Large algae less affected

In the polycultures the strain *C. sorokiniana* (12_A9) showed a higher presence in flask trials than in open pond cultivations despite its slow growth performance. One reason could be the fact that it was not outcompeted by fast growers and further, its larger cells size makes it less susceptible to predator attack. Despite larger cell size which can help algae to survive, the high variety of shapes illustrate an important strategy of grazing defence.

Protective spines

The presence of protective spines or bristles like those seen on *M. pusillum* (5_H4) and *D. intermedius* (1_C4) appears to make them less vulnerable to a range of predators. However an extraordinarily high degree of variability in morphological characteristics in cultures of *M. pusillum* is known [14].

Depending on the culture conditions *Micractinium* morphology contains both cells forming colonies and solitary *Chlorella*-like simple coccoids and therefore can exhibit a surprising morphological similarity to *Chlorella vulgaris*. Bristle formation can be induced or intensified by grazing pressure of predators which serves as protection against feeding activity e.g. the rotifer *Brachionus* [15]. In contrast it has been observed that *Micractinium* loses its bristles in a monoculture condition and becomes morphologically similar to *Chlorella* species in a monoculture condition [16]. Observations during the experiments showed both morphological appearances of *M. pusillum* (5_H4), solitary cells and colonies. However reasons behind the change during the experiments are not confirmed.

During the adaptation stage and when highly diluted in open ponds, single cells were seen including the presence of bristles. With continuing growth and increasing density of the culture, both morphological stages colonial and single cells were present, however almost no bristles were detected on the cells, suggesting the species is losing its protective spines when crowded. However no further contaminants despite smaller ciliates were detected.

Within the flask culture *M. pusillum* (5_H4) was mostly solitary and long needle shaped bristles formations were detected around the cells. This agrees with the literature that bristle formation and its intensity is dependent upon grazing pressure. *D. intermedius* (1_C4) cell morphology always showed stacks of 2-4 cells including spines and the length of spines seemed to increase during the flask cultivation most likely a defense reaction against protozoa similar to *M. pusillum* (5_H4). Nevertheless spines, bristles or ridge shapes don't protect successfully against all predatory organisms; for examples they are not effective obstacles for amoeba [17].

Auto-flocculation of general single cell morphology algae species has been observed as a result of grazing pressure shown clearly during the monocultivation of *Chlorella sp.* (11_H5) and partially during the flask polyculture. Algae flocs and debris particles provide a "hiding place" for small algae. Protozoa grazing along debris flocs searching for bacteria and algae might be likely to reach algae trapped along the outside of the flocs. This explains why almost no small coccoid algae cells are seen on the debris. However not all cells can be accessed and with decreasing grazing pressure, the algae species can establish their cell numbers again. With flocculation, a higher chance of the survival might be achieved but due to light limitation the growth and productivity is limited and should be avoided in commercial production. Therefore it is important to study these responses and the underlying mechanisms.

Types of predatory organisms observed

A diversity of predator species were observed during the time of pond cultivation trials which showed different impact on the algae cultures.

An example of the predatory organisms observed are:

- Ciliates (morphologically similar to *Vorticella*, *Tetrahymena*, *Stylonychia, Paramecium*),
- Flagellates (morphologically similar to *Peranema* (supplementary video 3.15 and 3.16) and *Chlamydomonas*),
- Heliozoans (morphologically similar to *Actinophryida* (supplementary video 3.16),
- Rotifers (morphologically similar to *Rotaria sp.* and *Brachionus*),
- Amoeba (morphological similar to *Paramoeba*),
- Fungi

Predators generally have preferred food sources which can include algae, but also bacteria or other microorganisms. Rotifers (e.g. *Rotaria*) can also negatively influence algae production systems. With the right conditions they can lead to the loss of the entire culture within days [15], however they were observed coexisting within the culture of *Scenedesmus* sp. (Pinjarra001) with no observable negative

effect upon the algae population. A long term experiment with the local algae (data not shown) showed a stable culture over one year without culture crash (monthly re-nutrification). Rotifers lived within the culture but had no impact on its stability and did not seem to ingest the algae. Furthermore no other foreign microorganism were microscopically detected, assuming the predator had a positive influence by keeping the culture “clean”. Therefore paradoxically, appropriate co-cultivation of "predators" can actually be used as a tool to sustain algal cultures.

Chlamydomonas-like microorganisms (flagellates) and small fast swimming *Paramecium* were in almost every open pond algae culture detected. They do not seem to attack algae but are more attracted in the presence of heavy bacteria-loaded cultures. For example *M. pusillum* (5_H4) showed *Chlamydomonas*-like organisms within the culture which were overgrown over time. The bacterial load within the culture increased with the disappearance of these microorganisms simultaneously, which indicates a potential beneficial impact.

Coexistence of ciliates and algae was especially observed during *C. sorokiniana* (15_E4) cultivations with almost no grazing impact on the culture. However the same *Tetrahymena* like organism (morphologically identified) observed in the *C. sorokiniana* (15_E4) culture seemed to be responsible for heavy aggregation of the algae *C. sorokiniana* (8_C4) indicating the chance of an inducible bioflocculation effect (see chapter 4).

3.5 Conclusion and outlook

Microalgal production facilities can provide a habitat for a wide variety of undesirable competitors, predators, and pathogens, especially in more exposed systems. Microorganism contaminants of an algae culture include other indigenous algae species, bacteria, parasites (virus, fungus) or predators (protozoans, aquatic invertebrates). These organisms can be detrimental for algae growth and in some cases can lead to full culture crashes. Species of the phylum *Rotifera* in particular can reportedly have a massive impact on algae cultures. To overcome this challenge, it is important to observe rotifer behaviours, habitat and prey preferences, as well as the mechanisms of predation (e.g. algae morphological constraints) and to analyse microalgal defense strategies (morphological, behavioural and chemical). Long term microscopic monitoring of algae cultures, predator occurrence and behaviour will be an integral part of each experiment.

It is very important to monitor the first signs of invasion by a predator and to conduct continuing investigations into population dynamics. Frequent observation is not only crucial to optimise production of the desired algae culture but also to monitor for predators and their impact on the culture, to understand warning signs, predict culture shifts and lethal culture crashes. Using observations from previous experiments we can discuss and develop future strategies to counteract culture loss.

A diverse variety of predators can be present in an algae production culture. The type of predator will determine the effect on the algae; in some cases it even can be beneficial (e.g. if used to clear unwanted contaminating algae). From previous and future experiments it will be possible to analyse which predators are most common and dominant in the culture and what impact they have. Using this information it can be determined how to act against them and optimise the culture production.

From previous cultivation experiments of the best production strains, both effective and poor defense mechanisms were observed depending on the algal species. Small single cell green algae benefit from their fast growth rates but are more vulnerable to predation. Larger and more morphologically diverse species are less readily predated. Each predator can only consume a limited amount of algae so it should be possible to predict the impact of a given predator population on the algal culture at any time. Therefore complex modeling systems can potentially be used to predict acceptable levels of predators in a culture. This modeling can be validated against experimental results.

The ability to influence or control predators is very useful. Several possible mechanisms could be used for this, but not all will be equally practical. For example, temperature is a physical variable that has been observed to affect rotifer invasion. Biological mechanisms could include algae responses (e.g. mucous production), hormonal effects on rotifer reproduction or viability, while chemical conditions (e.g. pH) or treatments to poison predators while leaving algae unaffected, may be useful.

One of the most interesting of these mechanisms was the observed flocculation of *C. sorokiniana* (8_C4) in the presence of a *Tetrahymena* like ciliate. This finding was further investigated (Chapter 4) to identify underlying mechanisms and has also led to a patent application describing a novel bio-flocculation process.

3.6 References

1. Wolf, J., et al., *Integrated medium design and microbial screening for high biomass production.* Algal Research, submitted.
2. Jakob, G., et al., *Surveying a diverse pool of microalgae as a bioresource for biotechnological applications.* Journal of Petroleum & Environmental Biotechnology, 2013: p. 4-5.
3. Radzun, K.A., et al., *Automated nutrient screening system enables high-throughput optimisation of microalgae production conditions.* Biotechnol Biofuels, 2015. **8**: p. 65.
4. Wood, A.M., R.C. Everroad, and L.M. Wingard, *Measuring Growth Rates in Microalgal Cultures*, in *Algal Culturing Techniques*, A.R. Anderson, Editor. 2005, Elsevier Academic Press. p. 269-285.
5. Zhang, Q., A.G.B. Simpson, and W. Song, *Insights into the phylogeny of systematically controversial haptorian ciliates (Ciliophora, Litostomatea) based on multigene analyses.* Proceedings of the Royal Society B-Biological Sciences, 2012. **279**(1738): p. 2625-2635.
6. Stephens, E., et al., *An economic and technical evaluation of microalgal biofuels.* Nature Biotechnology, 2010. **28**(2): p. 126-8.
7. Demirbas, A. and M.F. Demirbas, *Importance of algae oil as a source of biodiesel.* Energy Conversion and Management, 2011. **52**(1): p. 163-170.
8. Park, J.B.K., R.J. Craggs, and A.N. Shilton, *Wastewater treatment high rate algal ponds for biofuel production.* Bioresource Technology, 2011. **102**(1): p. 35-42.
9. Zamalloa, C., et al., *The techno-economic potential of renewable energy through the anaerobic digestion of microalgae.* Bioresource Technology, 2011. **102**(2): p. 1149-1158.
10. Sheehan J, D.T., Benemann J, Roessler P, *A look back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae. Close-Out report.* National Renewable Energy Lab, Department of Energy, Golden, Colorado, U.S.A., 1998. Report number NREL/TP-580-24 190.
11. Schenk, P.M., et al., *Second generation biofuels: high-efficiency microalgae for biodiesel production.* BioEnergy Research, 2008. **1**(1): p. 20-43.
12. Richmond, A., *Principles for attaining maximal microalgal productivity in photobioreactors: an overview.* Hydrobiologia, 2004. **512**(1-3): p. 33-37.
13. Song, L., et al., *Competition and succession between the oily alga Botryococcus braunii and two green algae Chlorella vulgaris and Chlamydomonas reinhardtii.* Journal of Applied Phycology, 2013. **25**(3): p. 847-853.
14. Luo, W., et al., *Genotype versus phenotype variability in Chlorella and Micractinium (Chlorophyta, Trebouxiophyceae).* Protist, 2006. **157**(3): p. 315-333.

15. Schluter, M., J. Groeneweg, and C.J. Soeder, *Impact of rotifer grazing on population-dynamics of green microalgae in high-rate ponds.* Water Research, 1987. **21**(10): p. 1293-1297.
16. Luo, W., et al., *Genotype versus phenotype variability in Chlorella and Micractinium (Chlorophyta, Trebouxiophyceae)*. Protist, 2006. **157**(3): p. 315-333.
17. Gong, Y.C., et al., *Vernalophrys algivore gen. nov., sp nov (Rhizaria: Cercozoa: Vampyrellida), a New Algal Predator Isolated from Outdoor Mass Culture of Scenedesmus dimorphus.* Applied and Environmental Microbiology, 2015. **81**(12): p. 3900-3913.

Chapter 4

Current harvesting and downstream technologies remaining expensive and representing a critical challenge for economically feasible commercial algae production systems.

Flocculation of algae cells offers a cheaper dewatering solution. It enables to use airlift or gravity to increase the biomass density and is applicable to large quantities.
However to reduce significant costs, the dewatering process needs to be rapid and highly controllable and the agent to induce bioflocculation inexpensive and required in low quantities.

The work presented displays a controllable and rapid flocculation mechanism using a wild type ciliate *Tetrahymena* and is presented in the word format that it was accepted by the Journal *Algal Research*, 2015.

Triggerable exocytosis of the protozoan *Tetrahymena* as a source of bioflocculation and a controllable dewatering method for efficient harvest of microalgal cultures

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Abstract

Microalgae offer a promising route to the production of high-value products, foods, animal feeds, and biofuels. Efficient algal dewatering strategies are therefore important for minimising energy and costs. Bio-flocculation is a potentially low-cost, low-energy harvesting strategy which can be facilitated by the microbial production of extracellular polymeric substances (EPS), but must be inducible since productive algal culture generally requires cells to be in suspension. Here we show that algal bio-flocculation can be controllably induced using the protozoan *Tetrahymena*. As little as 1:400 starved *Tetrahymena* to algal cells can be used to initiate rapid bio-flocculation. We demonstrate that stimulators of ryanodine receptors (caffeine and *p*-chlorocresol) trigger exocytosis in *Tetrahymena* and that inexpensive physicochemical stimuli (ammonium ions, shifts in pH and salinity) can also be effective. We suggest that triggered EPS secretion by protozoans in the starved state may explain apparently spontaneous bioflocculation of algae in both natural and artificial systems.

Highlights

- *Tetrahymena* is an efficient bioflocculant for algal cultures
- Bioflocculation can be controllably induced by inexpensive inducers
- A 1:400 Tetrahymena to algae cell ratio is sufficient to induce flocculation
- Biomass concentration can be efficiently dewatered >20-fold

Keywords

Microalgae, bioflocculation, exocytosis, Protozoa, Tetrahymena

4.1 Introduction

Microalgae provide a promising low-carbon emission biotechnology for the manufacture of foods, fuels and high value products. Forecasts by international peak organisations suggest that by 2050, a global population ~ 9 billion will require 70% more food [1], 50% more fuel [2], 50% more water [3] and ~50-80% CO_2 emissions reductions [4] to maintain political, social, food, fuel, water and climate security. Algae biotechnologies are positioned at the nexus of these challenges as they tap into the huge solar energy resource (~2600x global energy demand), and use CO_2 to produce food, fuels and clean water by expanding photosynthetic capacity onto non-arable land.

A significant challenge for energy- and cost-efficient algal biotechnology is dewatering, since the cells need to be kept in suspension in nutrient media during growth, but the resultant dilute solutions impose a significant energy burden on processes used to extract the biomass, while the equipment required represents additional cost and maintenance. Consequently, both cost effectiveness and energy balance benefit from simple and inexpensive dewatering techniques.

Flocculation enables gravity settling or flotation and has long been attractive as a dewatering technique, the major disadvantage being the need to add (and then recover) significant and expensive quantities of extrinsic flocculants such as minerals or synthetic polymers. Several mechanisms are commonly advanced: algal surface charge can be neutralised by flocculant ions, enabling cells to approach each other in stabilised aggregates; cells can be swept from the culture by mineral precipitations; and soluble polymers can act either as electrostatic patches or as bridges between cells [5]. Bridging effects can also be due to naturally occurring microbially produced polymers known as *extracellular polymeric substances* (EPS), which lead, through electrostatic, hydrophobic and physical interactions to biologically mediated flocculation ('bioflocculation'), typically encountered in activated sludge treatment in wastewater systems [6]; as well as being important for membrane fouling and biofilm formation [7] and potentially for a range of useful environmental applications including bioremediation [8]. EPS is a generic term and does not define the polymer chemistry, but typically EPS are carbohydrate, protein, lipid or mixed chemistries of these, displaying complex interactions with local environment (More et al. 2014). It is not the case that EPS always enhances flocculation – the specific chemistry and the ratio of EPS to biomass is important [8], while some algae secrete an extracellular EPS layer around the cell to maintain a biofilm.

Although some algal species flocculate spontaneously due to specific cell-cell interactions, unmanaged flocculation is a significant disadvantage during the growth stage and the number of

species available for use are limited. Bioflocculation has also been explored by mixing different species, one of which tends to flocculate and is used to capture the other (non-flocculating) strain [9]. For particular strains this can be a successful strategy; however, it requires the growth of significant quantities of the 'pro-flocculation' strain, which may not be intrinsically valuable, could reduce product quality, and can significantly add to capital and operating costs. It would therefore be advantageous to have a controllable, low energy flocculant strain which did not require for flocculation a comparable cell ratio to the target strain. Bacterial and fungal biomass types have been used as sources of EPS for bioflocculation [10] [11] [12] [13] [14] but typically require substantial flocculation times (up to 24h) and significant amounts of biomass to be mixed in or grown on carbon sources in the culture.

Lee et al. (2010) [15] estimated that with bacterial flocculation, a cost as low as AU$0.13 per tonne of biomass could be attained, with an energy requirement (mainly for mixing) of ~0.9 kWh per tonne of dry biomass flocculated in the particular system they investigated. They concluded that bioflocculation was a realistic prospect in terms of cost and energy balance but noted the extensive time (~20h) required for effective flocculation using bacterial biomass, and subsequently moved to electroflocculation [15].

Spontaneous algal bioflocculation occurs both in natural water bodies and in artificial bioreactor systems, but the reasons remain poorly understood. Many microorganisms produce EPS but the nature of the organisms, the timing of the events and in particular the biochemical signals responsible for inducing this spontaneous bioflocculation have till now been obscure [5].

In the course of outdoor pilot plant production at the Solar Biofuels Research Centre (SBRC) in Brisbane Australia, we observed variable flocculation between different high rate pond cultures of a *Chlorella* species, which we tentatively attributed to the presence of a ciliate (subsequently identified as a *Tetrahymena* species). However, since the simple presence or absence of the ciliate did not correlate straightforwardly with flocculation, some additional factor was clearly required.

Exocytosis in *Tetrahymena* is a well-studied phenomenon which is readily demonstrated using the polyanionic dye Alcian blue [16] [17]. The secreted material consists largely of granule lattice (Grl) proteins packaged in ~1µm mucocysts or dense core granules, which are triggered by the addition of the dye and this results in the formation of a mucous gel in the medium around the cell [18] [19]. Typically this gel condenses, forming a mucous, Alcian blue-stained proteinaceous capsule around the cell, from which it eventually escapes. As such, this capsule comprises a type of EPS.

Consequently, although bacteria and fungi are the best known sources of EPS, protozoans such as *Tetrahymena* can also contribute to the overall EPS load in natural water bodies and in human systems such as wastewater [20] management systems. Although the biological advantage of exocytosis for *Tetrahymena* has not been well established, one suggestion is that it forms a substrate upon which bacteria grow (or are trapped) and which then provides a food source for the *Tetrahymena*. We observed that this extracellular polymer also traps algal cells quite effectively and therefore can act as a bioflocculant.

This suggested that it was the triggering of exocytosis in recently starved *Tetrahymena* which initiated bioflocculation in our algal cultures and prompted investigation of whether *Tetrahymena* could be suitable for controllable algal dewatering and if so, what conditions could efficiently and cost-effectively induce this. We show that *Tetrahymena* offers a controllable, rapid, low-energy and cost-effective harvesting technology. Furthermore, the requirement for *Tetrahymena* (and presumably similar protozoans such as *Paramecium*) to be specifically in a starvation state, and then to receive an appropriate physicochemical signal, provides a two-step mechanism for EPS secretion and (given the modest protozoan cell density required) an explanation for why spontaneous bioflocculation occurs both in natural water bodies and algaculture systems.

4.2 Materials and Methods

Algae growth

The Australian wild type strain *Chlorella sorokiniana* (8_C4) was obtained from our laboratory's internal culture collection and was previously isolated from a fresh water habitat in Queensland, Brisbane [21]. This algae strain was cultivated in Tris acetate phosphate (TAP) medium [22] incubated in a 250mL Erlenmeyer flasks filled with 100mL medium (continuous shaking at 120 rpm, light intensity 400µE, C10 orbital platform shaker New Brunswick Scientific). It was used as a model organism for the flocculation experiments described here.

Isolation of Tetrahymena sp.

The protozoan *Tetrahymena sp.* was identified to the genus level by morphological comparison to taxonomic keys [23]. It was observed in previous open pond reactor experiments at the Solar Biofuels Research Centre (Brisbane QLD) together with cultivated algae *C. sorokiniana* (data not shown). Identification of nuclear DNA using fluorescent staining was performed using Sybr Green (Invitrogen; 0.05x stock concentration and 30 min dark incubation at room temperature), followed by fluorescence microscopy on an Olympus BX-51 microscope equipped with a Semrock GFP-4050A filter set. Excitation was conducted using an Exfo X-Cite 120 epi-lamp.

The *Tetrahymena* sp. was isolated by using a liquid dilution series technique in modified Neff's Medium [24] complemented with cefotaxime (0.66mM). Modified Neff's medium contains 0.25% proteose peptone, 0.25% yeast extract, 0.5% glucose and 33.3µM $FeCl_3$ and was used to maintain the stock cultures only. For reaching log phase and high cell densities the *Tetrahymena* culture was transferred into Super Proteose Peptone (SPP) medium containing 2% proteose peptone, 0.1% yeast extract, 0.2% glucose and 33.3µM $FeCl_3$ [25] and cultivated for 1-2 days.

Tetrahymena species identification by DNA sequencing

The cytochrome-c oxidase subunit 1 (cox1) gene was used to identify the species of Tetrahymena. The cox1 gene was amplified from genomic DNA using the cox1 primers and PCR protocol described by Chantangsi et al. [26]. A single amplicon resulted, which was sequenced in both directions (from each PCR primer) using Sanger sequencing with BigDye terminator v3.1 kit by the Australian Research Genome Facility (AGRF; http://www.agrf.org.au) using each PCR primer. The cox1 gene sequence was then searched against the NCBI nucleotide database using BLAST and the top hits were

aligned with the sequence using ClustalW analysis (MacVector) and subjected to manual inspection. All variant bases were rechecked against the sequence chromatogram.

Tetrahymena culturing and starvation

Flocculation experiments were conducted with *Tetrahymena* undergoing starvation. To reach starvation phase, 50mL of *Tetrahymena* culture grown to log phase was centrifuged (1 min @ 600g) and washed twice in starvation buffer (0.15mM sodium citrate, 0.1mM NaH_2PO_4, 0.1 mM Na_2HPO_4, 0.1 mM $MgCl_2$ and 0.5 mM $CaCl_2$) and finally re-suspended in 25mL volume of the same buffer [17]. *Tetrahymena* were incubated approximately 24 hours before use to ensure cells were in the starvation phase but still healthy.

Induction of exocytosis with Alcian Blue

Exocytosis in *Tetrahymena* was triggered with Alcian blue following the protocol of Turkewitz et al. (2000). Alcian blue (stock concentration 1 %) was added at a ratio of 1:40 to a suspension of starved *Tetrahymena* (typically 0.5–1 x10^6 cells mL^{-1}), to yield a final concentration of 0.025 %. After 1 minute the *Tetrahymena* cells were pelleted by low speed centrifugation (600g, 1 min) and the Alcian blue supernatant discarded. The cell pellet was then resuspended in starvation medium prior to further analysis or addition to algal suspensions.

Bioflocculation screening assays

The suitability of different inexpensive alkaline chemicals NH_4OH, NaOH, and neutral chemicals NH_4Cl (pH=7), $(NH_4)_2SO_4$ (pH=7), and NaCl as inducers of *Tetrahymena*-mediated algal flocculation was initially tested by visual screening in separate 12 well assay plates, except for NH_4OH which was tested in small bottles to prevent cross-contamination by volatile ammonia.

The first 10 wells were filled with algae cultures of *C. sorokiniana* in TAP medium and starved *Tetrahymena* culture, mixed together via pipette. Subsequently the test chemical in a range of concentrations was added individually to a series of wells. The final concentrations of chemical per well were, in succession, 5, 10, 30, 50, 100, 150, 300, 450 600 and 1000mM. The final algae concentration was set to OD_{750}= ~1.0 (~10^6 cells/mL) and the cell ratio of *Tetrahymena* to algae was set at 1:250 in a final volume of 0.6mL. The remaining two wells of each plate were used for controls; one with algae and chemical alone (300mM) without *Tetrahymena*, and the second with algae and *Tetrahymena* without added chemical.

The plate was immediately incubated on an orbital microwell plate shaker (Talboys, Incubating Microplate Shaker, Model 1000MP) at 200 rpm for 30 sec to ensure homogenous cell distribution. Subsequent mixing was reduced to 160 rpm and continued for 1 hour to enhance floc formation. Plates were photographed at intervals to guide later experiments.

Tetrahymena behaviour and floc formation was assayed under an inverted microscope (Nikon Ti-U, x200 and x 400 magnification) and high resolution photomicrographs taken with an Olympus BX-51 upright microscope using differential interference contrast (DIC x200, x400 and x600 magnification) microscopy.

Bioflocculation settlement assays

For quantification of settling rates a spectrophotometric settling assay was used. A spectrophotometer Varian Cary 50 UV-Visible equipped with 18 measuring slots was used to conduct the settlement assays. The cell concentration was measured using the optical density at 750nm and 680nm (OD_{750}, OD_{680}), however resulting OD_{680} curves were similar to OD_{750} and are not displayed here.

For all assays the algae-*Tetrahymena*-chemical ("ATC") mixtures were prepared in 2ml microcentrifuge tubes. Each component (400µl) was added in succession for a final volume of 1.2 ml. The tube was then inverted 5 times and mixed continuously on a RSM6DC rotary shaker (approx. 10 rpm) for 30 min. The concentration of algae prior to mixing with *Tetrahymena* culture was OD_{750}=3.0, leading to a final OD_{750}=1.0 in the reaction mixture.

After the 30 min pre-incubation, the samples were transferred to 10 x 4 x 45 mm polystyrene cuvettes (Sarstedt, Germany) by gentle pouring and the OD_{750} of each cuvette was immediately measured for time point t=0 min of the settlement. Further measurements were taken at 5, 10, 15, 20, 30, 45 and 60 min. During the settlement period the samples remained at the same spectrophotometer position, kept in the dark at room temperature. The OD_{750} was then plotted against time to estimate the rate of settlement. The recovery efficiency was calculated as follows:

$$\text{recovery efficiency}(\%) = \left[1 - \frac{OD_{750}(t)}{OD_{750}(t_0)}\right] \cdot 100$$

where $OD_{750}(t)$ is the turbidity at the time of the clarified zone and $OD_{750}(t_0)$ the turbidity of the initial culture. For t_0 the OD_{750} of the control algae-*Tetrahymena* ("AT") at time point t=0 min was used.

In contrast to a settlement assay performed in an Imhoff cone or a measuring cylinder, cuvettes have a short settlement path and the OD_{750} never falls below ~80% of its initial value even when settlement is complete. Consequently the cuvette assay gives only a relative recovery efficiency. For absolute recovery efficiencies, a settlement assay in a measuring cylinder was used (described below).

Algae-Tetrahymena ratio optimisation

To identify the minimum cell density of *Tetrahymena* required to efficiently flocculate the algae (a critical factor for cost of scale-up), different ratios of *Tetrahymena* to algae were tested (in triplicate) in the presence of a constant concentration of each chemical (300mM). The *Tetrahymena* to algae cell ratios used were 1:1000, 1:500, 1:400, 1:250, 1:125, 1:62.5 and 1:32.25. *Tetrahymena* cell dilutions were calculated from the algae and ciliate cell count and added to the algae culture before each chemical (chemicals tested were NH_4OH, $(NH_4)_2SO_4$ (pH=7) and NaCl).

Settlement controls were performed in parallel for each chemical, by testing each ratio of algae to *Tetrahymena* without a chemical trigger ("AT"), algae and chemical only ("AC"), *Tetrahymena* and chemical only ("TC") and the natural settlement of the untreated algae *C. sorokiniana* (i.e. local strain 8_C4).

Chemical concentration optimisation

Based on the result of the algae-*Tetrahymena* ratio experiments, the optimisation of chemical concentration was subsequently conducted with a cell ratio of 1:125. Five chemicals identified as effective flocculation inducers during microwell plate screening assays were analysed at different concentrations for flocculation and settlement efficiency. The concentration ranges for each chemical were: $(NH_4)_2SO_4$ and NH_4OH (300, 150, 100, 50, 30, 10 and 5 mM), NaCl (1000, 300, 150, 100, 50 and 30 mM), caffeine (30, 10, 5, 2.5, 1, 0.5, 0.1 mM) and 4-chloro-3-methylphenol (2.5, 1, 0.75, 0.5, 0.25, 0.1 and 0.05 mM).

Measuring cylinder assay

To test the results and reproducibility of the previous experiments in a larger volume, a flask trial of *Tetrahymena* mediated algae bioflocculation triggered by sodium chloride was conducted. Three identical 500 mL culturing flasks were prepared with a mixture of algae, *Tetrahymena* and NaCl to a final volume of 100 mL. The final algae OD_{750} was close to OD_{750}=1.0 and the *Tetrahymena* to algae ratio was 1:125. NaCl was added to the algae-*Tetrahymena* mixture to a final concentration of 100 mM. The flasks were immediately incubated on an orbital shaker (continuous shaking at 80 rpm, light

intensity 100µE, C10 orbital platform shaker New Brunswick Scientific) for 30 min. Although this pre-incubation was performed for uniformity, flocculation was visible in the flasks within 1-2 minutes.

After the 30min incubation time the cultures were transferred into three 100 mL cylindrical flask by gentle pouring, to avoid possible floc disruption. The settlement was then observed. A time lapse movie over 30 min (1 picture per second) was recorded (supplemental data).

To analyse the recovery after 30 min, the settlement layers were divided into 10mL fractions and optical density (OD_{750}, OD_{680}) measured. Cell density was also measured by microscopy from the top, middle and bottom fractions, using a haemocytometer.

Pre-triggering assay

To test for the ability of *Tetrahymena* to flocculate algae when exocytosis was induced before the addition to the algae suspension, plate assays were conducted using either NaCl or *p*-chloro-*m*-cresol as a triggering agent. Starved *Tetrahymena* were prepared at a concentration of 1.2 million cells mL^{-1} in starvation medium. An aliquot of 5x stock of either NaCl or *p*-chloro-*m*-cresol was added to the *Tetrahymena* suspension at a ratio of 1:4 resulting in a final concentration of 100mM (NaCl) or 0.5mM (*p*-chloro-*m*-cresol) which resulted in triggering exocytosis of the *Tetrahymena*. At various times (5 s to 10 min) after initiation of exocytosis, 100 µL aliquots were added in triplicate to successive wells of a 12-well plate, each of which contained 1mL of a suspension of *C. sorokiniana* adjusted to OD_{750} = 1.0 (2.2 x 10^6 cells). This resulted in a *Tetrahymena*-to-algae ratio of 1:236. Following addition to the well, the plates were incubated with gentle shaking (80 rpm) at room temperature for 40 min to allow flocculation to proceed. Finally plates were photographed for semi-quantitative scoring.

4.3 Results and Discussion

The observation at the Solar Biofuels Research Centre that only some outdoor cultures of *C. sorokiniana* flocculated (despite being ostensibly identical in composition) prompted an investigation of what factors might differ between them. Both cultures contained low concentrations of ciliated protozoans but it was found that the flocculating cultures contained cells which appeared to be stressed, as shown by a different morphology, faster movement, and by more extensive ingestion of algae. These ciliates also died rapidly after flocculation was initiated. The actual initiation of flocculation appeared to be triggered by the addition of small amounts of ammonium hydroxide, which was dosed into the systems as a pH-amendment and nitrogen source. The ciliated protozoan was tentatively identified as a *Tetrahymena* species (later confirmed upon isolation and subculture). Subsequent amplification of the cox1 gene was used to confirm that it was in fact Tetrahymena. The nearest species was strain RA9 (GenBank EF070322) which was isolated in Singapore from guppy skin, and the nearest named species is Tetrahymena tropicalis [26].

Although *Tetrahymena* can and do ingest *Chlorella*, they do not appear able to effectively digest them, and normally live on bacteria in the culture. We hypothesised that as the *Tetrahymena* in the algal culture exhausts the supply of bacteria, they enter a starved state and become primed for exocytosis, in which case the trigger for exocytosis may have been the ammonium ion itself, the ionic strength or the resultant pH change, although neither pH shifts nor ammonium ion have previously been reported as inducers (also known as "secretagogues") for *Tetrahymena* exocytosis.

Evidence that exocytosis of *Tetrahymena* was responsible for the algal flocculation was sought by isolating the protozoan as an axenic culture, by confirming its identity and ability to undergo exocytosis, and then by confirming its bioflocculation activity with algae.

Tetrahymena acts as a bioflocculant for algae and other single celled organisms

The isolation and identification of an axenic culture of *Tetrahymena* is described in the Methods section. Upon transfer to starvation medium, cells became thinner and moved more rapidly (Figure 4.1 a,b). The polyanionic dye Alcian blue is typically used to trigger exocytosis in starved *Tetrahymena* (Turkewitz 2004). We confirmed that this locally isolated *Tetrahymena* strain also responds to Alcian blue treatment, when starved, by inducing exocytosis (Figure 4.1 d,e).

Next, the ability of *Tetrahymena* exocytosis to trap and flocculate algae was demonstrated using Alcian blue as the inducer. Alcian blue-treated *Tetrahymena* were pelleted after 1 min treatment and

resuspended in a solution of algae at a cell ratio of 1:125. Microscopic examination (Figure 4.2 a,b) clearly shows that the exopolymeric mucous capsules act as a bioflocculant for the algal species (*C. sorokiniana*) from which the *Tetrahymena sp.* was isolated.

We also confirmed that ammonium hydroxide treatment, suspected of acting as a flocculation inducer in our large scale pond cultures, could trigger exocytosis in *Tetrahymena*, which has not previously been reported. Microscopy (Figure 4.2 c) reveals flocs that are similar, though not identical to, flocs generated by Alcian blue treatment of *Tetrahymena*. Small scale assays (Figure 4.2 d) show that as low as 30mM ammonium hydroxide in TAP medium produces flocculation in algae if *Tetrahymena* is present. In contrast, even up to 300mM NH_4OH no flocculation is observed if *Tetrahymena* is absent. Although high (pH >11) or low (pH <4) pH can produce algal flocculation, these assays were conducted in buffered medium to rule out algal flocculation attributable primarily to pH effects [27, 28].

Other algal species are also effectively captured (data not shown) indicating that the observed aggregation is not dependent on the actual algal species utilised. Not only did this explain the flocculation behaviour of the bioreactor cultures, but suggested that *Tetrahymena sp.* could be used as an economical and controllable bioflocculant.

A contribution of protists such as *Tetrahymena* to the formation of biofilms and flocs in wastewater systems has been previously postulated in wastewater treatment [29] [30] while Arregui et al. 2007 [20] showed that *Tetrahymena* triggered with Alcian blue were capable of flocculating latex beads after several days co-incubation. However the use of *Tetrahymena* for the specific flocculation of algae as described here is outstanding, both in the ability to regulate the process using exocytosis inducers and in its rapidity. To our knowledge, *Tetrahymena*, or other protists known to secrete extracellular polymeric substances such as *Paramecium* (Klauke et al. 1998), have not, to date, been used deliberately to produce a controlled flocculation of crop biomass such as microalgae, yeast or bacteria.

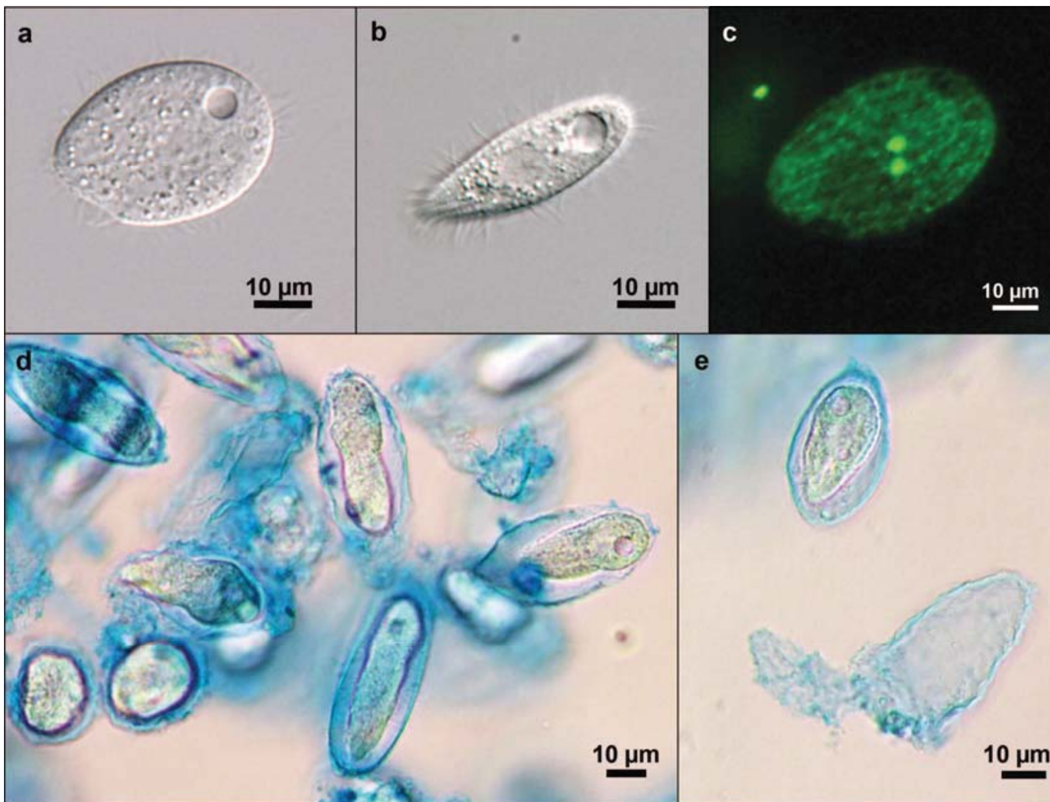

Figure 4.1: *Tetrahymena sp*. isolated from outdoor algal cultures in which spontaneous flocculation occurred. Non-starved (a) and starved (b) *Tetrahymena* cells are shown. Starved *Tetrahymena* are significantly thinner than non-starved cells, and swim more rapidly. The unique nuclear dimorphism of *Tetrahymena* is visualised by fluorescence microscopy using Sybr Green (c). Both nuclei are clearly visible in the cell centre, and secretory granules can be seen at the outer plasma membrane. Starved *Tetrahymena* sp. treated with the cationic dye Alcian blue (d). The exocytosis of extracellular polymer (normally transparent but here stained with Alcian blue) leads to the formation of a loose network around the cell, which in response to cell movement eventually creates a tight capsule around the cells, and also binds cells together in flocs (d). Over time the trapped ciliate breaks through the capsule leaving an empty protein shell in the culture (e).

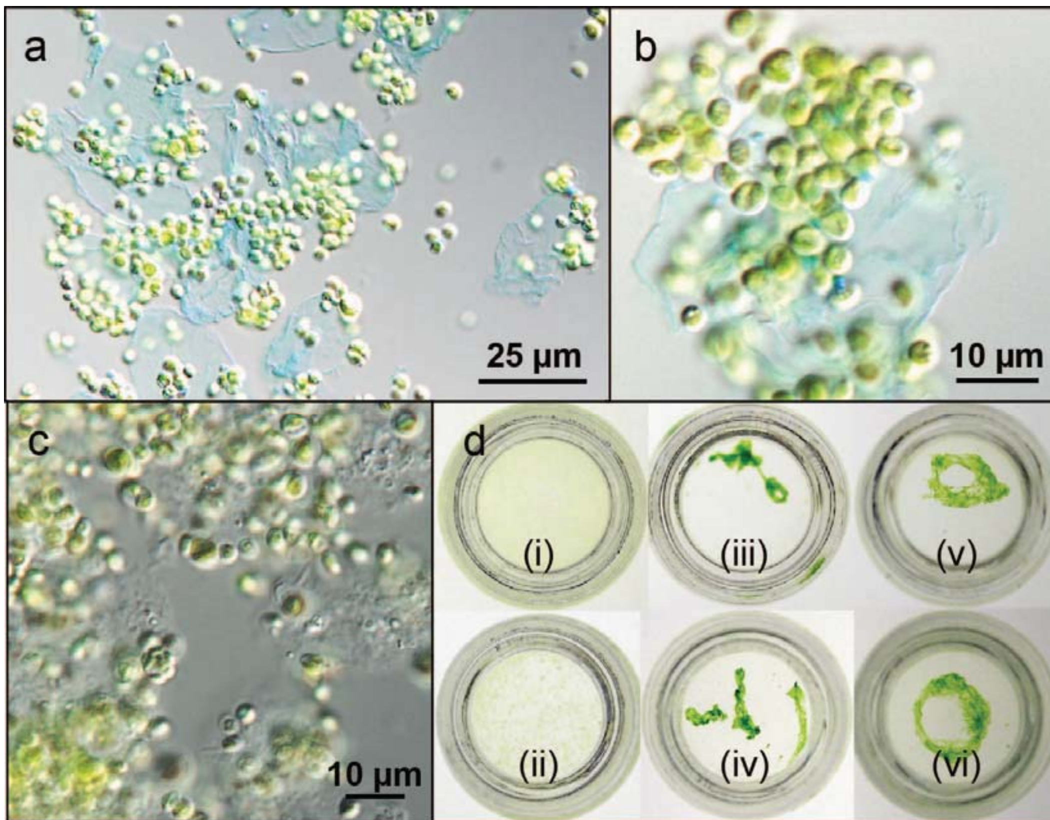

Figure 4.2: Flocculation of algae by *Tetrahymena* in response to exocytosis. Panel (a) shows the entrapping of *C. sorokiniana* in EPS produced by Alcian blue-initiated exocytosis of *Tetrahymena*. At higher magnification (b) it can be seen that the algae are held together primarily by the extracellular *Tetrahymena* polymer rather than by cell-cell interactions. Alcian blue carried over from the treatment stains the EPS a pale blue. In Panel (c) EPS produced by exocytosis of *Tetrahymena* treated with 30mM ammonium hydroxide generates a similar flocculation of *C. sorokiniana*, though here the EPS is unstained. In Panel (d) a series of well assays demonstrates that the flocculation of *C. sorokiniana* in TAP medium only occurs in the presence of *Tetrahymena* (cell ratio 1:250; assays recorded at 30min). No flocculation occurs in wells containing only algae with 300mM ammonium hydroxide (i). Slight flocculation occurs when algae and *Tetrahymena* are present without an inducer (ii). Robust flocculation occurs in the presence of algae, *Tetrahymena* and ammonium hydroxide at 30mM (iii) 50mM (iv) 300mM (v) or 450mM (vi).

Identification of inducers

Since Alcian blue is not a feasible inducer (secretogogue) for industrial scale applications, we searched for easily produced conditions that would trigger exocytosis but which would still be cost-effective at large scale and yet would not compromise subsequent biomass treatment. It has also been previously noted that exocytosis can also occur under high salt conditions (150mM [31]. We confirmed this using a screening assay in 24 well plates (Figure 4.3), demonstrating that as little as 75mM NaCl was sufficient to initiate flocculation.

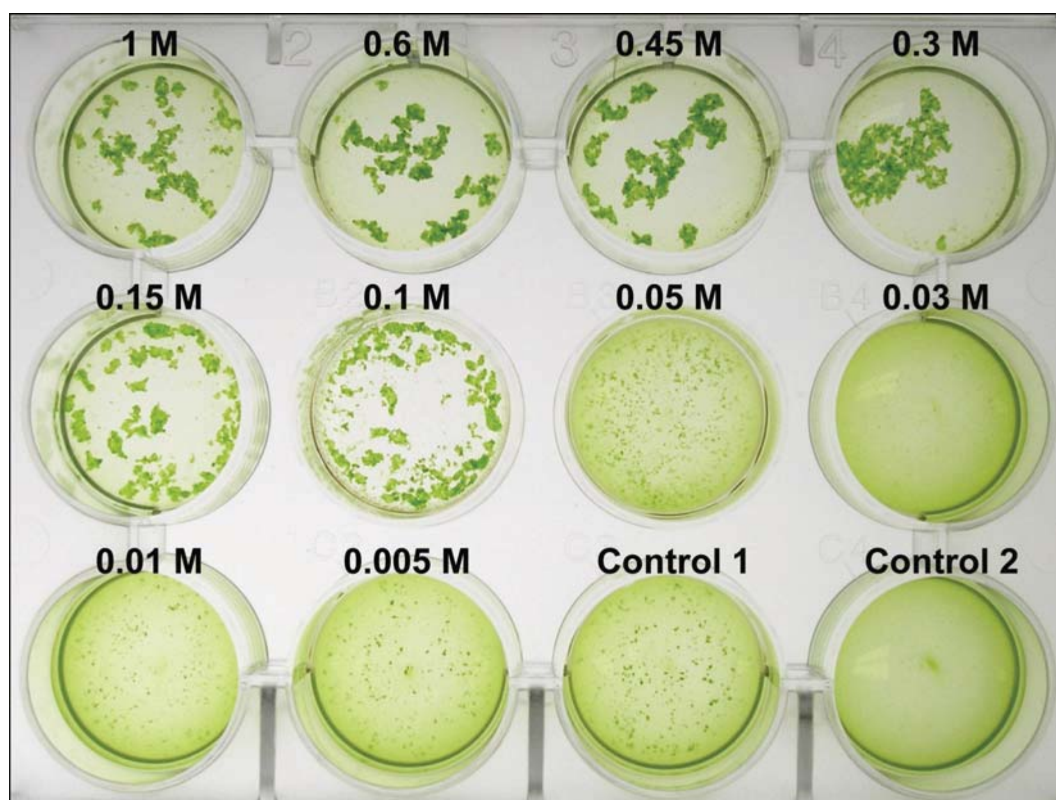

Figure 4.3: Representative microwell plate assay for NaCl as an inducer of exocytosis in starved *Tetrahymena* leading to algal bioflocculation. Each compound was tested and compared over times from 1 to 60 min; the panel shown is at 10 min. The concentrations of NaCl shown on each well are the final concentrations in the well. Slight flocculation of algae occurs in the presence of starved *Tetrahymena* alone (probably due to basal exocytosis) but is dramatically increased over 50mM NaCl. The ratio of *Tetrahymena* to algae was 1:250. The final cell density of algae was 2.5×10^8 cell mL^{-1}.

Although sodium hydroxide was also effective at triggering exocytosis, ammonium hydroxide produced bioflocculation at concentrations as low as 30mM, while ammonium chloride and ammonium sulphate were also effective at 50mM, lower than that observed for NaCl. High pH (e.g. NaOH) is capable of producing flocculation of algae even without the presence of *Tetrahymena* [32] [27], but the resulting flocs were better formed when *Tetrahymena* was present (data not shown).

Microscopic investigation of the flocs suggested that both the reagent and the ratio of *Tetrahymena* to algae led to variation in the properties of the flocs with those produced by ammonium hydroxide being tighter and more dense than flocs produced by NaCl. Although not fully quantified to date, this suggests that flocs with different properties may be preferred for specific harvesting methods; for example, less dense flocs may be better suited for dissolved air flotation while denser flocs may be preferred for settling methods.

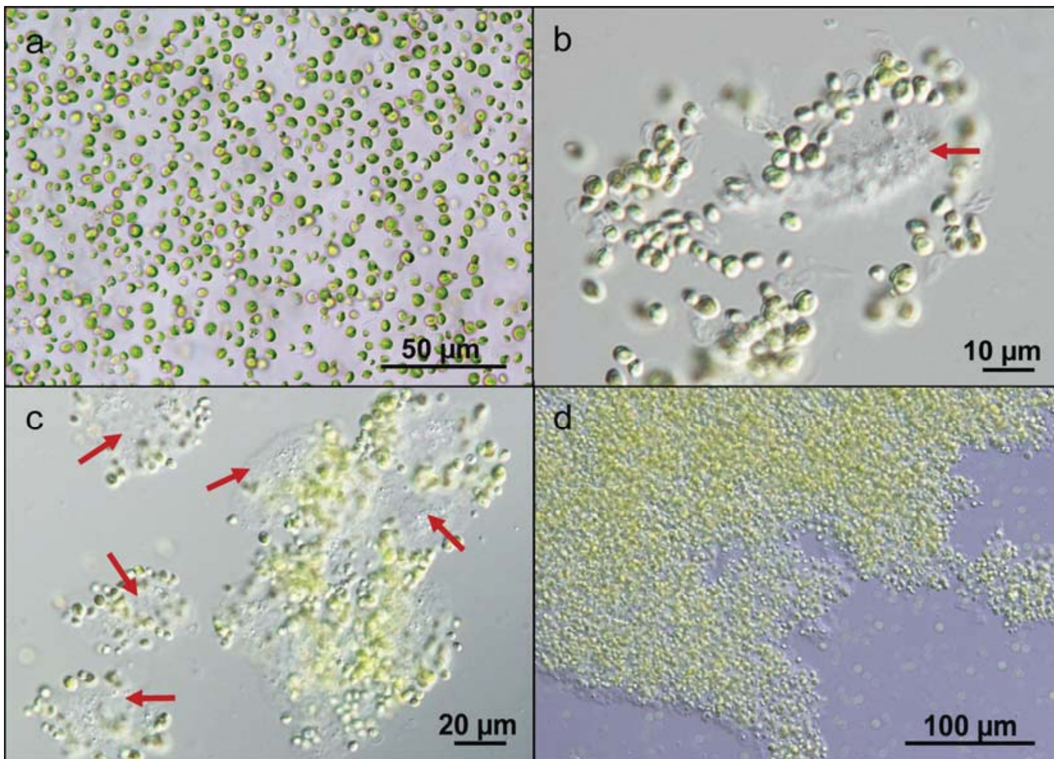

Figure 4.4: Floc development by *Tetrahymena* triggered with NaCl. Panel a shows *C. sorokiniana* in its natural non-flocculating state. After adding NaCl to a final concentration of 100mM to a 1:125 mixture of *Tetrahymena* and *C. sorokiniana*, algal cells immediately adhere to the EPS around the ciliate (b). *Tetrahymena*-*Chlorella* flocs then rapidly increase in size due to the aggregation of smaller flocs (c and d). *Tetrahymena* cells releasing EPS are indicated with red arrows. At this salt concentration, the *Tetrahymena* do not survive long and disintegrate so that the final flocs appear to contain mainly algal cells (d).

Bioflocculation settling assays

Quantitative settling assays of bioflocculation were conducted using a spectrophotometer to measure biomass settling in a cuvette, following the triggering of exocytosis. Both OD_{750} (measuring light scattering) and OD_{680} (scattering as well as chlorophyll absorbance) were used to follow the settling of *C. sorokiniana* 8_C4 in spectrophotometer cuvettes. Without mixing, this organism settles at a recovery of ~3-4% h^{-1}. An algal cell suspension containing the inducer but without *Tetrahymena* was used as a control for flocculation activity not attributable to *Tetrahymena*. The settlement response can be divided into two phases. Triggering of exocytosis is rapid, occurring in milliseconds [33] producing an immediate drop in OD_{750} as the initial flocs form rapidly, followed by a slow decrease as the flocs accumulate more algae and also sink slowly.

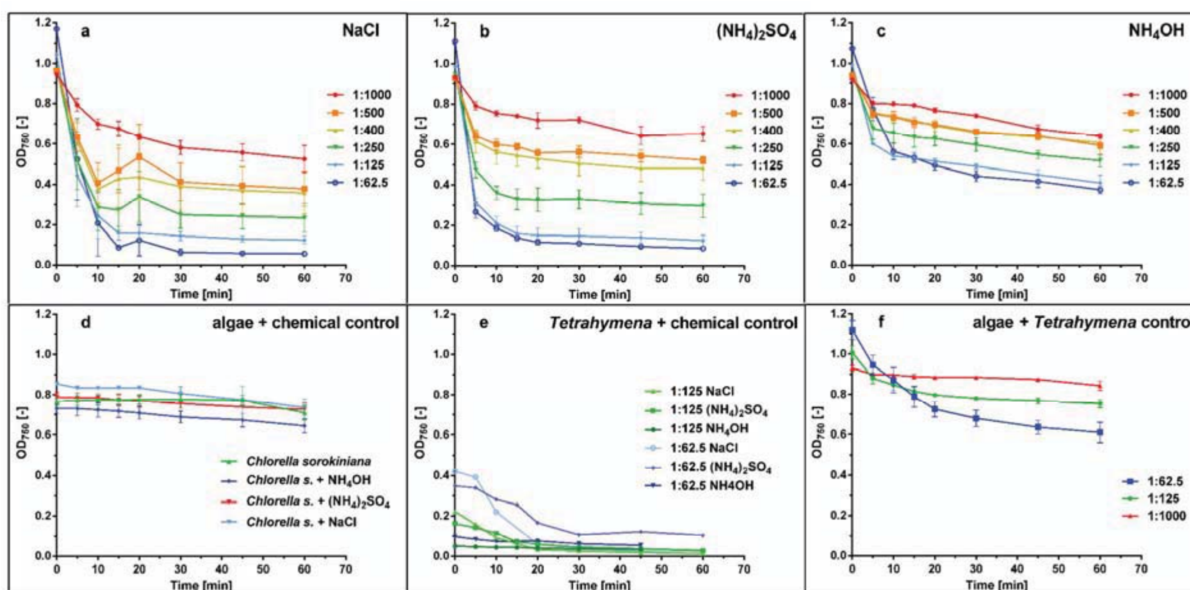

Figure 4.5: Settling assays to determine effective ratios of *Tetrahymena* to *C. sorokiniana* in the presence of three inducers of flocculation: (a) NaCl (300mM), (b) ammonium sulphate (300mM), and (c) ammonium hydroxide (300mM). Panel (d) shows the effect of the same chemical additions in the absence of *Tetrahymena*. Panel (e) is a control for OD_{750} due to *Tetrahymena* alone while panel (f) shows the settling of starved *Tetrahymena* in the presence of algae but without addition of an inducer.

Effect of Tetrahymena to algae ratio

The feasibility of using *Tetrahymena* as an algal bioflocculant depends on achieving a suitable effect with a relatively small amount of *Tetrahymena* biomass. Consequently, the effect of a variable ratio of *Tetrahymena* to algae was tested using suitable exocytosis-inducing conditions derived from screening assays (Figure 4.5). It was found that for all triggering stimuli examined, flocculation is satisfactory at ratios of 1:400 to 1:60, but that high *Tetrahymena* ratios (1:30 and above) inhibit flocculation while ratios below 1:500 become ineffective. Microscopic inspection of the flocs suggested that high *Tetrahymena*:algae ratios lead to small tight flocs largely consisting of *Tetrahymena* EPS, with the exclusion of much of the algal biomass. Subsequently, a ratio of 1:125 was routinely used in these experiments.

Mechanism of induction

The mechanism by which these agents trigger exocytosis is still under investigation; although not specifically identified in *Tetrahymena*, the final exocytosis process in the ciliate *Paramecium* involves mobilisation of internal calcium stores [34] which is thought to be mediated by ryanodine (RyR) receptors which are calcium channels. The small molecule 4-chloro-3-methylphenol (*p*-chloro-

m-cresol) is an agonist for these receptors while caffeine acts as a sensitiser to potentiate channel activation and both have been shown to promote exocytosis in *Paramecium* (Plattner 2013).

We confirmed in microplate assays (data not shown) that these agonists also produce immediate exocytosis in *Tetrahymena* leading to bioflocculation of algae, thereby supporting a ryanodine receptor-mediated exocytosis in *Tetrahymena*. The mechanism by which changes in pH, salinity and ammonium ion affect exocytosis (presumably also via RyR activation) is under investigation, but the demonstration here of both a highly specific receptor based mechanism and a simple bioassay, suggests that there are excellent prospects for the identification of more potent agonists that will prove feasible to use at very low concentrations for algal harvesting. We are currently exploring a range of stimuli both physical (thermal, electrical) and chemical (osmotic, pH, calcium ionophores and other compounds) to achieve this. There is also potential for genetic modification of the exocytosis system to allow sensitive artificial control, for example by linking RyR receptors to a different biological triggering system such as light (optogenetics).

Effect of inducer concentration

The minimum effective concentration of the inducing stimulus was then examined (Figure 4.6). It was shown that flocculation was induced effectively, if not optimally, at 100mM NaCl, 50mM $(NH_4)_2SO_4$, 50mM NH_4OH, 30mM caffeine and 0.5mM *p*-chloro-*m*-cresol.

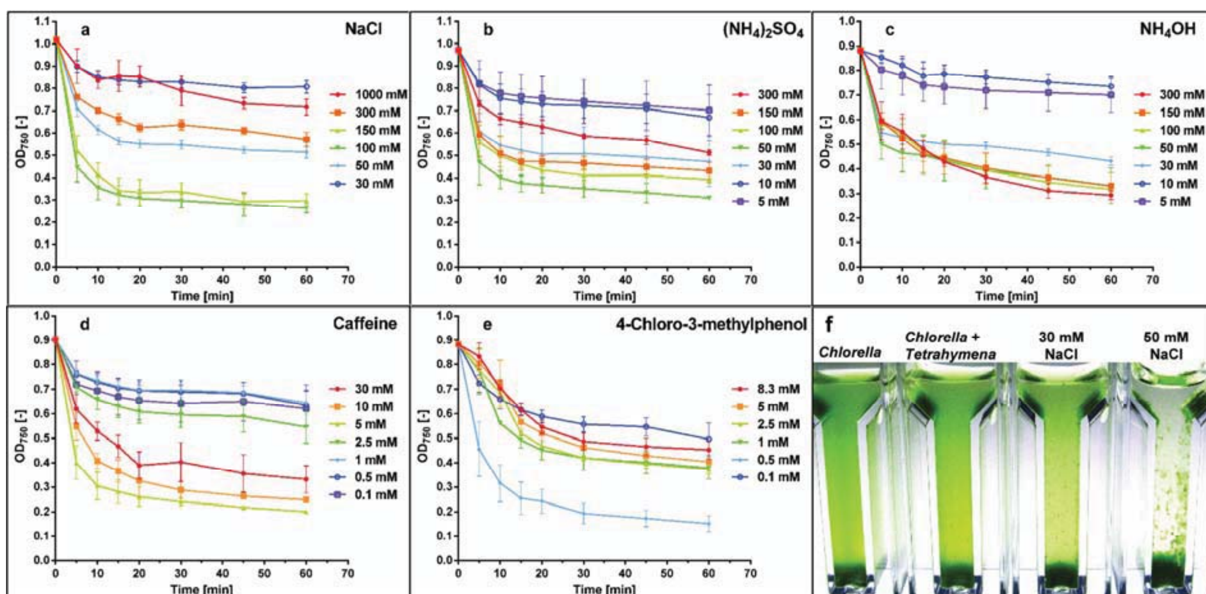

Figure 4.6: (a-e) Flocculation settlement experiments testing various inducer concentrations at a constant *Tetrahymena* to algae ratio (1:125) over a 60 min time course. Panel (f) shows the appearance of cuvettes including settled Algae-*Tetrahymena* culture at different NaCl concentrations (as shown on figure) after 60 min.

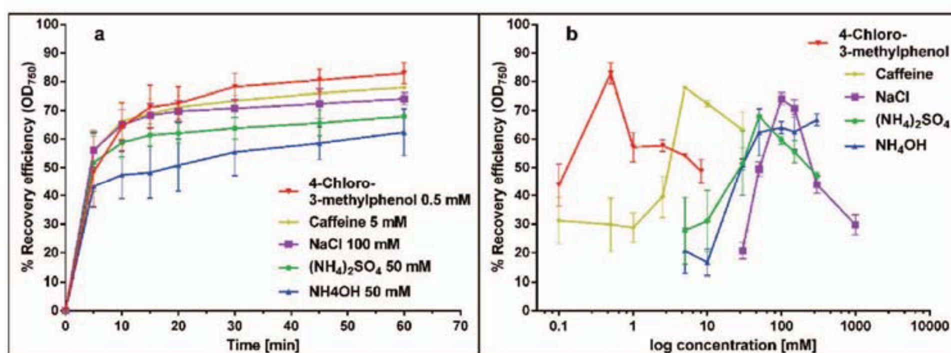

Figure 4.7: Comparison of the recovery efficiency of each tested chemical over time using its optimised concentration (a). The graph shows the highest recovery efficiency of 82% is achieved with *p*-chloro-*m*-cresol (0.5mM) after one hour. In panel (b) the effective concentration range of each chemical is compared using settlement data at 30min.

We were particularly interested in the use of sodium chloride since many algal strains grow in saline conditions. Seawater has a salinity of ~600mM, most of which is due to NaCl. Since as little as 30mM NaCl will trigger exocytosis, the simple addition of starved *Tetrahymena* to a saline algal culture was found to be sufficient to induce exocytosis and bioflocculation. For saline-tolerant strains, this avoids the need to add a specific trigger compound to induce flocculation and is consequently of great practical utility. Using a salt-tolerant *Tetraselmis* strain we confirmed that effective flocculation could be induced in this way.

The use of ammonium compounds is also of interest as ammonium hydroxide can be used as an industrial nitrogen source and pH modulator while ammonium sulphate is a typical nutrient reagent. This is relevant because not only must flocculation be induced upon harvesting, but the prevention of spontaneous flocculation during culture is also important.

Naturally it is unlikely that algae are uniquely susceptible to *Tetrahymena*-induced bioflocculation; consequently the application of this technique may extend well beyond the field of algaculture. Nonetheless, biomass harvesting is not such an energy- and cost-dependent issue for many other applications as it is for algae, especially in the case of biofuel systems where the net energy balance is a crucial variable and where harvesting imposes a significant energetic load.

Effectiveness of dewatering

Following the formation of algal flocs, aeration of the culture with fine bubbles leads to flotation of the flocs on the surface of the culture (Figure 4.8A) (see supplementary data video 4.1), indicating that dissolved air flotation could be a viable method for industrial applications of this technique. Conversely, as already shown, flocs readily settle (Figure 4.8B) so that dewatering can also be

achieved using gravity, as long as an effective method is available to remove the settled flocs from the supernatant. We examined the effectiveness of bulk floc settling at laboratory scale using measuring cylinder assays under standard conditions (1:125 ratio of *Tetrahymena* to algae, with 100mM NaCl as an inducer of flocculation). For settlement experiments, triplicate 100mL samples were allowed to settle for 30min following floc induction, and the supernatant collected in 10mL fractions down to the floc zone. The biomass contained in the resultant fractions (floc and supernatant) was then measured using OD_{750} measurements and cell counting. Time lapse video (exemplified in the supplementary data video 4.2) was also collected to illustrate the process. It was shown that 30min settling allowed harvesting of >95% of the biomass in the culture which was therefore concentrated ~20x. It is likely that this could be substantially improved at industrial scale.

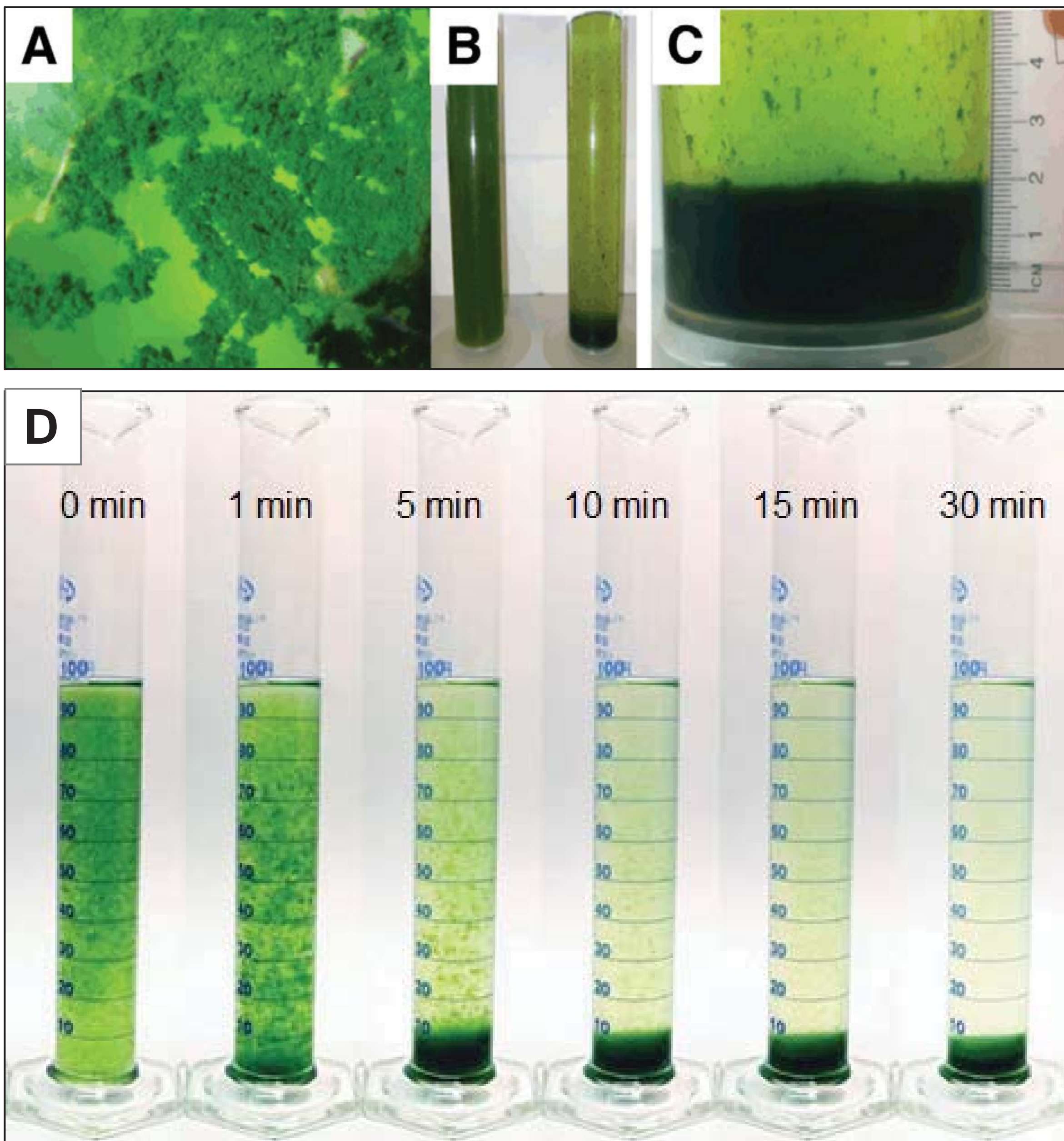

Figure 4.8: Flotation or gravity settling as methods for floc harvesting. A: Flotation of biofloc in an algal photobioreactor as a result of air bubbling (see supplementary data video 4.1). B: Gravity settling in graduated cylinders, comparing a non-flocculated with a flocculating culture. C: After 30min settling, the bulk of the biomass was present in the bottom 10mL of the flask, representing 1/10th of the flask volume. D: Settlement of a *C. sorokiniana* culture flocculated with *Tetrahymena* and 100 mM NaCl. Images of one cylinder at different time points are shown. Flocs sink rapidly and biomass accumulation is already visible after 1 minute. After 5 minutes the culture has significantly cleared in the first 90 mL and sedimentation process is nearly complete by 15min (see supplementary data video 4.2).

Feasibility of pre-triggering exocytosis or co-culture

Using the method described above, the inducer is added to the entire algal culture, to initiate floc formation. A preferable approach would be to pre-trigger a small volume of *Tetrahymena* culture, which can then be added to the bulk algal culture. Figure 4.9 shows that this approach is effective for two inducers, NaCl and *p*-chloro-*m*-cresol. In the case of NaCl, flocculation is less complete if initiated too soon after addition of exocytosis, but after 30 sec full flocculation is apparent. The final concentration of inducers in the algal suspension is 9.1mM for NaCl or 4.5 μM *p*-chloro-*m*-cresol respectively and demonstrates that final effective inducer concentrations can be very low, minimising material costs. Further work will establish the limits of this approach.

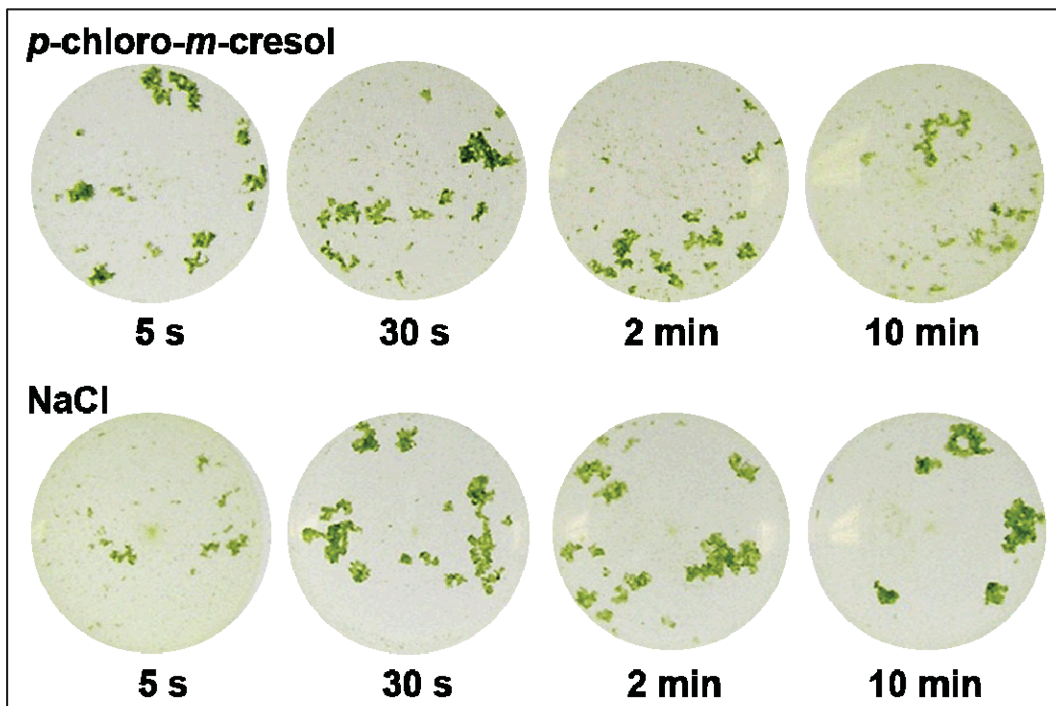

Figure 4.9: Plate assay for flocculation of *C. sorokiniana* using pre-triggered *Tetrahymena* with either *p*-chloro-*m*-cresol (0.5mM) or NaCl (100mM) and diluted 10-fold into the algal suspension at 5 s, 30 s, 2 min or 10 min after triggering exocytosis. Representative wells are shown.

Similarly, it is realistic to grow *Tetrahymena* alongside algae in a culture, allowing starvation at the point of harvesting, as long as the growth of *Tetrahymena* can be independently regulated and other

organisms do not interfere. This offers the possibility of a very low energy system since only the inducer would need to be mixed in. In the case of a low molecular weight compound such as *p*-chloro-*m*-cresol this would be assisted by diffusion, reducing the mixing energy. Conversely, in situations where protozoan exocytosis needs to be suppressed, the availability of specific RyR antagonists (e.g. procaine) may achieve this.

4.4 Conclusion

The use of protozoan exocytosis systems as a bioflocculant method for microalgal biotechnology systems has not been previously reported.

It is expected that this approach will be especially advantageous for algal applications, as energy efficiency is most critical in biofuel systems, and large scale cultures which exclude intrinsically expensive methods such as centrifugation. However, we anticipate that this approach is equally applicable to non-algal systems, including yeast, bacteria and cells from multicellular organisms grown in culture, such as mammalian cell suspension cultures. The main benefits of this system stem from the fact that:

1. Relatively little *Tetrahymena* biomass is needed to effectively flocculate algae, and *Tetrahymena* biomass is effectively used in the algal product.
2. Subsequent gravity settling leads to effective dewatering
3. The *Tetrahymena* biomass does not detrimentally affect the properties of the subsequent harvested biomass
4. The process is highly controllable and rapid
5. Flocculation triggers exist which are practical to employ at commercial scales
6. Other inducers may exist which are even more effective, or which may be engineered using genetic modification techniques
7. Pre-triggering or growth *in situ* may be possible, further reducing material requirements
8. For algae grown in saline culture the medium itself is sufficiently saline to trigger exocytosis and bioflocculation

The result is a bioflocculation-based harvesting system that is potentially inexpensive and low in energy inputs. We expect that for large scale algaculture, this will provide a way to reduce energy and capital costs which will bring the prospect of cost-effective algal biofuels one step closer.

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Author Contributions

GJ, IR, BH, ES, and MO conceived and designed the experiments. GJ conducted the experiments. GJ and IR analysed the experimental data. IR, GJ and BH wrote the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

4.5 References

1. How to Feed the World 2050. 2009, Food and Agriculture Organisation of the United Nations.
2. (IEA), I.E.A., World Energy Outlook 2011. 2011, International Energy Agency (IEA): Paris.
3. Environmental Outlook to 2050. 2014, Organisation for Economic Co-operation and Development.
4. IPCC, Climate Change 2007: Synthesis Reoprt. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, P. Core Writing Team, R.K. and Reisinger, A., Editor. 2007: IPCC, Gemeva, Switzerland.
5. Vandamme, D., I. Foubert, and K. Muylaert, Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. Trends in Biotechnology, 2013. **31**(4): p. 233-239.
6. Liu, Y. and H.H.P. Fang, Influences of extracellular polymeric substances (EPS) on flocculation, settling, and dewatering of activated sludge. Critical Reviews in Environmental Science and Technology, 2003. **33**(3): p. 237-273.
7. Lin, H.J., et al., A critical review of extracellular polymeric substances (EPSs) in membrane bioreactors: Characteristics, roles in membrane fouling and control strategies. Journal of Membrane Science, 2014. **460**: p. 110-125.
8. More, T.T., et al., Extracellular polymeric substances of bacteria and their potential environmental applications. Journal of Environmental Management, 2014. **144**: p. 1-25.
9. Salim, S., et al., Harvesting of microalgae by bio-flocculation. Journal of Applied Phycology, 2011. **23**(5): p. 849-855.
10. Oh, H.M., et al., Harvesting of Chlorella vulgaris using a bioflocculant from Paenibacillus sp AM49. Biotechnology Letters, 2001. **23**(15): p. 1229-1234.
11. Lee, A.K., D.M. Lewis, and P.J. Ashman, Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel. Journal of Applied Phycology, 2009. **21**(5): p. 559-567.
12. Manheim, D. and Y. Nelson, Settling and Bioflocculation of Two Species of Algae Used in Wastewater Treatment and Algae Biomass Production. Environmental Progress & Sustainable Energy, 2013. **32**(4): p. 946-954.
13. Prajapati, S.K., et al., Exploring Pellet Forming Filamentous Fungi as Tool for Harvesting Non-flocculating Unicellular Microalgae. Bioenergy Research, 2014. **7**(4): p. 1430-1440.
14. Sathe, S. and P.M. Durand, A low cost, non-toxic biological method for harvesting algal biomass. Algal Research-Biomass Biofuels and Bioproducts, 2015. **11**: p. 169-172.

15. Lee, A.K., D.M. Lewis, and P.J. Ashman, Harvesting of marine microalgae by electroflocculation: The energetics, plant design, and economics. Applied Energy, 2013. **108**: p. 45-53.
16. Tiedtke, A., Capsule Shedding In Tetrahymena. Naturwissenschaften, 1976. **63**(2): p. 93-93.
17. Turkewitz, A.P., et al., Regulated protein secretion in Tetrahymena thermophila. Methods in Cell Biology, Vol 62, 2000. **62**: p. 347-362.
18. Haddad, A., G.R. Bowman, and A.P. Turkewitz, New class of cargo protein in Tetrahymena thermophila dense core secretory granules. Eukaryotic Cell, 2002. **1**(4): p. 583-593.
19. Cowan, A.T., et al., Genetic, genomic, and functional analysis of the granule lattice proteins in Tetrahymena secretory granules. Mol Biol Cell, 2005. **16**(9): p. 4046-60.
20. Arregui, L., et al., Ciliate contributions to bioaggregation: laboratory assays with axenic cultures of Tetrahymena thermophila. International Microbiology, 2007. **10**(2): p. 91-96.
21. Jakob, G., et al., Surveying a diverse pool of microalgae as a bioresource for biotechnological applications. Journal of Petroleum & Environmental Biotechnology, 2013: p. 4-5.
22. Gorman, D.S. and R.P. Levine, Cytochrome F And Plastocyanin - Their Sequence In Photosynthetic Electron Transport Chain Of Chlamydomonas Reinhardi. Proceedings of the National Academy of Sciences of the United States of America, 1965. **54**(6): p. 1665-&.
23. Taylor, W.D. and R.W. Sanders, Chapter 3 - Protozoa, in Ecology and Classification of North American Freshwater Invertebrates (Third Edition), J.H.T.P. Covich, Editor. 2010, Academic Press: San Diego. p. 49-90.
24. Cassidy-Hanley, D.M., Chapter 8 - Tetrahymena in the Laboratory: Strain Resources, Methods for Culture, Maintenance, and Storage, in Methods in Cell Biology, C. Kathleen, Editor. 2012, Academic Press. p. 237-276.
25. Cassidy-Hanley, D.M., Tetrahymena in the Laboratory: Strain Resources, Methods for Culture, Maintenance, and Storage, in Tetrahymena Thermophila, K. Collins, Editor. 2012. p. 239-276.
26. Chantangsi, C., et al., Barcoding ciliates: a comprehensive study of 75 isolates of the genus Tetrahymena. International Journal of Systematic and Evolutionary Microbiology, 2007. **57**: p. 2412-2425.
27. Vandamme, D., et al., Flocculation of Chlorella vulgaris induced by high pH: Role of magnesium and calcium and practical implications. Bioresource Technology, 2012. **105**: p. 114-119.
28. Liu, J.X., et al., Freshwater microalgae harvested via flocculation induced by pH decrease. Biotechnology for Biofuels, 2013. **6**.

29. Watson, J.M., Mechanism of bacterial flocculation caused by protozoa. Nature, 1945. **155**(3931): p. 271-272.
30. Curds, C.R., The flocculation of suspended matter by ciliated protozoa. J Protozool, 1963. **10**((Suppl.)): p. 30-31.
31. Tiedtke, A., et al., Exocytosis, endocytosis and membrane recycling in *Tetrahymena-thermophilia*. Journal of Cell Science, 1988. **89**: p. 515-520.
32. Lee, S.J., et al., Effects of harvesting method and growth stage on the flocculation of the green alga Botryococcus braunii. Letters in Applied Microbiology, 1998. **27**(1): p. 14-18.
33. Knoll, G., C. Braun, and H. Plattner, Quenched flow-analysis of exocytosis in paramecium cells - time course, changes in membrane-structure, and calcium requirements revealed after rapid mixing and rapid freezing of intact-cells. Journal of Cell Biology, 1991. **113**(6): p. 1295-1304.
34. Plattner, H., Calcium Regulation in the Protozoan Model, Paramecium tetraurelia. Journal of Eukaryotic Microbiology, 2014. **61**(1): p. 95-114.

Chapter 5

Discussion and Conclusion

Considering the rise of human population (to >9 billion) and the continue global economic growth, by 2050 we will require 50% more fuel [1], 70% more food [2], 50% more fresh water [3] and CO_2 emission cuts of ~80% [4] to maintain political, social, fuel and climate security.

Due to high biomass yields and potential for CO_2 neutral fuel production [5], the development of commercially viable microalgae production systems is a promising option to tap into the huge energy resource of the sun and provide a path to a more sustainable future.

This challenging goal requires the identification of optimum microalgae production conditions. However this is a complex multidimensional space problem with many interdependent variables which is currently occupying a global research effort. Species selection, nutrient and CO_2 supply, the removal of oxygen, pH and temperature profiles are all known to be crucial variables requiring optimisation. Technical issues including system design, batch versus continuous cultivation, mono- versus polyculture and protection or resistance against external environmental factors (e.g. microorganism competition and predation) complicate industrial scale realisation.

I first focused on the sampling, isolating and successful maintenance of a reasonably broad biological diversity set of Australian microalgae (Chapter 2). This provided experience of handling a broad species diversity, helped to identify opportunities and avoided "locking in" system designs to suit only a small selection of common strains. Sampling sources included fresh water, brackish water and marine systems to obtain as broad and diverse a range of species and abilities as possible. An overview of the species identified from the different water sources is given in table 5.1. Ideally extreme conditions (e.g. extreme salinity, temperatures etc.) would have been desirable to test but these were beyond the practical scope of the work.

Table 5.1: Summary of identified microalgal species, isolated from the major categories of freshwater, brackish and saltwater habitat.

	Freshwater	Brackish water	Saltwater
ALGAL SPECIES ISOLATED AND IDENTIFIED	*Ankistrodesmus sp.* *Anabaena sp.* *Aulacoseira sp.* *Chlamydomonas sp.*, *Chlorella sp.* *Chlorella sorokiniana* *Chlorococcum sp.*, *Coleastrum sp.* *Closterium sp.* *Desmodesmus sp.* *Desmodesmus intermedius* *Elakatothrix sp.* *Euglena sp.* *Micractinium pusillum* *Micractinium sp.* *Merismopedia sp.* *Nannochloris sp.* *Scenedesmus sp.*, *Scenedesmus abundans* *Staurastrum sp.* *Stichococcus sp.*	*Chlorella sp.* *Chlorella sorokiniana* *Micractinium pusillum* *Micractinium sp.* *Navicula pelliculosa sp.* *Scenedesmus sp.*	*Chlorella sp.* *Chlorella sorokiniana*

Although clearly the full complex multidimensional space of species distribution is not been covered as this is beyond the scope of the PhD thesis, the results provided in chapter 2 demonstrate a streamlined process for microalgae recovery, and have resulted in a library of local algal strains for ongoing work, many of which are cryo-preserved [6].

Experience was gained in a range of algae purification methods including fluorescence activated cell sorting (FACS), micromanipulation and dilution techniques. Here the rapid isolation principle of FACS proves to be the most effective high-throughput method for physically robust and tolerant algal strains. The laborious technique of micromanipulation, on the other hand, has benefits for the targeted isolation of a higher species diversity. Almost half of the collection of around 150 maintained cultures were successfully preserved using cryo-preservation techniques reported in Bui et al (2013) [6].

With the focus on optimising microalgae production, the next phase of reducing the complexity of this multidimensional space was to analyse optimal nutrient conditions for each microalgae strain (Wolf et al. 2014) [7]. This identified the highest biomass producing strains at the optimal nutrient conditions, enabling selection of high biomass producing strains.

It was anticipated that the process of scaling up from laboratory to pilot scale culture systems would reveal differences in growth and culture complexity. To analyse this we selected the top 20 strains based on the maximum growth rates and of these chose the top eight most morphologically diverse for trials at pilot scale to sample a broad phylogenetic diversity to track their relative performance in polyculture. Furthermore we included an indigenous microalgae species which dominantly established itself in trials of open ponds containing only nutrified water at the pilot plant SBRC.

Many factors impact on the performance of microalgae exposed to the natural environment at pilot scale including CO_2 supply, oxygen levels, pH and temperature fluctuation, mono- versus polycultivation or contamination by other non-target microorganism (indigenous algae, predators, bacteria, fungi and viruses). Of the six HRPs constructed (displayed in figure 3.2, a), not all were available for every trial, as other experiments were being conducted simultaneously. There is also some variation expected between bioreactors, as physical mechanisms such as mixing, wind, splash, local heating or shear stresses which can impact on algal performance can vary between systems. These factors are not present or tightly controlled in the laboratory. Consequently in chapter 3 we first conducted single species trials in open pond systems using optimum nutrient conditions. The aim of this experiment was to compare the performance of the strains under these conditions and analyse whether there were other additional factors affecting optimum productivity that we had not yet considered. For example the effect of light and temperature fluctuation and its effect on specific species appearance and the complex interplay between each of these. Furthermore resulting competition and predation by foreign species (both algal and non-algal), led to inhibition or assistance in growth, as well as other effects such as aggregation of the target algal cells.
Based on these studies I found the top production candidates to be *M. pusillum* (5_H4), *Chlorella* sp. (11_H5), and *Chlorococcum* sp. (12_02). Besides their fast growth rates they were able to attain high cell densities and could be harvest using simple technologies (e.g. settlement). Furthermore especially *M. pusillum* (5_H4) and *Chlorococcum* sp. (12_02) show strong resistance against foreign microorganisms which limits the potential of most fast growing microalgae tested. This has therefore confirmed predation as another major factor that must be considered in the multidimensional space complexity.

The effect of polycultures was also tested. The rationale behind the use of polycultures is that different strains offer advantages at different times during culture evolution. However, it is also possible that historical effects could occur – for example the order of dominance of a strain could lead to elimination of other strains or chance dominance of particular strains. Most polycultures are systems where no attempt is made to control strain distribution, and the strains present are seeded from natural populations. This results in complex, poorly characterised assemblies of natural strains. Consequently, even when they perform well, it is difficult to demonstrate why this is the case. I therefore tested the effect and growth performance of a defined polyculture by mixing five morphologically different selected strains, which enabled tracking of the different subpopulations. This provided two key findings. First the polyculture outperformed all but one of the monoculture trials in terms of growth rate and productivity. Second, strains which are fast growing under laboratory conditions, but which result in poor monoculture performance due to grazing pressure by contaminant organisms, show strong growth and dominance within the polyculture. Possibly defenceless algal species find protective niches between species which do have protective mechanisms until grazing pressure diminishes. These findings require further study in defined systems such as those described here.

Future investigation and optimisation of algal pond cultivation towards optimised and profitable production need to be considered to strengthen the findings above. Some experimental difficulties could be resolved with better equipment and experimental design, for example monitoring the relationship between cell number, biomass and optical density. In so doing, flow cytometry would be a highly efficient tool for monitoring algal cell cycles, viabilities, strain-distribution and shifts, especially for polycultures, would give clearer insight on the culture behaviour and possibilities for predicting future changes. Work completed in the laboratory as these experiments were being conducted shows how optical density can be related to biomass via flow cytometry measurements [8]. Furthermore to confirm the observed findings and eliminating possible variables, the open pond experiments (mono- and polyculture) should be repeated in triplicates and under all seasonal weather conditions.

Interestingly predation also correlated with aggregation for example in cases of *Chlorella* sp. (11_H5) and *C. sorokiniana* (8_C4). This is important because the aggregation of algae greatly affects productivity due to light limitation. It needs to be suppressed during the growth cycle, but it also provides the basis for the most cost effective harvesting techniques which are also important from an economic perspective to developing high-efficiency microalgae production systems.

The large scale open pond trials revealed the presence of culture aggregation under specific conditions. This phenomenon was further investigated as a possible harvesting mechanism. Chapter 4 is therefore focused on the issue of aggregation which affects productivity and harvesting, both of which are economically important to the development of economic systems for scale up.

It was shown that *Tetrahymena,* a ciliate protozoan present as a natural contaminant in the cultures, could be used to flocculate and dewater microalgae in a very efficient and controllable manner. The idea of using a protozoan as microalgae flocculant was only once identified during the review process of this publication. Sathe *et al.* (2015) reported effective flocculation using the flagella organism *Peranema*, however the procedure differs to those of the ciliate Tetrahymena for example in terms of trigger mechanism, binding substance and possible limitation to microalgae only [9]. The promising nature of the use of protozoan exocytosis as a bioflocculant has not been previously reported and resulted in the submission of a patent application. Chapter 4 reports experiments carried out to demonstrate and characterise this effect, including the derivation of techniques to establish its basic feasibility as a practical system.

Importantly, besides the relatively low ratios (1:400) of ciliate organism needed relative to algal cells, a range of inexpensive triggers (ammonium ion, shifts in pH and salinity) in low concentrations can be used, including ryanodine receptors (caffeine and *p*-chlorocresol). As such this technique opens up a range of harvesting opportunities from wastewater to biofuel production systems and could replace or complement current expensive methods.

Consequently we regard the *Tetrahymena* mechanism as being more promising. Industrial scale microalgal cultures urgently need effective and inexpensive harvesting techniques especially for biofuel applications where the energy expended in harvesting is a large part of the overall energy input to the process. The low energy input, rapidity of flocculation and high level of control attainable with *Tetrahymena* make it a very promising candidate for such a harvesting mechanism.

In addition to development of a novel harvesting technique which could provide significant benefits in terms of economic return on energy balance of microalgae systems, an understanding of the factors affecting flocculation also proved to be critical for defining variables to improve biomass yield. For example, given the knowledge of how *Tetrahymena* can be used for algal bioflocculation, preventing or limiting contamination by *Tetrahymena* and other ciliated protozoans could result in more uniform

open pond cultures with a better light distribution and ultimately better productivities.

The unexpected interactions between *Tetrahymena* and one of the top producing algal strains *Chlorella sorokiniana* (8_C4) demonstrate the complexity of large scale outdoor cultures and illustrate the ongoing need to understand the evolution of ecosystems within algal production reactors. There are many opportunities with the current system to explore and model these interactions and their likely effects on bioreactors.

In summary, the present project has provided a library of Australian microalgae strains and streamlined techniques for their isolation, nutrient optimisation and management. The process of scaling the growth of these strains to large scale outdoor cultures has provided opportunities to redefine key issues for production strains, and explore the role of culture composition through the use of defined polycultures. Finally, investigation into the origins of culture bioflocculation has led to a new understanding of the role of ciliated protozoans in algal reactors and shown how they can be used to assist with the economically and energetically important step of dewatering.

References

1. International Energy Agency, *World Energy Outlook 2010*, in *IEA publications*. 2010: Paris.
2. *How to Feed the World 2050*. 2009, Food and Agriculture Organisation of the United Nations.
3. *Environmental Outlook to 2050*. 2014, Organisation for Economic Co-operation and Development.
4. Intergovernmental Panel on Climate Change (IPCC). *Climate Change 2014*. Fifth assessment report (AR5) 2014 [cited 2014 November 2014]; Available from: http://www.ipcc.ch/report/ar5/.
5. Stephens, E., et al., *Future prospects of microalgal biofuel production systems.* Trends in Plant Science, 2010. **15**(10): p. 554-564.
6. Bui, T.V.L., et al., *Impact of Procedural Steps and Cryopreservation Agents in the Cryopreservation of Chlorophyte Microalgae.* Plos One, 2013. **8**(11).
7. Wolf, J., et al., *Integrated medium design and microbial screening for high biomass production.* Algal Research, submitted.
8. Chioccioli, M., B. Hankamer, and I.L. Ross, *Flow Cytometry Pulse Width Data Enables Rapid and Sensitive Estimation of Biomass Dry Weight in the Microalgae Chlamydomonas reinhardtii and Chlorella vulgaris.* Plos One, 2014. **9**(5): p. 12.
9. Sathe, S. and P.M. Durand, *A low cost, non-toxic biological method for harvesting algal biomass.* Algal Research-Biomass Biofuels and Bioproducts, 2015. **11**: p. 169-172.